Functional Analysis of the Motor Circuit of Juvenile

*Caenorhabditis elegans*

by

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Abstract

*Caenorhabditis elegans* generates alternating dorsal-ventral bending waves across developmental stages. The overall level of dorsal and ventral muscle output is symmetric. In adults, this behavioral symmetry correlates with anatomy symmetry: distinct pools of cholinergic motor neurons activate dorsal and ventral body wall muscles, whereas distinct pools of GABAergic motor neurons contra-laterally inhibit dorsal and ventral muscles, respectively. However, in the first stage larva (L1), cholinergic motor neurons only make neuromuscular junctions to the dorsal muscle, and GABAergic motor neurons to the ventral muscle. How does an asymmetric motor circuit produce symmetric motor output?

In the past five years, my colleagues and I undertook anatomical and functional analyses of the L1 larva motor circuit to address this question. First, my colleagues fully reconstructed the connectivity of a complete L1 larva, from which we identified all candidate cellular components for muscle activity. Next, to elucidate the functional contribution of these candidates, I developed a novel all-optical manipulation methodology that allows fast and systemic probing of functional connections of neural circuits. Third, I led the effort to combine free-moving calcium
imaging, cellular ablation, chemogenetics, optogenetics, and all-optical interrogation to pinpoint mechanisms for the symmetric L1 muscle output.

Collectively, results from these studies show that all cells that make anatomic synaptic connections are insufficient to explain the symmetry of body wall muscle excitability in L1 C. elegans; instead, extra-synaptic transmissions play a crucial role in underling muscle activity regulation. In the L1 motor circuit, the anatomically asymmetric circuit is functionally compensated by extra-synaptic transmissions from both excitatory premotor interneurons and inhibitory motor neurons to operate as a symmetric circuit. Excitatory premotor interneurons can bypass the motor neurons layer to directly regulate excitability of muscle cells, thus assuming dual roles.

Animals with distinct wirings can produce similar motor patterns. Our findings in the juvenile C. elegans motor circuit show that structurally divergent neural circuits can functionally converge on the functional output, and that neurons can multiplex. This study further demonstrates a larger role of functional communications devoid of ultrastructural features in a developing neural circuit.
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Figure contributions

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1 Introduction: The neural circuit underlying of locomotor behaviors and technological advances in neurophysiology

1.1 Control of locomotion

1.1.1 Locomotion in various forms

The Merriam-Webster Dictionary defines locomotion as an act or the power of moving from place to place. Animals have developed various forms of locomotion during evolution, including undulation, combination of undulation and limbed-movement, and limbed-only movement (Jung and Dasen, 2015). Different forms of locomotion allow animals to adapt to diversified environments, ranging from aerial, aquatic, terrestrial, to fossorial.

1.1.1.1 Limbless undulatory movement

The axial-based undulation, such as that generated by snakes, propels the animal’s body in a wave-like motion (Gray, 1968; Mosauer, 1932). Alternating contractions of body segments along a body axis forms this flexural wave (Dasen, 2018). In most cases, including the anguilliform locomotion of eels and the serpentine locomotion of snakes, the wave propagates in an opposite direction towards that the animal moves (Breder Jr, 1926; Gray, 1933; Gray, 1946). The wave can also propagate in the same direction as the animal moves, as in the cases of alga Ochromonas, polychaete worm Nereis, and the fruit fly Drosophila larvae (Heckscher et al., 2012; JAHN et al., 1964; Spina et al., 2007).

How does undulation propel the animal in directional movements? The study of underlying mechanisms for propulsion of undulatory locomotion was pioneered by James Gray. Gray and others showed that, external forces that are tangential to the body surface retard the undulatory movement, whereas the anisotropic external resistances to the body surface and internal muscle tension collectively generate the propulsive force (Gasc et al., 1989; Gray, 1946, 1951, 1953; Gray and Lissmann, 1950; Gray and Lissmann, 1964; Guo and Mahadevan, 2008; Hu et al., 2009; Jayne, 1988; Mahadevan et al., 2004; Moon and Gans, 1998). The body curvature along
the anterior-posterior axis must vary in order to produce disequilibrium of forces along neighboring segments, and resulting propulsive force (Gray, 1946, 1953). Active moments produced by muscle contraction determine the velocity and shape of the undulatory locomotion (Guo and Mahadevan, 2008).

Versatile forms of undulatory movement can be observed in different environments. At least five forms of locomotion are found in snakes: serpentine, concertina, crotaline or side-widing, rectilinear, and side-pushing (Cundall, 1987; Gans, 1974; Gray, 1946; Gray, 1968; Jayne, 1986; Lissmann, 1950; Mosauer, 1932; Newman and Jayne, 2018). Grass snake Tropidonotus natri alone is able to perform serpentine movement under normal circumstances, concertina movement when subjected to a straight or circular tube, and crotaline movements when moving on a slippery surface (Gray, 1946). Diverse forms of undulatory locomotion also exist in fishes. anguilliform swimming for low speed fishes including eels, sub-carangiform swimming for higher speed fishes including trout, carangiform and thunniform swimming for high speed fishes including tuna (Lauder, 2015).

Roundworm Caenorhabditis elegans adjusts its undulating wavelength when swimming in liquid of various viscosities (Berri et al., 2009; Fang-Yen et al., 2010). Such changes allow these animals to adapt to their physical surroundings.

1.1.1.2 Limbed movement

The limbed locomotion drives body movement by appendages connected to the torso by joints. This form of locomotion, such as walking and flying, primarily exists in arthropods and vertebrates (Belanger, 2005; Ritzmann et al., 2004). Limbed movement typically involves fore- and hindlimb alternation, left-right coordination, flexor-extensor alternation, and interjoint, adding higher degrees of freedom compared to limbless undulatory movement (Akay et al., 2001; Bassler and Buschges, 1998; Bucher et al., 2003; Ghez and Sainburg, 1995; Hess and Buschges, 1997, 1999; Uuml et al., 1995).

Limbed movements can display a palette of adaptive gaits (Roberts, 1995). Icelandic horses for example, can walk, tölt, trot, gallop/bound, and in certain breeds pace, with increasing speed
Mice exhibit similar gait adaptation (Bellardita and Kiehn, 2015; Lemieux et al., 2016; Talpalar et al., 2013).

1.1.2 Properties of motor circuits

Motor circuits underlie locomotion. Motor circuits generate or maintain intrinsic rhythmic activities. Most motor circuits exhibit planar (left-right or dorsal-ventral) symmetry in both infrastructural wiring and functional output.

1.1.2.1 Central pattern generators

1.1.2.1.1 The discovery of central pattern generators

The rhythmic output of motor circuits is generated by central pattern generators (CPGs) - neurons and neural networks that intrinsically generate or sustain oscillatory activities independent of descending and sensory inputs (Friesen and Stent, 1978; Marder and Bucher, 2001; Selverston and Moulins, 1985). CPGs regulate both the frequency and patterning of the behavioral outputs (Kiehn, 2006, 2011).

Historically, there were two competing hypotheses for how motor rhythm is generated. Charles Sherrington proposed that the reflex chain, which uses sensory feedback from muscles to antagonize the opposing muscles, is the underlying mechanism for rhythmicity (Sherrington, 1907). Thomas Brown argued with recordings from the decerebrate cat that spinal cord neurons exhibited oscillatory activity independent of sensory feedback (Brown, 1911; Brown, 1914). Brown further formulated the half-center model for central oscillators, where two populations of neurons antagonize each other reciprocally and generate rhythmic output.

However, the field remained skeptical until the studies of arthropods nervous system. Torkel Weis-Fogh designed an intricate device that allowed precise recording of the tethered locust wingbeat, and put forth a reflex mechanism that drives the rhythm of wing stroke (Weis-Fogh, 1956). Donald Wilson used the same device to record action potentials. Results from his experiments negated this notion by showing that locusts could produce rhythmic motor patterns
after being completely deafferented (Wilson, 1961; Wilson, 1966; Wilson and Wyman, 1965). In these experiments, Wilson used a locust preparation that was composed of the head, thoracic nerve cord, trachea, and a ventral cuticular strip. A brief stimulation of the head nerve by wind puff or electrical shock was able to trigger a flight rhythm. Later experiments showed that in comparison with intact locusts, deafferented locusts exhibited lower frequency in both motor neurons oscillations and wingbeat (Pearson and Wolf, 1987; Wolf and Pearson, 1987). These results demonstrate conclusively that while reflex is not necessary for the rhythm generation, reflex can still modulate the rhythmicity.

Strongest supporting evidence of CPGs emerged from studies using the isolated nerve cords. Cornelis Wiersma et al. first observed bursts of motor neuron activity at isolated crayfish abdominal nerve cords (Hughes and Wiersma, 1960). Later, isolated nerves or spinal cords from the leech, lamprey, tadpole, salamander, rat, and cat were all shown to display rhythmic firing patterns that correlated with their in vivo motor patterns, a phenomenon termed fictive locomotion (Cohen and Wallen, 1980; Delvolve et al., 1999; Guertin et al., 1995; Juvin et al., 2007; Kiehn et al., 1992; Soffe and Roberts, 1982). Isolated pre-Botzinger complex from the rat brainstem generated robust oscillatory respiratory-related activities (Smith et al., 1991).

The most extensive characterization of CPGs results from studies in the crustacean stomatogastric ganglion (STG) (Bal et al., 1988; Hartline, 1979; Marder and Calabrese, 1996a; Maynard and Selverston, 1975). Two types of rhythms are generated from the STG: the pyloric rhythm that regulates the dilation and constriction of pylorus muscles; the gastric mill rhythm that regulates the closing of lateral teeth (Selverston and Moulins, 1985). For the pyloric rhythm, a group of interneuron (AB) and motor neurons (LP, PY, PD, IC, VD) produce the three-phase oscillation pattern lasting 1-2sec each cycle, with the order of AB-PD, LP-IC, PY-VD (Marder and Bucher, 2007) (Fig. 1.1). Interneuron AB and two motor neurons PD are electrically coupled (Fig. 1.1A). AB is intrinsically oscillatory and the source for pyloric rhythm. Intracellular recordings show that they form the pacemaker group that sets the dilator phase (Fig. 1.1B-C). The follower cells LP and PY fire sequentially and set the constrictor phase (Fig. 1.1C). IC usually fires together with LP, and VD with PY. Although chemical synapses in STG are cholinergic or glutamatergic, they are inhibitory (Fig. 1.1A). Reciprocal inhibition between STG neurons is common (Fig. 1.1A).
Figure 1.1: Triphasic pyloric rhythm of the stomatogastric ganglion

A) Cartoon depiction of the core cellular components generating pyloric rhythm in STG, with IC and VD neurons omitted. Neurons of the same type are overlapped. Cholinergic synapses are in
orange. Glutamatergic synapses are in blue. Both types of chemical synapses are inhibitory. Gap
junctions are drawn as zigzags.

B) Schematic of the locations of recordings on isolated stomatogastric nervous system (STNS)
from lobster *Homarus americanus*. CoG stands for commissural ganglion. OG stands for
esophageal ganglion. vlvn stands for ventral branch of lateral ventricular nerve. The intracellular
recordings with glass microelectrodes show neuron firings in the STG. The extracellular
recordings with pin electrodes show activities of motor nerves.

C) Three-phase rhythm produced by STG. AB and PD are synchronized, with LP and PY firing
sequentially afterwards. Adapted from Marder and Bucher, 2007.

### 1.1.2.2 Mechanisms that underlie rhythm generation by CPGs

Two main types of mechanisms underlie rhythm generation: one involves a pacemaker neuron
that sets the rhythm for follower neurons, as the case for the pyloric CPG; the other involves
reciprocally inhibitory half-centers, as the case for the gastric CPG. Some CPGs utilize a hybrid
of the two mechanisms (*Getting, 1989; Selverston and Moulins, 1985*).

For the pacemaker neuron driven rhythm, oscillation of membrane potential results from the
interplay between a plethora of ion conductance, mediated by leak channels, voltage-gated ion
channels, calcium, sodium and potassium-activated channels, and modulation by monoamines
and neuropeptides (*Harris-Warrick, 2002; Harris-Warrick, 2011; Harris-Warrick and Marder,
1991*). In many but not all species, glutamate release is needed to initiate the pacemaker rhythm
(*Cowley et al., 2005; Hochman and McCrea, 1994; Kiehn et al., 1996; Li et al., 2010; Reith and
Sillar, 1998; Roberts et al., 2008; Wallen and Grillner, 1987*). Major ion channels for pacemaker
neurons conduct an array of ionic currents, including persistent sodium current $I_{\text{Na}^+}$ (*Connors et
al., 1982; Dai et al., 2009; Hotson et al., 1979; Tazerart et al., 2007; Zhong et al., 2007; Ziskind-
Conhaim et al., 2008*), hyperpolarization-activated inward current $I_H$ (*Attwell and Wilson, 1980;
Bader et al., 1982; Bader et al., 1979; Barnes and Hille, 1989; McCormick and Pape, 1990; Pape,
1996*), transient low threshold calcium currents $I_T$ (*Andersen et al., 1964; Carbone and Lux,
1984; Fedulova et al., 1985; Huguenard, 1996; Jahnsen and Llinas, 1984; Llinas and Jahnsen,
1982; Llinas and Yarom, 1981a, b; Nowycky et al., 1985; Spedding and Paoletti, 1992*), sodium-

activated potassium current $I_{\text{KNa}}$ (Bader et al., 1985; Kameyama et al., 1984; Yuan et al., 2003), calcium-activated potassium current $I_{\text{KCa}}$ (Gardos, 1958; Knjjevic and Lisiewicz, 1972; Meech, 1972; Stocker, 2004), transient potassium current $I_{\text{A}}$ (Connor and Stevens, 1971; Gean and Shinnick-Gallagher, 1989; Huguenard et al., 1991; Noh et al., 2019; Rogawski, 1985), delayed rectifier potassium current (Adrian et al., 1970; Augustine, 1990; Clark et al., 2004; Nattel, 2008; Noble and Tsien, 1969a, b), sodium leak current (Lu and Feng, 2011). The interactions among these currents drive the membrane potential oscillations in the pacemaker neurons (Prinz et al., 2003; Ramirez et al., 2004).

For reciprocally inhibitory half-center oscillators, rhythm may be generated by multiple mechanisms, including intrinsic release and intrinsic escape, and synaptic release and synaptic escape (Sharp et al., 1996; Skinner et al., 1994; Wang and Rinzel, 1992). Dependence of synaptic voltage threshold, which is the threshold for cells to release neurotransmitters, is the key difference between the intrinsic and synaptic mechanisms. The intrinsic mechanisms can drive the oscillations independent of presynaptic threshold voltage, whereas the synaptic mechanisms heavily rely on presynaptic threshold voltage (Skinner et al., 1994; Wang and Rinzel, 1992). For the “release”-driven mechanism, active neurons reduce firing frequency due to frequency adaptation, releasing inhibition on the other neurons. For the “escape”-driven mechanism, inhibited neurons become progressively active, escaping inhibition and exerting inhibitory effect on the other neurons.

### 1.1.2.3 The organization of locomotory CPGs

#### 1.1.2.3.1 CPGs for limbless undulatory movements

Locomotory CPGs are anatomically diverse. Animals executing undulatory movements, such as leeches, lampreys, tadpoles, and *C. elegans*, have reiterated CPGs in the nerve cord or spinal cord (Cohen and Wallen, 1980; Friesen et al., 1976, 1978; Kahn and Roberts, 1982; Li et al., 2006; Wallen and Williams, 1984). Swimming in tadpoles and lampreys are considered to be driven by reciprocally inhibitory half-center CPGs, whereas in *C. elegans* by single neuron CPGs.
Tadpoles: Their swimming CPGs are proposed to reside in the hindbrain and rostral spinal cord (Kahn and Roberts, 1982; Li et al., 2006). Excitatory descending interneurons, inhibitory ascending interneurons, and local inhibitory commissural interneurons constitute CPGs in tadpoles (Dale, 1985; Dale and Roberts, 1985; Li et al., 2004a; Li et al., 2006). The excitatory descending interneurons are considered to be the pacemaker. Co-release of glutamate and acetylcholine from these interneurons render the half-center rhythmogenic, after blocking the left-right reciprocal inhibition (Kahn and Roberts, 1982; Li et al., 2004c; Li et al., 2006; Soffe, 1989; Soffe et al., 2009). NMDA receptors and electric coupling of these excitatory descending interneurons were proposed to contribute to their pacemaker rhythm (Li et al., 2009; Prime et al., 1999).

Lampreys: Their swimming CPGs are generally considered to locate along its spinal cord (Cohen and Wallen, 1980; Wallen and Williams, 1984). Excitatory premotor interneurons, inhibitory premotor interneurons, commissural inhibitory interneurons, and commissural excitatory interneurons form local CPGs on the left and right side of each segment (Buchanan, 1982, 1992, 1999a, b; Buchanan and Cohen, 1982; Buchanan and Grillner, 1987, 1988; Buchanan et al., 1989; Buchanan and Kasicki, 1995). Excitatory interneurons are suggested to be the pacemaker in lampreys (Cangiano and Grillner, 2005). The rhythmic bursting was still present after complete physical severing of the midline, indicating the independence of rhythmogenesis in half-centers (Cangiano and Grillner, 2003, 2005; Cangiano et al., 2012). A mixed strong reciprocal inhibition by inhibitory glycinergic interneurons and weak reciprocal excitation by glutamatergic interneurons couple the half centers (Alford and Williams, 1989; Cohen and Harris-Warrick, 1984; Hagevik and McClellan, 1994, 1999; McClellan and Hagevik, 1999). The left-right alternating rhythmic output is maintained primarily by the reciprocal inhibition (GRILLNER and WALLÉN, 1980; McPherson et al., 1994). Upon pharmacological blocking of contralateral inhibitory glycinergic interneurons, synchronous bursts of rhythm output were found in the half-centers, owing to the remaining weak commissural excitatory glutamatergic interneurons (Cohen and Harris-Warrick, 1984).

*C. elegans*: CPGs in very small invertebrates were harder to study largely due to difficulties of isolating nerve cords. The advancement of calcium imaging and *in vivo* cell manipulation techniques made these studies possible. Recent studies revealed that in this compact nervous system, excitatory motor neuron themselves are the CPGs (Fouad et al., 2018; Gao et al., 2018;
Xu et al., 2018). Motor neuron cell bodies are located along the ventral nerve cord and are both chemically and electrically synapsed by premotor interneurons in C. elegans (White et al., 1986). Unlike many other systems, there is no voltage-gated sodium channels in C. elegans; instead, it is the voltage-gated calcium channels that primarily drive its depolarization (Consortium, 1998; Gao and Zhen, 2011; Goodman et al., 1998; Lee et al., 1997; Liu et al., 2018; Richmond and Jorgensen, 1999; Shtonda and Avery, 2005). With the exception of olfactory neuron AWA and in some occasions AIA, most C. elegans neurons examined to date could not fire action potentials (Bargmann, 1998; Consortium, 1998; Dobosiewicz et al., 2019; Goodman et al., 1998; Kato et al., 2015b; Liu et al., 2017; Liu et al., 2018; Mellem et al., 2008; Xie et al., 2013). Remarkably, with such unique cellular properties, the motor neurons directing backward locomotion, and some motor neurons directing forward locomotion are intrinsically oscillatory (Fouad et al., 2018; Gao et al., 2018; Xu et al., 2018). The P/Q/N-type voltage-gated calcium channel UNC-2 is suggested to underlie the sustained depolarization phase of membrane oscillation in motor neurons (Gao et al., 2018). Ablation or inhibition of a portion of excitatory motor neurons failed to terminate the propagation of undulatory waves anterior or posterior to the ablated region, suggesting that the motor neurons’ oscillatory activity are distributed along the nerve cord (Fouad et al., 2018; Gao et al., 2018; Xu et al., 2018). Therefore, in C. elegans CPG circuits are functionally conserved, but structurally compressed in motor neurons (Gao et al., 2018; Wen et al., 2018).

1.1.2.3.2 CPGs for limbed movements

CPGs that underlie limed locomotion show more clustered distribution at body segments near their limbs (Hinckley et al., 2005; Romanes, 1951; Zagoraiou et al., 2009). Left-right, fore- and hindlimb, and flexor-extensor coordination are needed different motor patterns (Butt et al., 2002; Kiehn, 2006, 2011, 2016). The precise cellular candidates of CPGs in these animals remain elusive, but the rhythmicity is typically considered to be driven by spontaneous oscillatory excitatory interneurons (Kiehn, 2016).

In the mouse for example, its locomotor CPGs reside in the spinal cord, candidates include excitatory non-V2a SHOX2+ interneurons, excitatory HB9+ interneurons, and unidentified excitatory interneurons (Caldeira et al., 2017; Dougherty et al., 2013). The non-V2a SHOX2+
interneurons are suggested to drive V2a SHOX2+ interneurons, which subsequently activate motor neurons and produce the rhythmic motor output (Stepien et al., 2010).

1.1.2.4 Symmetry of motor circuits at different behavioral paradigms

For most bilaterian, their locomotory circuits exhibit a shared property in structural symmetry. It is a conserved feature in different species at different behavioral paradigms.

In the mouse for instance, oscillatory non-V2a SHOX2+ interneurons mediate left-right coordination by activating commissural interneurons. Distinct pathways are recruited at different locomotor frequencies separately (Crone et al., 2008; Crone et al., 2009; Dougherty et al., 2013; Talpalar et al., 2013; Zhang et al., 2008b):

1. non-V2a SHOX2+ interneurons → excitatory SHOX V2a → inhibitory commissural V0_D
2. non-V2a SHOX2+ interneurons → excitatory commissural V0_V → local inhibitory neurons
3. non-V2a SHOX2+ interneurons → excitatory commissural non-V0 (might be V3)

At low frequency locomotion, inhibitory V0_D interneurons are recruited to inhibit motor neurons in the opposing side; at higher frequency locomotion, excitatory V0_V interneurons are recruited to activate local inhibitory neurons on the opposing side, which subsequently inhibit the contralateral motor neurons; at very high frequency locomotion, excitatory non-V0 interneurons are recruited to activate contralateral motor neurons, and establish a left-right synchronous motor pattern. The presence of a two-step inhibitory pathway mediated by excitatory commissural interneurons has not been found in limbless animals. This is considered to be required for gait adaptation for limbed quadrupedal animals, with left-right alternation at low speed, and left-right synchrony at high speed (Bellardita and Kiehn, 2015; Ferreira-Pinto et al., 2018; Kiehn, 2016; Lemieux et al., 2016; Talpalar et al., 2013). This type of gearshift was also found in limbless zebrafish motor circuits. Zebrafish V2a interneurons and motor neurons are separated into slow, intermediate, and fast modules, and are recruited at different speeds (Ampatzis et al., 2014; Song et al., 2018). (add additional examples; briefly mention c. elegans and then mention the separate section) For each functional configuration, the left-right symmetry of the motor circuit is preserved.
In addition to left-right coordination, the coordination for fore- and hindlimbs also contributes to the symmetry. DMRT3$^+$ dl6 interneurons are implicated in both left-right and fore- and hindlimb coordination (Andersson et al., 2012). Both glutamatergic and GABAergic long projection neurons extending from the cervical to the lumbar spinal cord regulate the hindlimb muscles on ipsilateral and contralateral sides, ensuring fore- and hindlimb alternation (Ruder et al., 2016). As a result, motor neurons on each side either show alternating or synchronous activities, with equal summed activity levels of all limbs on each side under different circumstances.

The flexor-extensor coordination is mediated by reciprocal inhibition between V1 and V2b interneurons (Zhang et al., 2014). V1 interneurons primarily inhibit flexor CPGs, whereas V2b interneurons primarily inhibit extensor CPGs (Britz et al., 2015). The circuit layout is also largely symmetric on the flexor and extensor side.

Together, the motor circuits underlying left-right alternation, left-right synchronization, fore- and hindlimb alternation, and flexor-extensor alternation together secure the balanced output levels on left and right sides (Deska-Gauthier and Zhang, 2019).

1.1.3 Neuromodulation of CPGs

Neuromodulators tune the pattern, frequency and phasing of the CPGs by modulating synaptic strengths and/or membrane potentials (Christie et al., 1995; Harris-Warrick, 2011; Katz, 1995; Katz et al., 1989; Marder and Calabrese, 1996b; Marder et al., 1995; Skiebe, 2001).

In the crustacean STG, a plethora of neurotransmitters, monoamines, and neuropeptides from sensory neurons, neuromodulatory neurons, and motor neurons act together on the CPG (Christie et al., 1995; Harris-Warrick, 2011; Marder et al., 1995; Skiebe, 2001). In LP neurons of STG, neuropeptides proctolin, crustacean cardioactive peptide, Cancer borealis tachykinin-related peptide la, red pigment concentrating hormone, TNRNFLRFamide, pilocarpine, synergistically activate the same voltage-gated inward current through their receptors (Swensen and Marder, 2000). When the endogenous source of neuromodulation was blocked in STG, exogenous application of distinct neuromodulators, such as pilocarpine, serotonin, proctolin, and dopamine, induced highly differential forms of pyloric rhythm (Marder and Weimann, 1992). In the sea slug Tritonia, neuromodulation of serotonin is essential for its swimming CPG, where the central
component of its CPG dorsal swim interneurons release serotonin to cerebral cell 2 by neuromodulatory mechanism, the blocking of which terminates the rhythm generation (Katz and Frost, 1995a, b; McClellan et al., 1994).

1.1.4 Sensory feedback regulation of motor circuits

Sensory feedback can regulate activities of CPGs. In the tadpoles, sensory Rohon-Beard neurons sense touch on the trunk cuticle and activates the swimming CPGs via dorsolateral commissural interneurons and dorsolateral ascending interneurons (Li et al., 2002, 2004b; Sautois et al., 2007; Sillar and Roberts, 1988, 1992). In the lampreys, the stretch sensitive edge cells mediate the proprioceptive feedback onto CPGs (Grillner et al., 1982a; Grillner et al., 1982b; Grillner et al., 1984). In C. elegans, proprioceptive feedback can directly modulate activities of excitatory motor neurons, i.e. its CPGs (Wen et al., 2012).

1.1.5 Descending control of motor circuits

Descending pathways from higher brain regions onto locomotor CPGs and motor circuits can regulate the initiation, termination, speed, direction, and gait of locomotor output (Arber and Costa, 2018; Ferreira-Pinto et al., 2018). In the vertebrate systems, locomotor command flows from higher brain regions to the spinal cord via multiple pathways, such as basal ganglia → thalamus → motor cortex → spinal cord, and basal ganglia → mesencephalic locomotor region → reticular formation → spinal cord (Grätsch et al., 2019).

A centerpiece in the descending control is the mesencephalic locomotor region (MLR), an evolutionarily conserved midbrain brainstem region identified by Grigori Orlovsky and colleagues to initiate locomotor behavior upon electrical stimulation (Alam et al., 2011; Jordan, 1998; Mori et al., 1989; Ryczko and Dubuc, 2013; Shik and Orlovsky, 1976; Shik et al., 1966). The MLR sits in between higher brain regions and spinal cord, gating locomotor information from higher brain regions, sends signals to the spinal cord primarily through reticular formation, and also projects to higher brain regions including basal ganglia (Jordan, 1998; Martinez-Gonzalez et al., 2011; Ryczko and Dubuc, 2013). It is composed of cuneiform nucleus (CnF) and
pedunculopontine nucleus (PPN), with CnF eliciting escape responses, and PPN driving exploratory locomotion (Caggiano et al., 2018; Roseberry et al., 2016; Shik and Orlovsky, 1976; Skinner and Garcia-Rill, 1984). The glutamatergic neurons in MLR are reported to be both necessary and sufficient to initiate locomotion, and can regulate speed, but are not equally contributing to different gaits (Caggiano et al., 2018; Josset et al., 2018; Lee et al., 2014; Roseberry et al., 2016). At lower speed with left-right alternation, glutamatergic neurons in both nuclei can contribute to the locomotion. At higher speed with left-right synchronization, only glutamatergic neurons in CnF are responsible for the locomotion. The cholinergic neurons in MLR modulate locomotion, whereas GABAergic neurons inhibit locomotion (Caggiano et al., 2018; Dautan et al., 2016; Josset et al., 2018; Roseberry et al., 2016; Xiao et al., 2016).

The lower brainstem neurons are considered to bridge MLR and the spinal cord (Orlovskii et al., 1999). Take mice for example, in this region, glutamatergic neurons in lateral paragigantocellular nucleus (LPGi) possibly relay signals from glutamatergic neurons in CnF and can drive high speed locomotion, whereas glycineric neurons in LPGi can cause locomotion arrest in different forms (Capelli et al., 2017). By acting through an inhibitory glycineric neuron population in the spinal cord, glutamatergic Chx10+ V2a neurons in the reticular formation of rostral medulla and caudal pons can stop locomotion when activated, and increase locomotor activity when inhibited (Bouvier et al., 2015). Other than signals from descending neurons, behavioral arrest can also be elicited by ascending inhibitory output from GABAergic and glycineric neurons in reticular formation to the thalamus (Giber et al., 2015). A recent study showed that Chx10+ reticulospinal neurons in nucleus gigantocellularis regulate left/right steering in locomotion (Cregg et al., 2019).

In C. elegans, the endogenous oscillation of motor neurons is both positively and negatively regulated by descending interneurons (Gao et al., 2018). AVA premotor interneurons can potentiate A-type motor neurons and drive backward locomotion through a mixture of electrical and chemical synapses (Gao et al., 2018). AVB premotor interneurons can potentiate B-type motor neurons and drive forward locomotion through electrical synapses (Xu et al., 2018). However, upon ablation of premotor interneurons or severing electric coupling from premotor interneurons that mediate shunting effect, motor neurons become rhythmically active (Gao et al., 2018; Kawano et al., 2011).
In summary, CPGs in the motor circuit generate the locomotor rhythm and are subjected to regulations from sensory feedback and descending control. The layout of the motor circuit is symmetric along one body axis (Catela et al., 2015).

1.1.6 Different circuit configurations can generate similar locomotor patterns

Neural circuits that wire anatomically differently can generate homologous motor outputs, consistent with convergent evolution. The CPGs in the invertebrate leech consist of three inhibitory interneurons, whereas CPGs in the vertebrate lamprey are made up of two reciprocally inhibitory half-centers as described previously, but these two evolutionarily distant species manage to execute highly similar sinusoidal swimming behavior (Friesen, 1985, 1989; Friesen et al., 1976, 1978; Mullins et al., 2011; Weeks, 1982). The crab and lobster have differences in their motor neuron connectivities of the stomatogastric systems, but share homologous pyloric and gastric mill rhythms (Combes et al., 1999; Weimann et al., 1991). Most prominently, two sea slug species, the Melibe leonina and Dendronotus iris show species-specific interneuron connectivities, yet they produce conserved swimming flexions (Falgairolle et al., 2013; Li et al., 2009; Li et al., 2006; Parker and Grillner, 2000; Sakurai and Katz, 2017). In these two sea slugs, pharmacological blocking of synapses in either one species disrupts the normal motor pattern, but addition of artificial synapses created by dynamic clamp can essentially rewire the connectivity and transform the circuit from one species to another. The resulted circuit is able to restore the motor pattern (Sakurai and Katz, 2017). It appears that divergent circuit configurations may converge at the output, the motor patterns.

1.2 C. elegans locomotion and motor circuit

1.2.1 C. elegans model for motor circuit studies

Upon hatching from fertilized eggs, C. elegans larva undergo first (L1) to fourth (L4) larval stages before reaching the adulthood (Sulston, 1976). In the laboratory culture plates, this transparent nematode lies on the lateral side, and moves sinusoidally by alternating contraction and relaxation of dorsal and ventral muscles, and sequential propagation of the dorsal-ventral
bending waves from head to tail or vice versa (Corsi et al., 2015). *C. elegans* uses only 220 to 300 neurons during development to perform a large repertoire of locomotor behaviors, e.g. foraging, reversals, omega turns, pirouettes, head steering, head sweeping, escape (Gray et al., 2005; Stephens et al., 2008; Sulston, 1976; Sulston and Horvitz, 1977; Sulston et al., 1983; White et al., 1986; Yemini et al., 2013).

Since Sydney Brenner and colleagues established the *C. elegans* as a model organism in 1960-70s (Brenner, 1974; Kaletta and Hengartner, 2006; Kenyon, 1988), circuit probing tools such as electrophysiology, laser cell ablation, photo-induced cell ablation, cell death-induced cell ablation, optogenetics, calcium imaging have been implemented (Avery and Horvitz, 1989; Avery et al., 1995; Bargmann and Avery, 1995; Chalfie et al., 1985; Chelur and Chalfie, 2007; Dal Santo et al., 1999; Harbinder et al., 1997; Kawano et al., 2011; Kerr et al., 2000; Leifer et al., 2011; Liewald et al., 2008; Nagel et al., 2005; Qi et al., 2012; Raizen and Avery, 1994; Richmond, 2006; Shaham and Horvitz, 1996; Suzuki et al., 2003a; Wen et al., 2012; Xu and Chisholm, 2016). With its versatility in locomotor behaviors, amenability for genetic and optical manipulation, and the numeric simplicity of its nervous system, *C. elegans* is a compact and powerful system for investigation of mechanisms for locomotion at the molecular, cellular and circuit levels (Kaletta and Hengartner, 2006).

### 1.2.2 Anatomy of the *C. elegans* motor circuit

An important aspect of understanding neural circuit is to know the anatomic structure of synaptic connectivity. For *C. elegans*, this has been addressed by electron microscopy reconstruction of the entire nervous system at single synapse resolution.

#### 1.2.2.1 Reconstruction of neural connectivity by electron microscopy

Electrons, like other particles, show wave-particle duality (Einstein, 1905). The wavelength of high energy electrons, at the range of 0.0009–0.027nm, is much shorter than that of the visible light. The resolution limit of the electron microscope, proportional to the wavelength of the particle, is superior to that of the light microscope (Vernon-Parry, 2000). Therefore, electron
microscopy is more suitable for detection of ultrastructure details, such as synapses, in biological samples. However, because of the short wavelength, electrons are unable to penetrate thick materials. Accordingly, electron microscopy analyses of a sample requires technically demanding sectioning, collection, imaging, annotation, and reconstruction of numerous nm range ultra-thin slices (Baena et al., 2019). Until recently, these technical difficulties have discouraged attempts to reconstruct the wiring diagram of large biological samples. To date, adult C. elegans remains the only multicellular animal where a full connectome, reconstructed from pieces of multiple animals, has been reported (Cook et al., 2019; White et al., 1986).

Several key technical breakthroughs in electron microscopy have enabled the initiative of connectomics studies in multiple animals (Mulcahy et al., 2018). Chemical fixation and dehydration of samples is needed to visualize subcellular structures in conventional electron microscopy (Mersey and McCully, 1978). However, traditional sample preparation protocols, which typically involved slow penetration of fixatives in mechanically severed samples, induce deformation at a high frequency (Gilkey and Staehelin, 1986; Mulcahy et al., 2018; Smith and Reese, 1980). High-pressure freezing, followed by freeze-substitution by fixatives can preserve small samples in an uniform, near physiological state (Moor, 1987; Riehle, 1968) (Simpson, 1941). Automation for sectioning, collection, imaging, segmentation, and reconstruction have been developed and gradually refined in recent years (Hall et al., 2012; Hayworth et al., 2014; McDonald, 2014; Mulcahy et al., 2018; Rostaing et al., 2004). These advances allowed a revisit of the C. elegans wiring diagram reconstructed in a near physiological state with high efficiency.

1.2.2.2 Symmetry of the adult C. elegans motor circuit

In adult C. elegans, electron microscopy reconstruction of its motor circuit (White et al., 1986) revealed four main classes of motor neurons that innervate dorsal and ventral body wall muscles, the A-, B-, D-, and AS-type motor neurons (Fig. 1.2A). The cholinergic dorsal and ventral A type motor neurons, 9 DAs and 12 VAs execute backward movement by innervating contraction of dorsal and ventral muscles, respectively. The cholinergic dorsal- and ventral-muscle innervating B type motor neurons, 7 DBs and 11 VBs, drive forward locomotion by innervating dorsal and ventral muscle contraction, respectively. The GABAergic dorsal and ventral D type motor neurons, the 6 DDs and 13VDs receive inputs from the excitatory A and B type motor
neurons that innervate muscle groups contralaterally, and inhibit muscle groups of the opposite side (Chalfie et al., 1985; Kawano et al., 2011; Petersen et al., 2011; Sulston and Horvitz, 1977; Von Stetina et al., 2006; Walthall et al., 1993; Wen et al., 2012; White et al., 1976, 1986). The 11 cholinergic AS motor neurons innervate dorsal muscles and send input to the ventral muscle innervating D motor neurons (Bany et al., 2003; White et al., 1976).

In summary, in the adult *C. elegans*, dorsal body wall muscles make NMJs with cholinergic motor neurons DAs, DBs, ASs, and GABAergic motor neurons DDs, whereas ventral body wall muscles make NMJs with cholinergic motor neurons VAs, VBs, and GABAergic motor neurons VDs. Therefore, both dorsal and ventral body wall muscles receive excitatory and inhibitory inputs. While AS motor neurons were proposed to contribute to general motor activity with mechanisms remain to be fully resolved (Tolstenkov et al., 2018), the A-B-D motor circuit at adult stage is largely symmetric.

**1.2.2.3 Asymmetry of motor circuit structure in juvenile (L1) *C. elegans***

However, at the larval stage, the motor circuit structure in *C. elegans* is different. Four decades ago, the pioneering work by White *et al.* provided the first glimpse into a drastically organization of the L1 motor circuit (White et al., 1978). Their electron microscopy reconstruction of a portion of an L1 larva found that in the region that they examined, A- and B-type motor neurons were the sole NMJ inputs to dorsal muscles, and D-type motor neurons were the sole NMJ inputs to the ventral muscles (Fig. 1.2B-C).

In adults, the A- and B-type motor neuron are cholinergic (Johnson and Stretton, 1985; Pereira et al., 2015; Stretton et al., 1978), and D-type GABAergic (Gendrel et al., 2016; Johnson and Stretton, 1987; McIntire et al., 1993; Stretton et al., 1978). Assuming acetylcholine is excitatory and GABA inhibitory in L1 larvae, this structure would result in polarized, dorsal bend-biased output pattern, instead of a dorsal-ventral alternating output pattern exhibited by adults.

The L1 reconstruction by White *et al.* only included a small fraction of the body segment. Using high-pressure freezing and automated serial section EM, Witvliet *et al.* reconstructed several L1 larvae at different hours post hatching. These results (Witvliet *et al.*, in preparation; Mucalhy *et al.*, in preparation) confirmed the findings from White *et al.* that in L1s, DAs and DBs innervate
dorsal muscles, whereas DDs innervate ventral muscles in at L1 stage. They further discovered that in L1s, previously uncharacterized sub-lateral motor neurons SIAs and SIBs innervate both dorsal and ventral muscles in L1, and a head mesodermal cell (HMC) is electrically coupled with anterior dorsal and ventral muscles. Details of the newly reconstructed L1 larvae motor circuit will be described in Chapter 2.

Despite its small number of neurons and synapses, recent studies reveal that the *C. elegans* motor circuit bears remarkable similarities with those of large animals in motor circuit organization and operation (Catela et al., 2015; Kiehn, 2016; Kohsaka et al., 2012; Zhen and Samuel, 2015). At the CPG circuit level, *C. elegans* utilizes excitatory motor neurons as distributed intrinsic oscillators along the nerve cord, and inhibitory motor neurons that resemble the local commissural interneurons to mediate dorsal-ventral cross inhibition (Fouad et al., 2018; Gao et al., 2018; McIntire et al., 1993; White et al., 1986; Xu et al., 2018). At the level of modulation, both descending interneurons and proprioceptive feedback have been implicated in regulation of CPG activities (Gao et al., 2018; Wen et al., 2012; Xu et al., 2018). This resemblance lays the foundation for extrapolation of mechanisms for *C. elegans* motor circuit to that of evolutionarily distant organisms.
Figure 1.2 The organization of the adult and L1 *C. elegans* motor circuits

A) Cartoon depiction of cells that innervate dorsal and ventral body wall muscles in adult *C. elegans* based on EM reconstruction from White *et al.* (White *et al.*, 1986). Dorsal side is up, ventral side is down. Cholinergic neurons are in orange, and GABAergic neurons in blue.

B) Cartoon depiction of cells that innervate dorsal and ventral body wall muscles in L1 *C. elegans* based on the partial EM reconstruction from White *et al.* (White *et al.*, 1978). Dorsal side is up, ventral side is down. Cholinergic neurons are in orange, and GABAergic neurons in blue.
C) Cartoon depiction of premotor interneurons, and motor neurons in L1 *C. elegans* based on White *et al.* (*White et al., 1978*). Muscle cells are drawn in ellipses. Dorsal side is up, ventral side is down. Cholinergic neurons are in orange, and GABAergic neurons in blue. Gap junctions are represented by zigzags. Premotor interneurons AVB and PVC innervate DB motor neurons, whereas premotor interneurons AVA, AVD, and AVE innervate DA motor neurons. Figure adapted from White *et al.* (*White et al., 1978; White et al., 1986*).

### 1.3 Tools for neural circuit studies

#### 1.3.1 Functional imaging of neuronal activities by calcium reporters

Being able to detect, measure, and quantify neuronal activities is critical to the understanding of how neural networks operate. Until recently, electrophysiology has been the dominating approach to measure neuronal activities. Luigi Galvani was the first to show that electric activity underlies muscle contraction and nerve conduction using an electrode inserted to a frog leg (*Galvani, 1791*). His work became the bedrock for electrophysiology (*Piccolino, 1998*). To date, electrophysiology has become a sophisticated and precise means to measure and manipulate cellular excitabilities, and study properties of ion channels at up to single unit level (*Hodgkin and Huxley, 1945; Hodgkin and Huxley, 1939; Hodgkin and Huxley, 1952a, b, c; Hodgkin and Huxley, 1952d; Hodgkin and Huxley, 1952e; Hodgkin et al., 1952; Neher and Sakmann, 1976; Neher et al., 1978*). Electrophysiology protocols have been developed to measure or manipulate electrical properties of excitable cells either intracellularly or extracellularly using electrodes. The scale of activity measurement and manipulation ranges from single ion channels to the whole tissue. Main intracellular electrophysiology recordings include voltage clamp, current clamp, patch clamp, and dynamic clamp (*Cole, 1949; Fenwick et al., 1982; Marmont, 1949; Neher and Sakmann, 1976; Sharp et al., 1993*). With the advent of electrophysiology, fundamental neuroscience principles were discovered, such as the Hodgkin-Huxley model for action potentials (*Hodgkin and Huxley, 1939, 1952e*).

However, the application and implication of electrophysiology studies is not without limitations. Low-throughput, invasiveness, and generally incompatibility with genetic manipulations are among the key limitations. Alternative and complementary approaches have been sought after.
One such approach is to assess intracellular calcium dynamics as an approximation of changes in membrane potential for excitable cells (Grienberger and Konnerth, 2012; Looger and Griesbeck, 2012). The development and optimization of calcium indicators has gradually enable high-throughput, less-invasive, and high-specificity recording of neuronal activities in intact neural circuits.

Chemical calcium indicators, such as quin-2, fura-2 and indo-1 are small molecules consisting of calcium chelators EGTA or BAPTA and fluorescent chromophores (Gryniewicz et al., 1985; Pozzan et al., 1982; Tsien et al., 1982). They alter the fluorescent signal intensity upon chelating calcium ions and therefore can reflect calcium dynamics (Paredes et al., 2008). Although popular in cell culture studies, they are not easy to load and applied to studies for intact neural circuits and behaving animals.

Osamu Shimomura and colleagues discovered Aequorin in the jellyfish Aequorea victoria. This bioluminescent protein emits photons upon binding of calcium and oxidization of its chromophore coelenterazine (Ohmiya and Hirano, 1996; Shimomura et al., 1962). Although aequorin was soon replaced by other genetic calcium sensors due to its slow kinetics, low stability, low quantum yield, and the requirement for coelenterazine for photon emission (Brini, 2008; Shimomura, 1997; Shimomura et al., 1993), it was the first genetically encoded calcium indicator (GECI) that was expressed in specific cells to measure calcium dynamics (Inouye et al., 1985; Rizzuto et al., 1992)

In A. victoria, Shimomura and colleagues found another protein, the green fluorescent protein (GFP) that gives greenish fluorescence in solution upon UV illumination (Shimomura et al., 1962). Follow up studies showed that GFP emits green light upon blue light illumination with a covalently attached chromophore 4-(p-hydroxybenzylidene)imidazolidin-5-one (Johnson et al., 1962; Morise et al., 1974; Ormo et al., 1996; Shimomura, 1979; Yang et al., 1996). This chromophore is spontaneously formed post-translationally by cyclization and oxidization of the tripeptide Ser65-Tyr66-Gly67 sequence in the GFP protein (Heim et al., 1994). GFP is a single-component system that emits fluorescence without any co-factors. After the cloning of GFP sequence (Prasher et al., 1992), Martin Chalfie and Frederick Tsuji’s groups demonstrated that GFP is a safe fluorescent marker that can be exogenously expressed in many heterologous
cellular systems (Chalfie et al., 1994; Inouye and Tsuji, 1994). Roger Tsien’s groups dramatically improved the performance of GFP by targeted engineering of GFP’s chromophore. Specifically, the S65T mutation increases GFP’s emission amplitude to about fourfold, and the emission spectrum to that of the commonly used FITC filter sets (Heim et al., 1995). The F64L variant yields enhanced GFP signals (EGFP) that can be stably expressed in mammalian cells (Cormack et al., 1996). GFP paved the way to the advent of a new generation of genetically encoded calcium indicators (GECIs). By combining GFP and its variants with the calcium-binding domain of calmodulin, Roger Tsien and colleagues demonstrated the possibility to measure calcium dynamics through a single component genetically expressed reporter system (Chen et al., 2013; Heim and Griesbeck, 2004; Miyawaki et al., 1997; Nakai et al., 2001; Persechini et al., 1997; Tian et al., 2009).

They led the development of two types of GECIs. The first type is the Förster resonance energy transfer (FRET)-based GECIs, such as cameleon, D3cpV, and TN-XL (Heim and Griesbeck, 2004; Miyawaki et al., 1997; Nagai et al., 2004; Palmer et al., 2006). FRET in general involves the excitation of one fluorophore of a shorter wavelength, but detect the emission of the second fluorophore with a longer wavelength (Piston and Kremers, 2007). In FRET-based GECIs, these two fluorophores are connected by a calcium binding motif. Calcium binding brings the two fluorophores in close proximity, allowing the emission of one fluorophore to excite the second fluorophore. An increase of the emission from the second fluorophore can be used as an approximation of calcium level (Lin and Schnitzer, 2016). The second type is the single-fluorophore GECI, such as GCaMPs particularly GCaMP6 with circularly permuted GFP + calmodulin + calmodulin-interacting M13 peptide, which show significantly increased fluorescent signals upon binding of calcium ions by conformational changes (Chen et al., 2013; Nakai et al., 2001; Tallini et al., 2006; Tian et al., 2009).

Enabling system wide, in vivo recording of calcium dynamics of excitable cells in intact circuits, awake and behaving animals, these GECIs open the door to quantitative measurement of the activity and its dynamics of neural circuits in physiological states (Tian et al., 2009).
1.3.2 Establishing causal relationship in neuroscience by optogenetics

Calcium indicators report neuronally activity pattern that can be correlated with behavioral presentation. But correlation does not establish causality. The ability to manipulate neuronal activities is necessary to establish causality in neuroscience studies. Electrophysiology can serve this purpose, but its limitation in versatility and invasiveness prompted the search for alternative tools that allow reversible, less-invasive manipulation of neuronal activity in intact neural circuits and behaving animals.

1.3.2.1 The advent of optogenetics

The idea of using light to manipulate neuronal activities was probably first proposed by Francis Crick (Crick, 1999). Femtosecond laser was first shown to be able to stimulate neurons without inducing genetic modifications (Hirase et al., 2002). It is important, however, to develop a system that specifically neuronal groups can be genetically targeted to become responsive to light, so that researchers can control and examine the function of neural populations in a less-invasive and targeted manner.

The relative simplicity of invertebrate phototransduction system and divergence from that of the vertebrates prompted early promise of genetically targeted light control. Gero Miesenböck’s group pioneered the field of optogenetics. Expressing a complete set of ten proteins involved in Drosophila phototransduction, NinaE (rhodopsin), NinaA, arrestin-2, Ga, Gβγ, NorpA, TRP, TRPL, and InaD, in the Xenopus oocyte, they demonstrated that white light illumination led to membrane depolarize (Zemelman et al., 2002). Three proteins, NinaE (rhodopsin), arrestin-2, and Ga form the minimal unit, which they termed chARGe, to evoke depolarization in Xenopus oocyte and elicit action potentials in rat hippocampal neurons (Zemelman et al., 2002). This system demonstrates for the first time that it is possible to control activities in genetically targeted cell populations. However, because chARGe is a GPCR-based metabotropic signaling system that activate innate cation channels through G protein-coupled signaling cascades, significant and variable latencies were found in both light-on or light-off periods (Zemelman et al., 2002).
The group developed ionotropic systems to reduce the latency. With a ligand-gated ion channel supplied with caged agonists activatable by photolysis (Zemelman et al., 2003), such as a capsaicin-gated cation channel TRPV1 with caged capsaicin, or the ATP-gated cation channel P2X$_2$ with caged ATP, they exhibited the ability to trigger action potentials upon near-UV light illumination when expressed in rat hippocampal neurons. Despite tighter coupling between light stimulus and neuronal responses, however, latency was still in the order of seconds. Requirement of synthesized caged agonists and short-wavelength illumination posed additional limitation for these systems.

As the case of GFP, the field searched for a single-protein strategy that would significantly simplify the applicability of light control. Bacteriorhodopsin, a single protein light-gated outward proton pump, was discovered on the purple membrane of archaea Halobacterium halobium (Oesterhelt and Stoeckenius, 1971). Halorhodopsin, the second light-gated ion pump identified from H. halobium (erroneously thought to be a sodium pump initially), was shown to drive inward chloride flow upon light stimulation (Bamberg et al., 1993; Lanyi and Weber, 1980; Lindley and MacDonald, 1979; Matsuno-Yagi and Mukohata, 1977; Schobert and Lanyi, 1982). All-trans-retinal is the cofactor for bacteriorhodopsin and halorhodopsin to respond to photons (Aton et al., 1977; Braiman and Mathies, 1980; Lanyi, 1984; Ogurusu et al., 1981).

The existence of photocurrents and light-gated ion transporters in phototactic green algae was first demonstrated by works from Peter Hegemann, Oleg Sineshchekov, Vitaly Sineshchekov, and others. Inward Ca$^{2+}$ and H$^+$ photocurrents were detected and shown to be mediated by archaeal rhodopsins in the eyespot of Chlamydomonas reinhardtii, Haematococcus pluvialis, and Volvox carteri (Braun and Hegemann, 1999; Ehlenbeck et al., 2002; Harz and Hegemann, 1991; Holland et al., 1996; Litvin et al., 1978). These photocurrent were induced within 30-50µs of light flash in these green algae, indicating that the photoreceptor and the ion channel are either tightly linked or essentially the same protein (Braun and Hegemann, 1999; Holland et al., 1996; Sineshchekov et al., 1990). Soon in eukaryotic microorganism, opsin NOP-1, which is homologous to archaeal opsin, was identified in fungi Neurospora crassa (Bieszke et al., 1999). With these findings, it seems possible to directly utilize light-gated ion transporters to evoke membrane potential changes in genetically designated mammalian neurons.
Georg Nagel, Ernst Bamberg, Peter Hegemann and colleagues isolated the opsin from *C. reinhardtii* and named it Channelopsin-1 (Chop1), which shares homology with bacterioopsin in the hydrophobic retinal-binding region ([Nagel et al., 2002](#)). They made the breakthrough by showing that when heterologously expressed in *Xenopus* oocytes, Chop1 plus all-trans-retinal (collectively known as the Channelrhodopsin-1, or ChR1) induced passive proton-driven photocurrents upon green light illumination. Oleg Sineshchekov, John Spudich, as well as Tetsuo Takahashi and others identified the second rhodopsin in *C. reinhardtii* with a different kinetics and action spectra ([Sineshchekov et al., 2002](#); [Suzuki et al., 2003b](#)). This protein, later termed Channelrhodopsin-2 (ChR2), drove inward cation currents upon activation by low intensity blue light. ChR2 was found to be capable of depolarizing both *Xenopus* oocytes and HEK293 cells ([Nagel et al., 2003](#)). Both ChR1 and ChR2-induced depolarization with a much shorter latency than that of the chARGe or ligand-gated ion channels.

Can ChR1 or ChR2 drive neuronal excitabilities with high temporal precision? Because Na\(^+\) influx initiates action potentials in most excitable cells, the cation channel ChR2 rather than the proton channel ChR1 became the logical candidate. Indeed, Edward Boyden, Karl Deisseroth and colleagues demonstrated that, by expressing ChR2 in cultured rat hippocampal neurons, blue light illumination was able to trigger action potentials, even synaptic transmissions with millisecond precision ([Boyden et al., 2005](#)). In this system, the depolarization of membrane potential reached its peak only 2.3±1.1ms after the onset of illumination under voltage clamp. Under current clamp, the latency to the first spike peak was only 8.0±1.9ms. Due to the fast kinetics of ChR2, spike trains recapitulated the frequency of light stimuli even at 30Hz. The parallel study by Stefan Herlitze and Peter Hegemann reached similar conclusion about ChR2 ([Li et al., 2005](#)). The coupling between light stimuli and membrane potentials was extremely tight compared with all existing approaches. The landmark demonstration of a single-component, temporally precise light control method, later termed optogenetics, was widely adopted and eventually revolutionized the field of neuroscience.

These studies, as well as works by Zhuo-Hua Pan, Georg Nagel, Alexander Gottschalk and others, demonstrated for the first time that light activation of ChR2 could elicit behavioral responses in intact animals ([Arenkiel et al., 2007](#); [Bi et al., 2006](#); [Li et al., 2005](#); [Nagel et al., 2005](#); [Wang et al., 2007](#)). These advances opened door for manipulating neuronal activities with
light control (Boyden, 2011; Deisseroth, 2011; Kim et al., 2017; Miesenbock, 2009, 2011; Tye and Deisseroth, 2012; Yizhar et al., 2011a).

Conveniently, ChR2 can function in vertebrate neurons without supplement of the cofactor all-trans-retinal because, derived from vitamin A, it is abundantly present in vertebrate neurons (Zhang et al., 2006). In invertebrates such as *C. elegans*, a dietary supplement of all-trans-retinal is needed for ChR2 to function (Nagel et al., 2005).

1.3.2.2 An explosion of next generation optogenetic tools

Various modified cation channels with different activation spectra from ChR2, such as the red-shifted channels, e.g. VChR1, C1V1, Chrimson, Chronos, ChRger1-3, have since been engineered to facilitate less invasive and independent control of neural populations by different light wavelengths (Bedbrook et al., 2019; Klapoetke et al., 2014; Yizhar et al., 2011b; Zhang et al., 2008a). Opsins with different properties were developed to facility different applications. For example, an opsin with faster dynamics (ChETA) was engineered to accommodate higher frequency stimulations (Gunaydin et al., 2010). Opsin with step-function opsin, which stay open long after the light stimulation, was constructed to achieve sustained stimulations (Berndt et al., 2009). A soma-localized opsin soCoChR enabled single-cell resolution stimulations (Shemesh et al., 2017).

Reagents that allow light-induced inhibition of neurons have also been developed. Herlitze and colleagues introduced a way of optogenetic inhibition of neurons in the same paper in which they tested ChR2 in neurons (Li et al., 2005). They expressed vertebrate rat rhodopsin 4, which couples G protein activation with the opening of an inward rectifying potassium channel to hyperpolarizes neurons. Later, more widely used opsins were discovered or engineered. One class is the blue light-activated inward anion pumps or channels, e.g. halorhodopsin (NpHR), iC1C2, ChloC, and ACR (Berndt et al., 2014; Govorunova et al., 2015; Han and Boyden, 2007; Wietek et al., 2014; Zhang et al., 2007). Another class is blue light-driven outward proton pump, including Mac, archaerhodopsin (Arch), AchT, and eBR (Chow et al., 2010; Gradinaru et al., 2010; Han et al., 2011; Waschuk et al., 2005). Red-shifted inhibitory opsins, such as eNpHR3.0 and Jaws were developed (Chuong et al., 2014; Gradinaru et al., 2010), intent to be used
combinatorically with blue light-activated cation opsins. The light-driven outward sodium pump KR2 from the flavobacteria *Krokinobacter eikastus* is the first of its kind, which hyperpolarizes membrane potentials without altering the pH or chloride concentration (Inoue et al., 2013; Kato et al., 2015a). Topological inversion of opsins reverses the direction of ion fluxes and could potentially double the types of opsins (Brown et al., 2018).

The tool development for both activation and inhibition of neurons, by various wavelengths of light and with differential kinetics, empowered scientists to investigate neural circuits across species with light (Adamantidis et al., 2015; Fenno et al., 2011; Josselyn, 2018).

1.3.3 Combining optogenetic manipulation and calcium imaging

With the advent of tools for calcium imaging and optogenetics, optical monitoring and manipulating neural circuits at a systemic level is within reach. It would be ideal to combine optogenetic manipulation with calcium imaging: to simultaneous manipulate and monitor neuronal activities *in vivo* allows mapping functional neural circuits at a systemic level (Alivisatos et al., 2013). However, the overlapping excitation spectra of opsins and GECIs remains a major challenge because illumination of the GECI reporters was sufficient to activate opsins (Guo et al., 2009).

Considerable work has been invested into solving this issue. This can be categorized into two generally approaches: first, to engineer opsins or GECIs so they have non-overlapping excitation and emission spectra (Akerboom et al., 2013; Kim et al., 2016; Packer et al., 2015; Rajasethupathy et al., 2015; Rickgauer et al., 2014; Zhao et al., 2011); second, to design optical devices that independently illuminate opsins and GECIs in the same sample (Bovetti et al., 2017; Guo et al., 2009; Packer et al., 2015; Rickgauer et al., 2014; Shipley et al., 2014; Szabo et al., 2014).

Both methods have limitations. ‘Red’-shifted opsins are not as specific in the excitation spectra as initially reported. Reducing background illumination intensity for GCaMP reduces the response of red-shifted opsins, but at the cost of much reduced signal-to-noise ratio of calcium signals. Red-shifted GECIs not only have much slower signal dynamics and lower signal-to-
noise ratio when compared to GCaMP, they were even reported to exhibit fluorescence change independent of calcium changes (Kim et al., 2017).

The second method requires complicated light-delivery setups to precise targeting of cells of interest, and can only manipulate neural circuits where the functionally connected cells do not spatially overlap (Bovetti et al., 2017; Guo et al., 2009; Packer et al., 2015; Rickgauer et al., 2014). For moving animals, tracking algorithms are needed, adding another layer of complexity that prevents its wide application (Shipley et al., 2014; Szabo et al., 2014).

The history of GFP, optogenetics, and genome-editing all showed that a simple but robust approach is necessary for general application (Deisseroth, 2015; Doudna and Charpentier, 2014; Tsien, 1998). It is imperative to find an alternative all-optical interrogation method that is accessible to most labs and applicable to widest circumstances. I developed a simple method to achieve all-optical interrogation with bi-directional manipulations (see Chapter 2).

### 1.4 Extrasynaptic transmission

Synaptic transmission, where chemical and electrical communications between neurons take place at the site of pre- and post-synaptic contact, are the focus of classic neurobiology (Bajjalieh and Scheller, 1995; Lin and Scheller, 2000; Rizo and Sudhof, 2002; Sudhof, 2004, 2012; Sudhof and Malenka, 2008). However, fast neurotransmitters such as acetylcholine, glutamate, glycine, and GABA, have all also been reported to exert their functions without classic chemical synaptic structures, named extra-synaptic transmission (De-Miguel and Fuxe, 2012; Fuxe et al., 2012; Vizi et al., 2010; Zoli et al., 1998).

#### 1.4.1 The discovery of extrasynaptic transmission for fast neurotransmitters

The presence of receptors for acetylcholine outside of synaptic sites was first implied at the rat diaphragm neuromuscular junctions, where membrane depolarizations were detected as far as 520±28μm away from the site of iontophoretic application of acetylcholine (Miledi, 1960). By similar methods, extra-synaptic receptors for all tested neurotransmitters were suggested to be present in the soma of leech neurons (Sargent et al., 1977). In mammalian neurons,
immunostaining often revealed mismatches in the localization between receptors and corresponding nerve terminals (acetylcholine and GABA) (Agnati et al., 1986; Herkenham, 1987). For instance, most ionotropic GABA_A receptor subunits reside not only in the synaptic region, but also the extra-synaptic regions (Brunig et al., 2002; Craig et al., 1994; Fritschy et al., 1998; Nusser et al., 1995; Nusser et al., 1998; Somogyi et al., 1996). The α1β2γ2 GABA_A receptor showed expression outside of the synaptic regions by post-embedding immunogold method and electron microscopy (Nusser et al., 1995). For metabotropic GABA_B receptors, their localization was also reported to be distant from synapses (Kulik et al., 2006; Kulik et al., 2003; Lacey et al., 2005; Lopez-Bendito et al., 2004).

More direct evidence that extra-synaptic transmissions may occur under physiological conditions emerged with electrophysiological studies. When acetylcholinesterase was blocked, acetylcholine was found to act on distant receptors in the frog and snake neuromuscular junctions were recorded by voltage clamp recording (Hartzell et al., 1975; Magleby and Terrar, 1975). Glycine acting on adjacent synapses was also recorded in Mauthner cells of goldfish (Faber and Korn, 1988). Glutamate extra-synaptic transmission was similarly recorded in calyceal synapses of chicken embryos (Trussell et al., 1993).

By dual electrophysiological recordings, extrasynaptic activation of GABA was found in the guinea pig hippocampal CA1, the rat neocortex, cerebellar mossy fiber glomerulus, and hippocampal CA3 (Isaacson et al., 1993; Mitchell and Silver, 2000a; Rossi and Hamann, 1998; Scanziani, 2000; Vogt and Nicoll, 1999). In these experiments, stimulation of GABAergic neurons resulted in inhibition of neighboring neurons that were not synaptically connected and this effect was dependent on GABA. In the rat cortex, neurogliaform cells, a type of GABAergic inhibitory interneurons, could even evoke inhibitory postsynaptic potentials in neurons 2.7±1.6µm away by unitary extra-synaptic transmission of GABA (Olah et al., 2009). Both ionotropic GABA_A receptor and metabotropic G-protein-coupled GABA_B receptor could be activated by the spillover of GABA (Isaacson et al., 1993; Mitchell and Silver, 2000a; Olah et al., 2009; Rossi and Hamann, 1998; Scanziani, 2000).

Glutamate spillover in mammals was reported in guinea pig hippocampal CA1 and dentate gyrus, rat olfactory bulb, rat hippocampal CA1, rat cerebellar mossy fiber glomerulus (Arnth-Jensen et al., 2002; Asztely et al., 1997; DiGregorio et al., 2002; Isaacson, 1999; Kullmann et al.,
Similar to the case of GABA, ionotropic NMDA and AMPA receptors, as well as metabotropic G-protein-coupled mGluRs could be activated by spillover glutamate (Arnth-Jensen et al., 2002; Asztely et al., 1997; DiGregorio et al., 2002; Isaacson, 1999; Kullmann et al., 1996; Lozovaya et al., 1999; Min et al., 1998; Mitchell and Silver, 2000b; Scanziani et al., 1997; Semyanov and Kullmann, 2000; Szapiro and Barbour, 2007).

Lastly, in the rat hemidiaphragms, acetylcholine was found to diffuse from neuromuscular junctions (Petrov et al., 2014). Inhibition of acetylcholinesterase and butyrylcholinesterase led to enhanced diffusion of acetylcholine from neuromuscular junctions, and activation of adjacent Schwann cells via nicotinic acetylcholine receptors.

These studies catapulted extra-synaptic transmission of fast neurotransmitters as a more general phenomenon, and inspired mathematical modellings for diffusion of fast neurotransmitters (Barbour and Hausser, 1997; Fuxe and Borroto-Escuela, 2016; Huang, 1998; Isaacson, 2000; Rusakov and Kullmann, 1998; Taber and Hurley, 2014).

1.4.2 Functional imaging of neurotransmission

The most direct demonstration of extra-synaptic transmission would be to visualize the extrasynaptic transmission under physiological conditions in intact behaving animals. Significant attempt has been made towards this goal (Wang et al., 2018).

YbeJ, a periplasmic binding protein from *Escherichia coli* that can bind glutamate and undergo significant conformational changes, was the first reported *in vitro* glutamate sensor when conjugated with a small molecule fluorophore acrylodan (de Lorimier et al., 2002). Based on the YbeJ protein sequence, the genetically encoded FRET-based fluorescent indicators for glutamate, FLIPE, GluSnFR, and SuperGluSnFR, were developed (Hires et al., 2008; Okamoto et al., 2005; Tsien, 2005). These indicators are not bright enough for *in vivo* measurements unfortunately. A fluorescent dye labeled protein sensor EOS was shown to detect glutamate *in vivo*, but the requirement of dye conjugation limits its live application (Namiki et al., 2007; Okubo et al., 2010). The single-protein based fluorescent indicator iGluSnFR constructed by
combining YbeJ and circularly permutated GFP significantly increased the signal-to-noise ratio when compared to the FRET-based ones (Marvin et al., 2013). Several variants of iGluSnFR with enhanced performance were subsequently engineered (Helassa et al., 2018; Marvin et al., 2018).

FRET-base fluorescent indicators CNiFERS were designed to visualize acetylcholine by detecting downstream calcium changes (Markovic et al., 2012; Nguyen et al., 2010; Ziegler et al., 2011). A fluorescent indicator GAcH, consisted of a G-protein-coupled acetylcholine receptor and circular permutated GFP, was reported to outperform all previous indicators (Jing et al., 2019; Jing et al., 2018). Another acetylcholine indicator iAChSnFR was also developed and being tested in multiple species (Borden et al., unpublished).

The first fluorescent indicator for GABA, the GABA-Snifit is also a FRET-based sensor (Masharina et al., 2012). However, it is a multi-component system using protein and synthetic fluorophores, and has relatively low GABA affinity. Using the similar strategy as for iGluSnFR, iGABASnFR was created to monitor GABA levels (Marvin et al., 2019).

It is important to point out that most, if not all developed neurotransmitter indicators remain to be examined for their ability to detect synaptic and extra-synaptic neurotransmission in vivo. In combination with calcium imaging, a simultaneous observation of neuronal activity and neurotransmitter dynamics will be ideal to investigate whether and how extra-synaptic transmission take place under physiological conditions. Further, simultaneous manipulation of neuronal activity using optogenetic tools and monitoring of neurotransmitter would allow us to establish causative relationship between neuronal activities and extra-synaptic transmission.
2 Results: Hidden functional connectivities that underlie the symmetric locomotor behavior of the juvenile L1 *C. elegans*

2.1 Abstract

From birth to adulthood, the nematode *C. elegans* generates forward or backward movement by propagating symmetric dorsal-ventral bending waves along its body. In the adult, symmetric connectivity to dorsal and ventral muscles is formed by different sets of cholinergic and GABAergic motor neurons that drive muscle contraction and relaxation, respectively. The symmetry of these motor neurons paired with each side of the nematode body, as well as the symmetry in their synaptic connectivity, lead to balanced dorsal and ventral bending waves. However, the original electron microscopy reconstruction of part of a juvenile *C. elegans* (L1 larva) revealed an asymmetry: its dorsal muscles were exclusively innervated by cholinergic motor neurons and its ventral muscles were exclusively innervated by GABAergic motor neurons. Here, I identify the functional and connectomic mechanisms that allow symmetric locomotor output before the development of the mature symmetric wiring diagram. We use optogenetics, functional imaging, and connectomics to map synaptic excitation and inhibition throughout the juvenile motor circuit. I find that extrasynaptic and proprioceptive mechanisms, in conjunction with the asymmetric connectivity of excitatory cholinergic and inhibitory GABAergic motor neurons, coordinate the symmetric muscle activities that drive locomotion.

2.2 Introduction

2.2.1 The conundrum of asymmetric motor circuit and symmetric behavior in the L1 larvae

Locomotion of aquatic and terrestrial animals is generated by motor circuits. Some vertebrates are capable of preserving patterned motor output while the body and motor circuit undergoing development (Catela et al., 2015). Similarly, the invertebrate nematode *Caenorhabditis elegans* generates stereotypical sinusoidal movement on a solid surface throughout its four stages of larval development and adulthood.
The dorsal-ventral symmetry in the motor pattern is well supported by the anatomic structure of the adult motor circuit. Two sub-groups of cholinergic A type motor neurons, 9 DAs and 12 VAs, execute backward movement by stimulating the dorsal and ventral muscle contractions, respectively. Two sub-groups of cholinergic B type motor neurons, 7 DBs and 11 VBs, innervate dorsal and ventral muscles, respectively, during forward movement. The GABAergic D type motor neurons are similarly divided into two subgroups (6 DDs and 13VDs) that receive inputs from the A and B type motor neurons, and mediate contra-lateral muscle inhibition (Kawano et al., 2011; Petersen et al., 2011; Sulston and Horvitz, 1977; Von Stetina et al., 2006; Walthall et al., 1993; Wen et al., 2012; White et al., 1986). These plus 11 AS motor neurons that innervate dorsal body wall muscles, and the 6 VC motor neurons that are involved in egg-laying, constitute the motor circuit (Bany et al., 2003; Tolstenkov et al., 2018; White et al., 1976).

Yet, VA, VB, VD, AS, and VC motor neurons emerge after the L1 stage, leaving L1 larva with only a subset of motor circuit – DAs, DBs, and DDs (White et al., 1978). This composition, which includes only the dorsal muscle innervating motor neurons in the adult motor circuit, predicts that functional rearrangement must occur in the motor circuit during development. Indeed, the partial EM reconstruction of the L1s suggest that DDs make NMJs to the ventral instead of dorsal muscles in L1, and remodel to innervate dorsal muscles in later larval stages, as the VD motor neurons take place of ventral muscle innervation (Kurup and Jin, 2016; White et al., 1978). Still, the L1 motor circuit remains to be anatomically asymmetric – with only GABAergic inputs to ventral muscles, and cholinergic inputs to dorsal muscles, respectively. Since the L1 larvae perform sinusoidal movement, it raises a question on how an asymmetrically wiring motor circuit manages to generate symmetric activity output, specifically, the underlying of ventral body bend.

### 2.2.2 Potential mechanisms that underlie the dorsal-ventral symmetry of body wall muscle output in the L1 larvae

Potential mechanisms that have been previously proposed (Gjorgjieva et al., 2014), and implicated by the full EM reconstruction (Witvliet et al., in preparation) for ventral body bend in the L1 larvae are listed below:

1. The ventral structure resembles a spring that bends without additional input.
2. The ventral body wall muscle is intrinsically and constitutively active.
3. Ventral body wall muscle is proprioceptive and innervated by periodic dorsal bends.
4. GABA from the D-type motor neurons is excitatory to ventral body wall muscles.
5. D motor neurons release excitatory neurotransmitters to innervate ventral muscles.
6. SIA and SIB motor neurons stimulate ventral body wall muscles.
7. HMC innervates ventral muscles via gap junctions with the dorsal muscles.
8. A- and B-type motor neurons stimulate ventral muscle cells by ephaptic coupling.

I used behavioral assays, genetic mutants, cell ablation, calcium imaging, optogenetics, and all-optical interrogation to address these possibilities.
## 2.3 Methods

### 2.3.1 Strains used in this work

<table>
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<tr>
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ZM10715  hpEx4236[Pceh-24::loxP::BFP::loxP::GtACR2::wCherry + Punc-17::Cre]; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
ZM10580  hpEx4179[Pacr-2(s):loxP::BFP::loxP::GtACR2::wCherry + Pceh-24::loxP::BFP::loxP::GtACR2::wCherry + Punc-17::Cre]; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
ZM10339  hpIs717[Pacr-2(s):loxP::BFP::loxP::Chrimson::wCherry + Punc-17::Cre]; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
ZM10340  unc-25(e156) III; hpIs717[Pacr-2(s):loxP::BFP::loxP::Chrimson::wCherry + Punc-17::Cre]; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
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ZM9711  unc-54(e190) I; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
CB156  unc-25(e156) III
CB407  unc-49(e407) III
ZM9172  unc-25(e156) III; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
ZM10451  unc-49(e407) III; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
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ZM9313  hpIs625[Ptrr-39::Arch::wCherry]; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
ZM10176  unc-25(e156) III; hpIs593[Ptrr-39::Chrimson::wCherry]; ljIs131[Pmyo-3::GCaMP3::UrSL::RFP]
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ZM10585  hpEx4177[Pmyo-3::GtACR2::wCherry]; hpIs595
ZM10440  unc-49(e407) III; hpIs592[Ptrr-39::Chrimson::wCherry]; hpIs595
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ZM10281  hpIs740[Ptwwk-40(s)::GCaMP6::wScarlett]
2.3.2 Electron microscopy of L1 larvae

N2 *C. elegans* were harvested for L1 larvae, and subjected to high-pressure freezing and staining. The sections were stained, imaged, and tracked according to as described (Mulcahy et al., 2018); Witvliet et al., to be submitted).

2.3.3 Synchronization of L1 larvae

For all L1 *C. elegans* experiments, the stage of the larvae is synchronized by the following protocol: One day before experiment, check plates of worms for experiment and make sure there will be plenty of eggs laid the next day. On the day of experiment, pick 10~50 eggs at 3-fold
stage from a healthy plate onto a new plate with OP50. More eggs will ensure enough L1 larvae for experiment. Carefully remove any larvae that are collaterally transferred by picking. Let the eggs hatch for 1hr at 23℃. Usually there are hatchlings at 1hr; if not, repeat the previous step until there are hatched larvae. Remove unhatched eggs. The remaining hatchlings are L1 larvae with a maximum of 1hr difference in stages.

2.3.4 Crawling assays

2.3.4.1 Crawling behavior assay

Individual larva is picked onto an unseeded plate. Within 2min, the plate is inverted and mounted onto the upright microscope, located under 4x objective through the eyepiece and focused in the live view panel of ImageJ through camera (Kawano et al., 2011). By adjusting the focal plane so that the larva is slightly out of focus, and using proper exposure time for the camera, the larva can be in stark contrast with the background. The most ideal imaging condition can result in dark larva object and white background with no visible trajectory. This image quality facilitates post-imaging image analysis. Then record and track the movement of larva by manually controlling the motorized stage with joystick. The length of recording is typically 68sec at a frame rate of 26Hz.

After one recording is completed, transfer the larva onto a seeded plate or remove the larva if no longer needed. Reuse the unseeded plate or use another unseeded plate for the next recording.

With enough recordings, proceed to the post-imaging image analysis. I developed the MATLAB program that allows analyses of body angle propagations and velocity. The program requires human input to determine the head and tail of the larva on the first frame of the recording. Then it segments the images, and isolates the contour of the larva. Afterwards, it divides the contour into 100 segments along the anterior-posterior axis, for each frame. Because the larva frequently entangles, the head and tail can be flipped multiple times during the analysis. The program provides the function of adjusting head and tail orientations by manual curation. The user can either flip the orientation on one frame, or all frames thereafter. After manual curation is completed, the program outputs the body angle propagation, as well as the velocity profile for the recording.
2.3.4.2 Crawling with optogenetic stimulations

These experiments use animals expressing opsins in certain cells/tissues. For control group, individual larva from regular plate is picked onto an unseeded plate. For experimental group, individual larva from parents incubated on ATR plate for at least two generations is picked onto an unseeded plate. Within 2min, the plate is inverted and mounted onto the upright microscope, located under 4x objective through the eyepiece and focused in the live view panel of ImageJ through camera (Kawano et al., 2011). By adjusting the focal plane so that the larva is slightly out of focus, and using proper exposure time for the camera, the larva can be in stark contrast with the background. The most ideal imaging condition can result in dark larva object and white background with no visible trajectory. This image quality facilitates post-imaging image analysis. Then record and track the movement of larva by manually controlling the motorized stage with joystick. The length of recording is 75sec at a frame rate of 26Hz.

After one recording is completed, transfer the larva onto a seeded plate or remove the larva if no longer needed. Reuse the unseeded plate or use another unseeded plate for the next recording. Plates for control and experimental groups are separated to avoid ATR cross-contamination.

The optogenetic stimulations are provided by a LED light source which is controlled in-house developed plugin in ImageJ via Arduino (Kawano et al., 2011). User can define the lighting sequence at will. The lighting sequence in this series of experiments is: no LED light for 10sec pre-stimulation, 4 rounds of LED light stimulations with each round consisting of 10sec light-on and 5sec light-off epochs, no LED light for 10sec post-stimulation.

With enough recordings for both control and experimental groups, proceed to the post-imaging image analysis. I developed the MATLAB program that allows analyses of body angle propagations and velocity. The program requires human input to determine the head and tail of the larva on the first frame of the recording. Then it segments the images, and isolates the contour of the larva. Given that the image lighting condition changes every time the LED light is on, dynamic thresholding is used to correctly segment the images. Afterwards, it divides the contour into 100 segments along the anterior-posterior axis, for each frame. Because the larva frequently entangles, the head and tail can be flipped multiple times during the analysis. The
program provides the function of adjusting head and tail orientations by manual curation. The user can either flip the orientation on one frame, or all frames thereafter. After manual curation is completed, the program outputs the body angle propagation, as well as the velocity profile for the recording. The program detects the lighting condition change and automatically annotate the light-on and light-off phases. It highlights the light-on phases with green color.

2.3.5 Swimming assay for dorsal and ventral bending preference

The experiments are carried out in a miniature well fabricated and cured on PDMS substrate in Hang Lu’s lab at Georgia Institute of Technology. A droplet of M9 solution is mounted onto the center of the well. The larva is then transferred into the M9 droplet. After mounting the coverslip, the PDMS substrate is then placed onto a glass slide, and located by 4x objective through the eyepiece of the microscope. With proper camera exposure time, the larva can be visualized with contrast to the background under a higher magnification. Then record the thrashing behavior with ImageJ (Kawano et al., 2011). The length of recording is typically 68 sec at a frame rate of 26 Hz.

In most of the cases, the anus and/or gonad is visible in at least certain frames of the recording. These anatomical landmarks help determine the dorsal/ventral orientation of the larva. With enough recordings, human curation of the recording is done by annotating dorsal, neutral, ventral body postures for each frame. The dorsal body posture is defined by a convex shape towards the dorsal side in the region between the neck and tail. The ventral body posture is defined by a convex shape towards the ventral side in the region between the neck and tail. The neural body posture is any shape that does not belong to the previous two groups. After annotations, the total time one larva spends in each posture in one recording is normalized by the total time of the recording.

Because *unc-104*(ut60), *unc-64*(js115), *cha-1*(ok2253) are all L1 lethal, they are all genetically balanced by balancers (generated from laboratories of Erik Jorgensen (unpublished), Michael Nonet, and *C. elegans* Gene Knockout Consortium respectively) (Consortium, 2012; Saifee et al., 1998). For these three mutants, I isolated homozygotes from the heterogeneous L1 larvae population by their corresponding phenotypes (small size, paralyzed body), and then proceeded
to swimming assays. *unc-18*(sks1) and *unc-13*(s69) are both viable, therefore I directly subjected their L1 larvae to the swimming assay.

2.3.6 Photo-ablation of cells

The animals expressing miniSOG protein in certain cells/tissues are used in these experiments. A specialized dark LED chamber is manufactured by Thomas Sun at Lunenfeld-Tanenbaum Research Institute to deliver light and enough oxygen for the experiments. 4 LED lights sit at the top of the chamber. An electric fan is at the back of the chamber. A large glass slide can hold the worm plate at 3 different distances from the LED source. A touchpad controller allows users to determine the time for illumination.

Larvae are pooled onto a fresh seeded plate at 1hr post-hatching. This plate of larvae is then subject to blue LED treatment for 40min-1hr at room temperature, with no lid on the plate. The closest distance is chosen to maximize the effect of ablation. After blue light treatment, the plate is wrapped by tinfoil. Wait for 4hr and then proceed to experiments. Cells ablated are normally vacuoles at this stage, which are indicative of successful ablation.

Collect individual larva after experiment onto individual plate, and label the plate with the corresponding recording number. Wait for 2 days until the larvae reach L4 or adult stage, and examine if the fluorescent markers for cells of interest disappear. If no fluorescent markers remain, then the successful ablation is achieved.

2.3.7 Laser-ablation of cells

Before laser-ablation, adjust the position of laser according to its instructions. The larvae need to be immobilized for laser-ablation. Larvae at 1hr post-hatching are mounted onto a droplet of 10% sodium azide solution on the 2% agarose pad. After the larvae are paralyzed, mount the coverslip. Locate the larvae under 10x objective, and then switch to 63x objective. Turn on the blue light to visualize the GFP markers, and move the cells of interest to the center. Ablate the cells of interest by repetitive laser pulses. Explosion or collapsing of the cell can be seen if the ablation is successful. Repeat this step for all larvae on the agarose pad.
Collect the larvae from the agarose pad, and transfer them onto seeded plates. The effect of sodium azide will diminish. Wait for 4 hr and then proceed to experiments. Cells ablated are normally vacuoles at this stage, which are indicative of successful ablation.

Collect individual larva after experiment onto individual plate, and label the plate with the corresponding recording number. Wait for 2 days until the larvae reach L4 or adult stage, and examine if the fluorescent markers for cells of interest disappear. If no fluorescent markers remain, then the successful ablation is achieved.

**2.3.8 Calcium imaging of neurons**

Larvae expressing genetically-encoded calcium indicators (GECIs) in neurons of interest are used in this experiment. After synchronization, wait for 4-5 hr before imaging. The larvae are mounted onto a droplet of M9 or halocarbon oil on agarose pad, depending on the ideal velocity for imaging different neurons. For M9 solution, it usually gives the optimal velocity when the majority of liquid is absorbed by the agarose pad before placing the coverslip. For halocarbon oil, the coverslip can be placed when the oil droplet is still spreading.

After placing the coverslip, the slide is placed onto a Zeiss Axioskop 2 Plus upright microscope, which uses Zeiss 4x, 10x, 20x, 40x, and 63x objectives. The microscope is customized with a motorized stage controlled by Applied Scientific Instruments MS-2000-WK multi-axes stage controller. A MAG Biosystems DV2 Dual-Channel Simultaneous Imaging System is mounted on top of the microscope. A Hamamatsu ORCA-R2 digital CCD camera is mounted on top of DV2 beamsplitter, and connected Hamamatsu Camera Controller C10600. The camera controller is connected to a Mac desktop. The X-Cite Series 120 is used to illuminate the samples. Detailed description of the system can be found in Kawano *et al.* ([Kawano et al., 2011](#)).

To locate the larvae, observe the sample slide under 4x objective. Switch to 20x, 40x, or 63x objective to image neurons of interest according to the size of neurons. Adjust the focus and camera exposure from the live view panel of the ImageJ. Avoid saturations by checking the maximum pixel brightness. A shorter exposure time will minimize the smear of fluorescent signals during fast movement.
Estimate the velocity of the larva in the live view panel: if the larva is moving too fast, wait until it slows down, or simply find another larva for imaging. A slower movement will dramatically stabilize signal fluctuations on the RFP channel, and minimize the signal artifact induced by movement. An in-house developed ImageJ plugin is used to record the calcium dynamics of neurons (Kawano et al., 2011). The frame rate is typically 10Hz, or 26Hz. The length of the recording is typically 68s or 90s. Track the larvae movement manually by moving the joystick that controls the motorized stage. Upon completion of recording of one larva, proceed to another until enough number of larvae have been imaged.

After data acquisition, proceed to the image analysis. I developed a MATLAB program based on Quan Wen’s script (Wen et al., 2012) that allows fast neuron tracking and signal processing, with the function of manual curation. After curation of neuron tracking, the program produces the dynamics of GECI and velocity of larva. It also pools calcium dynamics in forward and backward locomotion modes separately. If needed, the body angle change can also be analyzed, and compared with the calcium dynamics.

Distinct promoters are employed to drive the expression of GCaMP6 (Chen et al., 2013) in specific type of motor neurons. Punc-4 for DAs (Lickteig et al., 2001; Miller and Niemeyer, 1995), Pacr-2(s) for DBs (Jospin et al., 2009), Punc-25 for DDs (Jin et al., 1999), Pceh-24 for SIAs and SIBs (Altun-Gultekin et al., 2001; Schwarz and Bringmann, 2017), Ptwk-40 for premotor interneurons AVA, AVE, and AVB (Hung et al., in preparation). The fluorescence intensities of a worm codon-optimized mCherry (wCherry) (Shaner et al., 2008) is fused with GCaMP6s and used as a correction for motion artifact.

Seven DA motor neurons (DA1~7) cell bodies are spaced apart along the ventral nerve cord, while the two most posterior members (DA8~9) are close to each other, reside in the tail, and visualized as a single object during recordings. With 20x or 40x objectives, the majority of the DA motor neurons can be captured in the field of view. Seven DB motor neurons (DB1~7) soma are distributed along the ventral nerve cord. Because Pacr-2(s) is expressed in DBs and relatively weakly in DAs, and DB1~3 are relatively clustered, I imaged activities of DB4~7 which are well separated. Six DD motor neurons (DD1~6) soma are located along the ventral nerve cord. Their anterior puncta are where the presynaptic sites and visible at 63x objective. Therefore, I imaged calcium activities of the puncta for DD4~5. Four SIAs and four SIBs soma are clustered in the
ventral ganglia region in the head. It is difficult to separate individual cell bodies of these sublateral neurons. Their activity patterns are similar most of the time. As such, I used averaged activities of anterior neurons and posterior neurons in the field of view for these sublateral neurons.

2.3.9 Calcium imaging of muscles

Larvae expressing GCaMP3 or GCaMP6 in body wall muscles are used in this experiment. After synchronization, wait for 4-5hr before imaging. The larvae are mounted onto a droplet of M9 or halocarbon oil on agarose pad, depending on the ideal velocity for imaging. For M9 solution, it usually gives the optimal velocity when the majority of liquid is absorbed by the agarose pad before placing the coverslip. For halocarbon oil, the coverslip can be placed when the oil droplet is still spreading.

The same imaging system described above is used for recording muscles. After placing the coverslip, the slide is observed under 4x objective to locate the larvae. Switch to 20x objective to image muscles. Adjust the focus and camera exposure from the live view panel of the ImageJ. Avoid saturations by checking the maximum pixel brightness. A shorter exposure time will minimize the smear of fluorescent signals during fast movement.

Estimate the velocity of the larva in the live view panel: if the larva is moving too fast, wait until it slows down, or simply find another larva for imaging. A slower movement will dramatically stabilize signal fluctuations on the RFP channel, and minimize the signal artifact induced by movement. An in-house developed ImageJ plugin is used to record the calcium dynamics of neurons. The frame rate is typically 10Hz, or 26Hz. The length of the recording is typically 68s or 90s. Track the larvae movement manually by moving the joystick that controls the motorized stage. Upon completion of recording of one larva, proceed to another until enough number of larvae have been imaged.

After data acquisition, proceed to the image analysis. I developed a MATLAB program that allows fast muscle segmentation and signal processing. The program requires human input to determine the head and tail of the larva on the first frame of the recording. Then it segments the images, and isolates the contour of the muscles. Afterwards, it divides the contour into 100
segments along the anterior-posterior axis, for each frame. It also calculates the skeleton of the muscles, and thus produces the middle line. The middle line further divides the segments into a total of 200 segments, with 100 segments on dorsal and ventral sides respectively. The average value for pixels within each segment is considered as the muscle activity for this segment. The body wall muscle activity propagation can therefore be analyzed. The angles formed by intersecting vectors are also calculated. A comparison between muscle activity and body posture propagation pattern becomes available.

A muscle specific *myo-3* promoter (Miller et al., 1983) is used to drive the expression of a calcium indicator, GCaMP3. Fluorescent signal change in tagRFP (Shaner et al., 2008), co-transcribed but separately translated with GCaMP3 via a SL2 slicing leader (Huang and Hirsh, 1989) was used as a correction for motion artifact.

2.3.10 All-optical interrogation

To carry out all-optical interrogation, a strain expressing opsin in upstream cells and GECI in downstream cells is required. To generate such as strain, first construct strain A expressing opsins in potential upstream cells, with intersectional Cre-loxP system if necessary; second, construct strain B expressing GECIs in potential downstream cells; last, genetically cross the strain A with strain B to obtain the desired strain.

On day of experiment, synchronize L1 larvae of the crossed strain. For ATR experimental group, particularly the worms with Cre-loxP, it is ideal to use larvae from worms grown on 2x or 4x concentration of ATR plate for multiple generations to maximize the strength of optogenetic stimulations. Mount L1 larvae onto slides with little M9 solution in order to immobilize the larvae. Mount the slide onto the microscope, and turn on the same imaging system as described above. Wait for about 1 min. Without turning on the LED light source, start recording at 10 Hz or 26 Hz. Manually turn on and off the LED light during recording. The on phase is usually longer than 3 sec, and off phase is typically around 5-20 sec. The randomness is intentional, as this approach should randomize the initial activity state of both the cells being manipulated and recorded. Once the recording is completed, either repeat the recording on the same larva or
another larva. After imaging, proceed to the data analysis pipeline as described above. Typically a 3 or 6sec-time window is used for analysis of each epoch.

In experiments searching for neurons contributing to muscle activities, I noticed several cases of promoter expression patterns leaking into body wall muscles. This would cause misinterpretation of the function of neurons. To circumvent this problem, I used the intersectional Cre-loxP system as described before. For manipulation of DAs and DBs, I first used Pacr-2(s) to drive opsins. This promoter is reported to be specific to A- and B-type motor neurons (Jospin et al., 2009). However, I found that Pacr-2(s) is also expressed in the body wall muscles at L1 stage. Therefore, I used Pacr-2(s)::loxP::BFP::loxP::GtACR2;Punc-17::Cre to avoid body wall muscle expression. For manipulation of SIAs and SIBs, we first used Pceh-24 to drive opsins. This promoter is reported to be specific to SIAs, SIBs, SMDVs, pharyngeal and vulval muscles (Altun-Gultekin et al., 2001; Schwarz and Bringmann, 2017). However, I found that Pceh-24 is also expressed in the body wall muscles at L1 stage. Therefore, I used Pceh-24::loxP::BFP::loxP::GtACR2;Punc-17::Cre to avoid body wall muscle expression. For inhibition of premotor interneurons, I first used Pnmr-1 to drive GtACR2. This promoter is reported to be specific to premotor interneurons at adult stage (Kawano et al., 2011). However, I found that Pnmr-1 is also expressed in the body wall muscles at L1 stage for non-integrated transgenic lines. As an alternative, I used Prig-3::loxP::BFP::loxP::GtACR2;Ptwk-40::Cre;Psra-11::GtACR2 to silence AVA+AVB (plus AIY due to expression pattern of Psra-11). The combined expression pattern is not expressed in the body wall muscles.
2.4 Results

2.4.1 Complete electron microscopy reconstruction of a complete L1 larva (adapted from Witvliet et al.)

High-pressure freezing protocol achieves significantly improved structural preservation (Dahl and Staehelin, 1989; Moor, 1987; Mulcahy et al., 2018; Riehle, 1968). Using high pressure freezing protocol, automated serial sectioning (Schalek et al., 2012), and annotation program CATMAID (Saalfeld et al., 2009; Schneider-Mizell et al., 2016), Witvliet et al. have completely reconstructed the motor circuit of L1 larvae at 1hr, 14hr and 15hr post-hatching (Fig. 2.1).

These unpublished results confirmed that the anatomic structure of the L1 larvae is asymmetric, where the A- and B-type motor neurons innervate dorsal body wall muscles and the D-type motor neurons, and the D-type motor neurons innervate only ventral body wall muscles (Fig. 2.1A, C-E). These studies also revealed additional cells that make neuromuscular junctions to L1 muscles, including the SIA and SIB motor neurons that innervate dorsal and ventral body wall muscles on the lateral sides (Fig. 2.1A, F-H). Head mesodermal cell (HMC), whose function is relatively unknown, has several gap junctions with dorsal and ventral muscles in the anterior body wall (Fig. 2.1A-B).
Figure 2.1 EM identifies cells that innervate body wall muscles in *C. elegans* L1 larva 
(Credit: Witvliet, Mulcahy, et al.)

A) Cartoon depiction of cells that innervate dorsal and ventral body wall muscles in N2 wild-type *C. elegans* L1 larva based on EM reconstruction of an 15hr post hatching L1 larva. Arrows indicate chemical synapses, and zigzag lines are electrical synapses. Numbers indicate the total sections that contain synaptic structures, which reflect the strength of connectivity.

B) EM micrograph showing electrical synapse between HMC and a muscle cell. Arrow denotes electrical synapse. A DD motor neuron is visible nearby.

C) Dyadic chemical synapse between a DA motor neuron and dorsal muscle cells plus a DD motor neuron. Arrow head indicates active zone of chemical synapse. Synaptic vesicles can be seen in that DA motor neuron.

D) Dyadic chemical synapse between a DB motor neuron and dorsal muscle cells plus a DD motor neuron. Arrow head indicates active zone of chemical synapse. Synaptic vesicles can be seen in that DB motor neuron.

E) Chemical synapse between a DD motor neuron and ventral muscle cells. Arrow head indicates active zone of chemical synapse. Synaptic vesicles can be seen in that DD motor neuron.

F-H) Chemical synapses between SIB and muscle, SIA and SIB, SIA and muscle respectively. Arrow head indicates active zone of chemical synapse. Synaptic vesicles can be seen in the corresponding presynaptic sublateral motor neurons.

2.4.1.1 A-type motor neurons innervate dorsal muscle, project anteriorly, and receive inputs from backward promoting premotor interneurons

There are nine A-type motor neurons in *C. elegans* L1 larval stage; all innervate dorsal muscle cells. These A-type motor neurons maintain this configuration throughout development, and are thus named dorsal A, or DA motor neurons (White et al., 1978; White et al., 1986). Cell bodies of DA motor neurons are all localized along the ventral nerve cord. All DA motor neurons send their commissures towards the dorsal muscle. After reaching the dorsal side, their processes extend anteriorly. They form dyadic synapses that include neuromuscular junctions with dorsal muscle cells and D-type motor neurons (Fig. 2.1A, C, Fig. 2.2A, Fig. 2.3A). Therefore, DA motor neurons should regulate both dorsal muscle cells and D-type motor neurons.
Each DA motor neuron possesses a long process that extends from the cell body on the ventral nerve cord, which allows the DA motor neurons to receive inputs from premotor interneurons AVA, AVD, AVE (Fig. 2.2A, Fig. 2.3A). According to studies in adult *C. elegans*, premotor interneurons AVA, AVD, and AVE, plus A-type motor neurons all direct reversals ([Chalfie et al., 1985; Gray et al., 2005; White et al., 1976, 1986]). With this wiring preserved across developmental stages, it is reasonable to speculate that DA motor neurons are also involved in backward locomotion in L1 larvae.

**2.4.1.2 B-type motor neurons innervate dorsal muscle, project posteriorly, and receive inputs from forward promoting premotor interneurons**

All seven B-type motor neurons in L1 send their axons to the dorsal muscle cells (Fig. 2.1A). These B-type motor neurons preserve this structure to adulthood ([White et al., 1978; White et al., 1986]). They are hence named dorsal B, or DB motor neurons. Cell bodies of DB motor neurons are also ventrally localized. DBs extend their axons to the posterior side after projecting commissures to the dorsal side. DB motor neurons also form dyadic synapses with dorsal muscle cells and D-type motor neurons (Fig. 2.1A, D, Fig. 2.2B).

On the ventral side, DB motor neurons send out processes posteriorly (Fig. 2.2B). These processes receive inputs from premotor interneurons AVB and PVC, as in the adult circuit (Fig. 2.3A). AVB, PVC and DB motor neurons together contribute to forward locomotion ([Chalfie et al., 1985; White et al., 1976, 1986]). Because this circuitry is maintained in L1 larvae, it is likely that they direct forward locomotion in L1 larvae as well.

**2.4.1.3 D-type motor neurons innervate ventral muscle and receive inputs from the A- and B-type motor neurons**

Six D-type motor neurons in L1 larva all innervate ventral muscle cells (Fig. 2.1A). Hence, they must reverse the polarity and remodel into dorsal innervation after the L1 larval stage ([Hallam and Jin, 1998; He et al., 2015; Howell et al., 2015; Kurup and Jin, 2016; Meng et al., 2017; White et al., 1978]). Because of their dorsal innervation layout in adult, these ventral innervating D-type motor neurons in L1 larval are still called dorsal D, or DD motor neurons.
The somas of DD motor neurons reside in the ventral nerve cord. DD motor neurons send out processes to the anterior of their somas. The processes of DD motor neurons resemble the shape of letter “I”. Their ventral processes are axons, and dorsal dendrites (Fig. 2.1A, E, Fig. 2.2C). DD motor neurons are downstream to DA and DB motor neurons (Fig. 2.3A). Consequently, they should be directly innervated by motor neuron.

With this layout, DD motor neurons probably release neurotransmitters to the opposing ventral side upon receiving signals from DA and DB motor neurons on the dorsal side. In adults, DD motor neurons are inhibitory to body wall muscles (McIntire et al., 1993). In L1, DD motor neurons are likely inhibitory (Han et al., 2015). Therefore, DD motor neurons may cross-inhibit the ventral muscle cells during both directional movements in C. elegans L1 larvae.

2.4.1.4 Sub-lateral motor neurons innervate dorsal and ventral muscle, and receive inputs from forward directing premotor interneurons

Sub-lateral motor neurons, including four SIAs and four SIBs also innervate body wall muscles (Fig. 2.1A). Two SIA and SIB motor neurons extend their axons along the dorsal sublateral cord on both left and right sides (SIADL, SIADR, SIBDL, SIBDR). The other two SIA and SIB motor neurons send out their axons along the ventral sublateral cord on both left and right sides (SIAVL, SIAVR, SIBVL, SIBVR). SIA motor neurons elongate all the way to the end of the body wall. SIB motor neurons terminate their axons at around 2/3 of the body length. In addition to NMJs, SIA also synapses onto the SIB motor neurons (Fig. 2.1A, F-H, Fig. 2.2D).

SIA and SIB innervate muscle cells in a distinct pattern from that of the DA, DB, and DD motor neurons. Muscle cells are organized into bundles on both dorsal and ventral sides (Hedgecock et al., 1987). These bundles are divided into four quadrants, with two rows of muscle cells for each quadrant (Sulston and Horvitz, 1977). SIA and SIB motor neurons only innervate the outer rows of muscle cells for each quadrant, whereas DA, DB and DD motor neurons cover both rows along their targeted muscle cell bundles (Fig. 2.2A-D, Fig. 2.4).

Both SIA and SIB motor neurons receive synapses from the PVC premotor interneurons (Fig. 2.3B), a component for the adult forward motor circuit (Chalfie et al., 1985). SIA and SIB
therefore may contribute to both dorsal and ventral body wall muscle contraction during forward locomotion.

2.4.1.5 HMC makes gap junctions to anterior dorsal and ventral muscles

HMC is not a neuron because of contractile fibrils (Sulston et al., 1983; White et al., 1976). Its cell body sits on the dorsal side of the terminal bulb of pharynx. It sends out two branches along the dorsal and ventral cords to the anterior body wall muscles (Fig. 2.1A-B, Fig. 2.2E). Both processes terminate at around 1/4 of the body wall length. Extensive gap junctions are formed between HMC and muscle cells on both sides. This structure suggests that HMC may electrically couple dorsal and ventral muscle cells, at least for the anterior portion of the body wall.
Figure 2.2 Anatomy of cells innervating body wall muscles at L1 stage (Credit: Witvliet, Mulcahy, et al.)

A-C) Anatomy of one DA, DB, and DD motor neuron with presynaptic, postsynaptic, somatic, and commissural regions labeled. Presynaptic regions are shown as red spheres; postsynaptic regions are teal. Soma of the neuron is a bigger sphere. Cholinergic neurons are in warmer color, GABAergic are in cooler color. DA projects its axon to the dorsal-anterior side, DB projects to the dorsal-posterior side. DD extends its axon to the ventral-anterior side. Dorsal on top, anterior to the left.

D) Anatomy of SIA and SIB motor neurons with respect to the muscle cells. SIAs are in orange, SIBs are in green, and muscle cells are in yellow. Both SIAs and SIBs extend further along the anterior-posterior axis. SIAs reach the tail end, whereas SIBs terminate at around 2/3 of the body wall length. Dorsal on top, anterior to the left.

E) Anatomy of HMC with respect to the muscle cells. HMC is in red, and muscle cells are in yellow. HMC only reaches approximately 1/3 of the body wall length. Dorsal on top, anterior to the left.
Figure 2.3 Connectivity of neurons innervating body wall muscles at L1 stage (Credit: Witvliet, Mulcahy, et al.)

A) Connectivity of the DA, DB, and DD motor neurons in L1 larva. Chemical synapses are drawn as black arrows. Gap junctions are drawn as gray zigzag lines. The strength of connectivity is in proportion to the line width. DAs receive input from premotor interneurons AVA, AVD, and AVE that are considered to direct backward locomotion according to studies in the adult. DBs receive input from premotor interneurons AVB and PVC that are considered to direct forward locomotion according to studies in the adult. DDs receive input from DAs and DBs that should enable its function in both directional movements.

B) Connectivity of SIA and SIB motor neurons in L1 larva. The only known premotor interneuron that innervates SIAs and SIBs is PVC, which directs forward movement in adult. Compared to DAs and DBs, the strength of this connection is relatively weak.
Figure 2.4 Detailed wiring diagram of all cells innervating muscles at L1 stage (Credit: Witvliet, Mulcahy, et al.)
Detailed connectivity of cells innervating muscles (green rhombuses labeled with indices). Neurites are drawn in solid black lines, soma in black dots. Solid lines with a mark in the middle indicate dorsal-ventral commissures. The presynaptic sites of chemical synapses are marked by rhombuses. The postsynaptic sites of chemical synapses are marked by arrows. The numbers associated with each chemical synapse indicate the number of presynaptic sites for this connection. Gap junctions are marked by pink T-shapes. Vesicle-filled varicosities are marked by blue circles with black edge.
2.4.2 Dorsal-ventral symmetry in undulatory locomotion across development

2.4.2.1 Locomotor output is symmetric in L1 larva

Forward and backward locomotion in *C. elegans* is driven by undulatory retrograde and anterograde bending waves, respectively. I assessed the symmetry in these dorsal-ventral bending waves using video microscopy. In crawling animals that lie flat on agar surfaces, it is straightforward to quantify the curvature of the body centerline over time and along its length. The wave propagation pattern was similar between L1 and L4 larva (Fig 2.5A). The amplitude was not different on either side of the body for L1 larva (Fig 2.5B).

L1 larvae also spent equal amounts of time with dorsal and ventral bending postures in our swimming assay (Fig. 2.5C, see Methods). Taken together, I uncovered no evidence of dorsal-ventral asymmetry in locomotory dynamics between L1 and adult animals.
Figure 2.5 L1 larvae generate symmetrical level of dorsal/ventral muscle output

A) Crawling body bending angle change of the same worm in N2 wild-type L1 and L4 larval stages. The animal body is divided into 33 segments along the anterior-posterior axis. Angles between each segment is shown along the y axis in the graph. The x axis shows the time. The head is oriented at the top, the tail at the bottom. The blank areas denote frames in which the larva body is entangled and cause image segmentation errors.

B) Amplitude of body bending during crawling for L1 larva. Each point represents the summed posterior body segment (2/3 of the body length, i.e. the posterior 22 segments of 33 segments from the image segmentation, as an estimate for the body wall muscles) angles in one frame for either side. This represents the same L1 larva recording shown in A). Because under the imaging condition, the dorsal and ventral side of L1 larva is not distinguishable, side one and side two are used to designate the side. The angles greater than 0 are grouped into side one, whereas angles
smaller than 0 are grouped into side two. n = 22, p = 0.2479, median of differences -89.61 (Wilcoxon matched-pairs sign rank test).

C) Body posture distribution scored manually for N2 wild-type L1 larvae thrashing in M9 buffer. The comparison is only between dorsal and ventral bending. There is no statistical difference between the time L1 larvae spend in dorsal and ventral bending postures. n = 10, p = 0.4258, median of differences 0.001667 (Wilcoxon matched-pairs sign rank test for all comparisons, because the only comparison is between dorsal and ventral bending proportions).

2.4.2.2 Muscle output during crawling in L1 larvae

Although the undulating body of the L1 larva shows no dorsal-ventral asymmetry that can be observed in swimming or crawling behavior, I further sought to confirm whether muscle activity is symmetric between the dorsal and ventral sides. Asymmetric motor circuit activity or muscle activity might compensate for a biomechanical asymmetry, for example. To quantify body wall muscle activity levels in L1 larvae, I imaged calcium dynamics in animals that expressed G-CaMP3 throughout the body wall muscles of freely moving L1 larvae (Pmyo-3::G-CaMP3) (Miller et al., 1983).

In undulating animals, I found that muscle activity peaks in the region of dorsal or ventral contraction with each undulation (Fig. 2.6A). As each bending wave travels from head to tail or from tail to head, the muscle activities along dorsal and ventral body wall muscles also propagate in phase with the body bend. The activities of corresponding muscle cells on either side of the body are antiphase, consistent with opposing relaxation and contraction of muscles on either side of the body with each bend (Fig. 2.6A). The average magnitude of dorsal and ventral calcium dynamics is the same on either side of the L1 animal, suggesting symmetry in the dorsal and ventral body wall muscle output during locomotion (Fig. 2.6B).
Figure 2.6 Muscle activities are balanced on dorsal and ventral sides in L1 larvae

A) Muscle activities in dorsal and ventral sides. The calcium imaging strain used here is AQ2953. Top: Muscle activity change detected by GCaMP normalized by RFP and analyzed by segmentation of body. Pixels in each segment are averaged for fluorescent intensities and treated as the muscle activity on that segment (inset). The head is approximately 35% of the body length, the body wall 65%. Patched and complementary activity patterns are seen on both dorsal and ventral sides. Bottom: Change of muscle activities along the anterior-posterior axis. Complementary activity dynamics is found between dorsal and ventral muscles.

B) Average body wall muscle activities on dorsal and ventral sides for AQ2953. The muscle activity of the posterior 65% of the body is averaged. No statistical difference is detected between dorsal and ventral muscles in average level of activity. n = 41, p = 0.0848, median of differences 0.05980 (Wilcoxon matched-pairs sign rank test).
2.4.3 **Motor neuron calcium imaging establishes correlation between cellular activities and locomotion in L1 larva**

To study the function of the motor neurons, the first step is to understand whether and how their activities are in correlated with L1 larva movements. Calcium imaging of motor neurons during locomotion is used to establish correlation (see Methods). On the basis of cellular connectivity, corresponding neurotransmitter types, as well as previous ablation studies in adult *C. elegans*, several predictions can be made for the motor neurons in L1 larvae: cholinergic DA motor neurons should correlate with backward locomotion, cholinergic DB motor neurons should correlate with forward locomotion, whereas GABAergic DD motor neurons should correlate both directional movements (*Chalfie et al., 1985; Duerr et al., 2008; Johnson and Stretton, 1987; McIntire et al., 1993; Stretton et al., 1978; Sulston et al., 1983; Walrond et al., 1985*). I also consider the SIA and SIB sublateral motor neurons, each of which is cholinergic, innervates both dorsal and ventral body wall muscles, and is reported to be associated with left-right turning during sleep (*Pereira et al., 2015; Schwarz and Bringmann, 2017*).

2.4.3.1 **Activity of A-type motor neurons correlates with anterior dorsal contraction during backward locomotion**

To image DA motor neurons activities during locomotion, I expressed cytoplasmic GCaMP and mCherry in the A-type motor neurons under the control of the *Punc-4* promoter (*Lickteig et al., 2001; Miller and Niemeyer, 1995*). The DA1-DA7 cell bodies are located in the ventral nerve cord and are well separated along the body. Two additional DA motor neurons (DA8 and DA9) are next to one another in the tail (Fig. 2.7A).

I quantified calcium dynamics in the cell bodies of these DA motor neurons during forward and backward movement. As expected from analyses of adult locomotion, DAs exhibited higher activity during backward movement than during forward locomotion (Fig. 2.7A). During backward movement, activity of DA motor neurons propagates from posterior to anterior. At each transition from forward to backward movement, the first DA motor neuron that becomes active is always located near the region of the body that is most dorsally bent. This suggests that the waves of DA motor neuron activity that drive locomotion do not necessarily commence at the posterior end. Instead, the DA motor neurons themselves might sense the curvature of the body by
proprioception as suggested by electron microscopy (White et al., 1986), as well as mathematical modelling (Erdös and Niebur, 1990; Niebur and Erdos, 1988a; Niebur and Erdos, 1988b; Niebur and Erdos, 1991). Detailed analysis showed that the activity of each DA motor neuron cell body is associated with the dorsal curvature of a region of the worm body that is immediately anterior (Fig. 2.8A-B). This observation is consistent with the anatomy of the DA motor neuron axon projections. Each axon from each DA(N) motor neuron projects anteriorly from its cell body and is located in the same body segment as the cell body of the DA(N-1) motor neuron.

2.4.3.2 Activity of B-type motor neurons correlates with posterior dorsal contraction during forward locomotion

To image DB motor neurons activities during locomotion, I expressed cytoplasmic GCaMP and mCherry in the B-type motor neurons under the control of the Pacr-2(s) promoter, which is a 1.8kb fragment of Pacr-2 that is specifically expressed in DA and DB motor neurons (Jospin et al., 2009). The DB1-DB7 cell bodies are located in the ventral nerve cord. DB1-DB3 are relatively clustered, whereas DB4-DB7 are well separated along the body (Fig. 2.7B).

I quantified calcium dynamics in the cell bodies of DB4-DB7 motor neurons during forward and backward movement. As expected from analyses of adult locomotion, DBs exhibited higher activity during forward movement than during backward locomotion (Fig. 2.7B). During forward movement, activity of DB motor neurons propagates from anterior to posterior. At each transition from backward to forward movement, the first DB motor neuron that becomes active is always located near the region of the body that is most dorsally bent. This suggests that the waves of DB motor neuron activity that drive locomotion do not necessarily commence at the anterior end. Instead, the DB motor neurons might sense the curvature of the body by proprioception, similar to DA motor neurons. Detailed analysis showed that the activity of each DB motor neuron cell body is associated with the dorsal curvature of a region of the worm body that is immediately posterior (Fig. 2.8B). This observation is consistent with the anatomy of the DB motor neuron axon projections. Each axon from each DB(N) motor neuron projects posteriorly from its cell body and is located in the same body segment as the cell body of the DB(N-1) motor neuron.
Figure 2.7 DA and DB motor neurons correlate with different modes of locomotion

A) DA motor neurons correlate with backward locomotion. The strain used here is ZM8428. **Top left:** DAs are labeled by Punc-4 (Miller and Niemeyer, 1995). **Top right:** Sample calcium dynamics of DA3-9 of one larva during locomotion. **Bottom right:** Overlapped traces of DA3-9
during locomotion from the same recording, with backward locomotion highlighted in grey. The colors of traces match those DA motor neurons marked in the top left panel. **Bottom left:** Comparison between activities in forward and backward locomotion for DAs. Larvae n = 12, DA neurons (including DA3-9) n = 81, ****p<0.0001, median of differences 0.08280 (Wilcoxon matched-pairs sign rank test).

B) DB motor neurons correlate with forward locomotion. The strain used here is ZM9128. **Top left:** DBs are labeled by Pacr-2(s) ([Jospin et al., 2009](#)). DAs are also labeled under this promoter, and marked by grey circles. **Top right:** Sample calcium dynamics of DB4-7 of one larva during locomotion. **Bottom right:** Overlapped traces of DB4-7 during locomotion from the same recording, with backward locomotion highlighted in grey. The colors of traces match those DB motor neurons marked in the top left panel. **Bottom left:** Comparison between activities in forward and backward locomotion for DBs. Larvae n = 14, DB neurons (including DB4-7) n = 30, ****p<0.0001, median of differences -0.2995 (Wilcoxon matched-pairs sign rank test).

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**Figure 2.8 DA and DB motor neurons correlate with dorsal contraction**

A) Activity of a motor neuron correlates optimally with body angles near its presynaptic region. **Left:** Cartoon depiction of DA axon projection orientation. DA6 is used as an example. The
presynaptic regions of DA6 is near the soma of DA5. *Right:* DA activity change compared with angle changes in regions anterior, overlapping, and posterior to the soma, as marked by the blue lines in the left panel. Activity is in orange, angle in blue. The shortest time lag is between DA6 activity and angle change near the soma of DA5. Higher values indicate dorsal contraction.

B) Shortest time lag is in accord with the orientations of motor neuron axon projections. *Left:* Time lag between neuronal activities and angle changes in regions anterior (N-1), overlapping (N), and posterior (N+1) to DAs soma. *Right:* Time lag between neuronal activities and angle changes in regions anterior (N-1), overlapping (N), and posterior (N+1) to DBs soma.

2.4.3.3 Activity of D-type motor neurons correlates with ventral relaxation in both directional movements

DD motor neurons synapse onto the ventral muscle. Their synapses are located in the largest puncta anterior to the soma. These puncta are on the ventral side. By driving the GCaMP under promoter *Punc*-25, all DD motor neurons can be imaged for their calcium dynamics (Fig. 2.9B). I recorded the calcium dynamics of these puncta during locomotion.

DD motor neurons receive synaptic inputs from both DA and DB motor neurons and should therefore be activated by DB during forward and DA during backward locomotion. Indeed, in both directional movements, the activities of DD motor neurons oscillated (Fig. 2.9A, C). There was no statistical difference between the activity level of DD motor neurons in forward and backward locomotion (Fig. 2.9C). Taken together, it is found that DD motor neurons correlate with ventral relaxation during both directional movements.

With calcium dynamics results from DA, DB, and DD motor neurons, I found neuronal contingents that correlate with backward locomotion, forward locomotion, and cross-inhibition respectively in L1 *C. elegans*. Nonetheless, I have not identified motor neurons that are correlated with ventral muscle contraction.
Figure 2.9 DD motor neurons correlate with ventral relaxation

A) The strain used here is ZM7656. *Top*: Sample calcium dynamics of DD4-5 of one larva during locomotion. *Middle*: Overlapped activity traces of DD4-5 during locomotion from the same recording in solid lines, and angle changes in dashed lines, with backward locomotion highlighted in grey. The colors of traces and angle changes match those DD motor neurons puncta marked in the top right panel. Higher values for angles indicate ventral relaxation, and vice versa. *Bottom*: Velocity change of the same larva during locomotion.
B) The strain used here is ZM7656. DDs are labeled by Punc-25 (Jin et al., 1999). The soma is marked in grey, whereas the puncta anterior to each soma is used in calcium imaging analysis.

C) Top: Activity of DD motor neurons pooled in forward and backward locomotion separately. The traces are obtained by grouping neuronal activities during either forward or backward locomotion from complete recordings. Neuronal activities during forward locomotion are colored in orange. Neuronal activities during backward locomotion are in blue. Then normalize each trace to its first data point, so that the relative change of neuronal activity upon moving directional change can be visualized. Because the larvae show a tendency to move forward, the orange lines are normally longer than blue lines. Bottom: Comparison between activities in forward and backward locomotion for DDs. Larvae n = 12, DD neurons (including DD4-5) n = 17, p = 0.1743, median of differences 0.04363 (Wilcoxon matched-pairs sign rank test).

2.4.3.4 Activity of sublateral motor neurons correlates with forward locomotion

SIAs and SIBs innervate both dorsal and ventral muscles according to EM reconstruction. They both receive weak inputs from a forward-directing premotor interneuron PVC.

By expressing GCaMP under Pcelh-24 (Altun-Gultekin et al., 2001; Schwarz and Bringmann, 2017), both SIA and SIB motor neurons can be recorded for their calcium dynamics. Because the somas of sublateral motor neurons are clustered, the calcium dynamics here reflect pooled activities of SIA and SIB motor neurons. Consistent with their connectivity, SIA and SIB motor neurons have relatively increased activities during forward locomotion (Fig. 2.10). Therefore, SIA and SIB motor neurons might provide excitabilities to both dorsal and ventral muscles during forward locomotion.

All together, DA and DB motor neurons correlate with dorsal contraction, and DD motor neurons with ventral relaxation in both directional movements; sublateral motor neurons correlate with forward locomotion. These results are all in line with the anatomy of neurons. Still, it is not immediately clear what is the source for ventral muscle contraction in both directional movements. Causal studies are needed to answer this question.
**Figure 2.10 SIA and SIB motor neurons correlate with forward locomotion**

A) The strain used here is ZM9401. *Top:* Calcium dynamics of SIA and SIB motor neurons. Backward locomotion is marked by gray bars. *Bottom:* Velocity change of larva during locomotion in the same recording.

B) The strain used here is ZM9401. *Top:* Activity of SIA and SIB motor neurons pooled in forward and backward locomotion separately, as described above. *Bottom:* Comparison between activities in forward and backward locomotion for SIAs and SIBs. Larvae n = 12, SIA and SIB neurons n = 26, ****p<0.0001, median of differences -0.1400 (Wilcoxon matched-pairs sign rank test).
2.4.4 An all-optical interrogation protocol establishes functional causality in neural circuits

To establish causality, I manipulated the circuit activity by optogenetic stimulation or inhibition of specific motor neuron groups, and simultaneously recorded corresponding muscle activity via calcium imaging with a simple protocol.

2.4.4.1 Rationale for a simple and systematic method for simultaneous manipulation and monitor of neural circuit activity

Simultaneous optical manipulation and monitoring of neural circuit activity has been technically challenging because widely-used opsins and GCaMP have overlapping excitation spectra (Guo et al., 2009; Klapoetke et al., 2014). This spectral overlap makes it difficult to observe the neuronal activities by calcium imaging before optogenetic stimulation. The excitation light used to acquire a GCaMP signal is enough to directly activate opsins. To circumvent this problem, previous studies have used either spatially restricted stimulation (Guo et al., 2009; Rickgauer et al., 2014) or spectrally separated opsins/calcium indicators, such as C1V1, bReaChES, or RCaMP (Akerboom et al., 2013; Kim et al., 2016; Packer et al., 2015; Rajasethupathy et al., 2015; Rickgauer et al., 2014). These methods have their limitations. The first method only lets one manipulate circuits where cells are easily spatially resolved. In this study, upstream motor neurons are colocalized with downstream muscle cells, making it impossible to illuminate only motor neurons but not muscle cells. Spatial patterning also requires sophisticated setups to control light to illuminate a restricted space (Guo et al., 2009; Rickgauer et al., 2014). In moving animals, the spatial patterning must be combined with tracking algorithms to follow the neurons of interest (Shipley et al., 2014). The second method uses red-shifted opsins C1V1/bReaChES or calcium indicator RCaMP that, compared to ChR2/Chrimson or GCaMP, are generally less effective than the original reagents. Also, even red-shifted opsins can be stimulated with the excitation light used with GCaMP. It is possible to reduce illumination intensity for GCaMP to avoid activating opsins, but this reduces the signal-to-noise ratio of calcium imaging. RCaMP has slower temporal dynamics and reduced signal-to-noise ratio than GCaMP (Kim et al., 2017).

Because C. elegans does not generate ATR by itself, there are experiments that compare calcium levels upon optogenetic stimulation in worms with and without ATR as a simple method to
achieve all-optical interrogation (Collins et al., 2016). However, this approach misses the initial change of calcium dynamics. Thus, the comparison can only be done between different worms (i.e., worms with ATR vs. worms without ATR), not within the same worm (i.e., a worm with ATR before stimulation vs. the exact same worm with ATR during stimulation). Additionally, the calcium recordings in those experiments typically last for minutes, which might bring secondary effects.

I proposed a simple method for simultaneous optogenetics and calcium imaging using existing reagents and standard fluorescence microscopy. I take advantage of the fact that GCaMP does not respond instantaneously with changes of cellular activity (Chen et al., 2013) (Fig. 2.11A). In dissociated neuronal culture, it takes 0.137±0.004s for GCaMP3 and 0.480±0.024s for GCaMP6s respectively to rise from its baseline to peak intensity level after 10 action potential stimuli; conversely, it needs 0.597±0.008s for GCaMP3 and 1.796±0.073s for GCaMP6s respectively to decay from its peak to half-peak level (Chen et al., 2013). The temporal dynamics of GCaMP intensity over the course of hundreds of milliseconds can be captured by most standard fluorescence microscope setups.

In experiments reporting optogenetic activation of upstream neurons and calcium imaging of downstream neurons, GCaMP intensity remains unchanged for a short period immediately after stimulation light for opsins is delivered (Guo et al., 2009; Packer et al., 2015; Rickgauer et al., 2014). Thus, the first frames of calcium imaging after stimulation light is applied represent the level of GCaMP intensity before stimulation. The calcium dynamics following this short period will reflect the optogenetically-induced activity, which can be directly compared with the baseline signals from the first few frames. I can thus perform simultaneous optogenetic illumination and calcium imaging by simply turning on the excitation light and quantifying the temporal dynamics of the calcium response from the first frames (that report baseline) to subsequent frames (that might indicate excitation or inhibition).

2.4.4.2 Verification of the methodology in known neural circuits by bidirectional manipulation

As a proof-of-principle experiment, I tested this method in DAs/DBs-DDs circuit. Based on EM reconstruction in the past (White et al., 1978) and ours, DAs and DBs innervate DDs in L1 C.
DAs and DBs are reported to be cholinergic (Alfonso et al., 1993). Because acetylcholine is primarily an excitatory neurotransmitter (Del Castillo et al., 1967; Delcastillo et al., 1963), activation and inhibition of DAs and DBs are predicted to activate and inhibit DDs, respectively. When I optogenetically activated/inhibited DAs and DBs by Chrimson/Arch, and simultaneously recorded DDs activity change by GCaMP6s in L1, the temporal dynamics of the changing GCaMP6s intensity were captured with a 10Hz (Fig. 2.11B-C). The time course of the calcium response in the DD neurons demonstrate synaptic excitation by the DA/DB neurons. Therefore, this approach is experimentally feasible.

As such, I will use this method to probe the causal relationship between neurons and muscles in *C. elegans* at L1 stage henceforth.
Figure 2.11 Simultaneous all-optical interrogation is achieved by a simple design
A) Time course response of genetically-encoded calcium indicator GCaMP is relatively slow. As marked by the dashed lines, the peak of the fluorescent signals arrives hundreds of milliseconds after the onset of the stimulations. This panel is copied and slightly modified from publication (Chen et al., 2013).

B) DD motor neurons are stimulated by optogenetic activation of DA and DB motor neurons. The strain used here is ZM9154. The total time of stimulation and recording is 3sec in these graphs. Top: Cartoon shows that DAs and DBs synapse onto DDs in L1, based on connectome analysis (Fig. 3A, C, D). Chrimson is expressed in DAs and DBs, and GCaMP in DDs. Recordings of GCaMP intensity change upon light stimulation can show the effect of DAs and DBs have on DDs. Middle: Raw traces of DDs activity change upon DAs and DBs activation without ATR (control) or with ATR. Values are normalized by GCaMP/RFP on the first frame. Upper panels are overlapped traces with mean values in a darker color and thicker line. Lower panels show individual traces. Bottom: Statistical analyses between normalized DDs activities on first and last frame for control and ATR groups for DAs and DBs inhibition. Control group larvae n = 5, stimulations for all DD neurons n = 20, p = 0.1536, median of differences -0.05202. ATR-fed group larvae n = 9, stimulations for all DD neurons n = 23, ****p < 0.0001, median of differences 1.634. (Wilcoxon matched-pairs sign rank test for both comparisons).

C) DD motor neurons are deactivated by optogenetic inhibition of DA and DB motor neurons. The strain used here is ZM9585. The total time of stimulation and recording is 3sec in these graphs. Arch is expressed in DAs and DBs, and DDs activity change is shown in a similar layout as in A). Control group larvae n = 6, stimulations for all DD neurons n = 25, p = 0.0903, median of differences -0.08540. ATR-fed group larvae n = 9, stimulations for all DD neurons n = 21, ****p < 0.000, median of differences -0.4649. (Wilcoxon matched-pairs sign rank test for both comparisons).
2.4.5 **Body wall muscles require neuronal inputs for activation in L1 larva**

First, I asked whether L1 muscles might be capable of contraction without synaptic input. Myogenic activity has been reported in the embryonic muscle cells of other invertebrates including *Drosophila* (Crisp et al., 2008). An UNC-104 null mutant in which all chemical synaptic transmission is disrupted, for example, can exhibit body wall muscle contraction in the L1 stage (Hall and Hedgecock, 1991). Several loss-of-function synaptic transmission mutants – including *unc-104, unc-13, snb-1, unc-64, unc-17, cha-1* – exhibit coiled postures at the L1 stage, also implying myogenic body wall muscle contraction (Alfonso et al., 1993; Hall and Hedgecock, 1991; Kohn et al., 2000; Nonet et al., 1998; Rand, 1989; Rand and Russell, 1984; Saifee et al., 1998). I asked if any dorsal/ventral bias in muscle activities might occur in synaptic transmission mutants with L1 larvae. The symmetry in L1 larva locomotion might be a result of an asymmetry in the myogenic activities of dorsal and ventral muscle cells that counterbalances the observed asymmetry in the wiring diagram.

I quantified locomotory behavior and muscle calcium imaging in L1 animals in a variety of mutants. By chemogenetics, as well as the all-optical interrogation method described in Chapter 2.4.4, I examined whether the muscle excitability requires neuronal input.

2.4.5.1 **Disrupting neurotransmission does not alter bending symmetry**

With the swimming assay (see Methods), I quantified locomotory behavior in L1 animals in a variety of neurotransmission mutants: *unc-104*(ut60), *unc-13*(s69), *unc-18*(sks1), *unc-64*(js115), *cha-1*(ok2253) (Hall and Hedgecock, 1991; Park et al., 2017; Rand and Russell, 1984; Richmond et al., 1999; Saifee et al., 1998). By quantifying dorsal/ventral symmetry in the bending movements of these animals in our swimming assay, I found no difference in the relative durations of dorsal and ventral bending in all mutants tested (Fig. 2.12).
Figure 2.12 Muscle output remains balanced on dorsal and ventral sides with neurotransmission disrupted

A) Preference of dorsal and ventral bending in liquid thrashing assay. Mutants disrupting synaptic neurotransmission are subjected to M9 buffer and manually scored for the time spent in dorsal, neutral, and ventral postures (see Methods). Dotted grey lines connect three postures of the same larva during a complete recording. No statistical difference between dorsal and ventral bending time is detected in any of the mutants tested (Wilcoxon matched-pairs sign rank test for all comparisons, because the only comparison is between dorsal and ventral bending proportions). For \textit{unc-104(ut60)}, the strain used is JC153, n = 13, p = 0.1294, median of differences -0.2644.

B) For \textit{unc-13(s69)}, the strain used is EG9631, n = 10, p = 0.7617, median of differences -0.03333.

C) For \textit{unc-18(sks1)}, the strain used is UHN21, n = 19, p = 0.9032, median of differences 0.000.

D) For \textit{unc-64(js115)}, the strain used is NM979, n = 18, p = 0.1826, median of differences 0.000.

E) For \textit{cha-1(ok2253)}, the strain used is VC1836, n = 16, p = 0.0654, median of differences 0.1653.
2.4.5.2 Synaptic transmission mutants exhibit symmetry in dorsal and ventral muscle activity

Next, I examined whether calcium dynamics in muscle cells of synaptic transmission mutants was also symmetric. I performed calcium imaging of body wall muscles in freely moving unc-13(s69) and unc-18(sks1) mutants. Both dorsal and ventral muscle cells exhibited robust spasm-like calcium activities in unc-13(s69) and unc-18(sks1). The magnitude of these calcium dynamics was indistinguishable when comparing the dorsal and ventral sides of the animal (Fig. 2.13). These data confirm that muscle activity can occur even when synaptic transmission is severely disrupted, but that the intrinsic activities of the dorsal and ventral body wall muscles are largely symmetric.
Figure 2.13 Muscle activities remain balanced on dorsal and ventral sides with neurotransmission disrupted

A) Muscle activities in unc-13(s69). The strain used here is ZM9139. Left: Muscle cell dynamics on the dorsal and ventral sides in one larva, and the angle change for the same recording. The y axis is oriented such that the top indicates head, the bottom indicates tail. The x axis represents time. See wild-type sample traces in Figure 2.2D. Middle: Overlapped traces for average dorsal and ventral activities from all recordings. Each light-colored trace represents the average muscle activity along the time course for either dorsal or ventral side of one larva during a complete recording. The average values of these traces along the time course are shown in darker and thicker lines. Right: Comparison between mean dorsal and ventral activities for each recording. n = 8, p = 0.5469, median of differences 0.02899 (Wilcoxon matched-pairs sign rank test).

B) Muscle activities in unc-18(sks1). The strain used here is ZM9527. The layout is similar to B). n = 14, p = 0.9515, median of differences -0.002450 (Wilcoxon matched-pairs sign rank test).

2.4.5.3 Impaired calcium and potassium signaling does not alter bending symmetry

To further rule out any possible differences in ionic environment between dorsal and ventral muscles, I tested the behavioral outputs of genetic mutations that disrupt calcium and potassium dynamics. Calcium and potassium are the major contributors to the membrane potential of muscle cells in *C. elegans*. The muscle activities would probably require ion channels, e.g. voltage-gated calcium channels, calcium-activated potassium channels (BK channels), as demonstrated from studies on smooth muscles (Arner and Pfister, 1999; Brenner et al., 2000; Davis and Hill, 1999; Nelson et al., 1995; Nelson et al., 1990). If the ventral body wall muscle is intrinsically more active, disrupting these channels could affect more on the ventral side, and cause dorsally-biased bending. To screen for any ion channels that are potentially involved in the generation of myogenic activities, I subjected an array of calcium or potassium signaling mutants, or channel inhibitor-treated wild-type, to liquid thrashing assays.
I tested the role of calcium with *cca-1(ad1650)*, *egl-19(n582)*, EGL-19 inhibitor nemadipine-A, *unc-36(e251)*, *tag-180-ok779*, *itr-1(sa73)* (Clandinin et al., 1998; Frokjaer-Jensen et al., 2006; Jeziorski et al., 2000; Kwok et al., 2006; Laine et al., 2011; Lee et al., 1997; Perez-Reyes et al., 1998; Schafer et al., 1996; Steger et al., 2005; Trent et al., 1983). I tested the role of potassium with *shk-1(ok1581)*, *slo-1(js379)*, *slo-1(ky389)*, *slo-2(nf100)*, *unc-58(bln221)* (Davies et al., 2003; Fawcett et al., 2006; Lim et al., 1999; Reiner et al., 1995; Wang et al., 2001; Yuan et al., 2000).

Interestingly, no mutants or channel-inhibitor treated L1 larvae displayed any obvious bias in thrashing (Table 1). Notably, EGL-19 inhibitor nemadipine-A-treated wild-type larvae formed a straight line and did not display motility. This screen suggests that the dorsal and ventral body wall muscles are probably not essentially different in their hypothesized myogenic activity.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation or Compound</th>
<th>Gene/Compound</th>
<th>Behavior</th>
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<tbody>
<tr>
<td>JD21</td>
<td><em>cca-1(ad1650)</em></td>
<td>T-type VGCC subunit α₁</td>
<td>Thrashing with no preference</td>
</tr>
<tr>
<td>MT1212</td>
<td><em>egl-19(n582)</em></td>
<td>L-type VGCC subunit α₁</td>
<td>Thrashing with no preference</td>
</tr>
<tr>
<td>N2</td>
<td>Nemadipine A 10uM, 24hr</td>
<td>Blocker for EGL-19</td>
<td>No thrashing, forming almost straight line</td>
</tr>
<tr>
<td>CB251</td>
<td><em>unc-36(e251)</em></td>
<td>VGCC, subunit α₂δ</td>
<td>No preference</td>
</tr>
<tr>
<td>VC550</td>
<td><em>tag-180-ok779</em></td>
<td>VGCC, subunit α₂δ</td>
<td>No preference</td>
</tr>
<tr>
<td>NM1081</td>
<td><em>unc-68(r1158)</em></td>
<td>Ryanodine receptor, intracellular calcium channel</td>
<td>Thrashing with no preference</td>
</tr>
<tr>
<td>JT73</td>
<td><em>itr-1(sa73)</em></td>
<td>IP3 receptor, intracellular calcium channel</td>
<td>No preference</td>
</tr>
<tr>
<td>RB1392</td>
<td><em>shk-1(ok1581)</em></td>
<td>Voltage-gated potassium channel, Shaker/Kv1 family</td>
<td>No preference</td>
</tr>
<tr>
<td>NM1968</td>
<td><em>slo-1(js379)</em></td>
<td>Calcium-activated, BK-type channel, Slo family</td>
<td>No preference</td>
</tr>
<tr>
<td>CX3933</td>
<td><em>slo-1(ky389)</em></td>
<td>Calcium-activated, BK-type channel, Slo family</td>
<td>No preference</td>
</tr>
<tr>
<td>LY100</td>
<td><em>slo-2(nf100)</em></td>
<td>Calcium-activated, BK-type channel, Slo family</td>
<td>No preference</td>
</tr>
<tr>
<td>JIP1154</td>
<td><em>unc-58(bln221)</em></td>
<td>TWK channel family</td>
<td>No preference</td>
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Table 1. Thrashing assay for mutant for calcium or potassium signaling, or wild-type L1 larvae treated with a calcium channel blocker
L1 larvae of a variety of calcium and potassium signaling mutants were screened for dorsal and ventral bending preference by liquid thrashing assays. Manual curation was used to score dorsal and ventral bending preference. Column 1 lists the strains used. Column 2 lists the names of the mutations, or drug used for the corresponding strains. Column 3 briefly describes the function the gene involved. Column 4 describes the behavioral output in liquid for corresponding rows. No obvious dorsal/ventral bending bias was detected in any of the mutants tested, or drug treated L1 larvae.

2.4.5.4 Constitutive pan-neuronal inhibition silences body wall muscles

It is questionable as to whether the synaptic transmission is completely abolished in mutants tested (Richmond et al., 1999). Would trace level of synaptic release in these mutants be sufficient to elicit muscle cell depolarization? Additionally, because neurotransmission mutations might affect development (Maruyama et al., 2001), muscle cells could adopt a property different from what they would in wild-type. A clear-cut acute inhibition of the nervous system in the wild-type is paramount to understand whether muscle activities are myogenic or not in C. elegans L1 larvae.

To achieve inhibition of all neurons, I utilized histamine-gate chloride channel HisCl1 (Pokala et al., 2014). By expressing HisCl1 under a pan-neuronal promoter and incubating L1 larvae on histamine plates for a brief period of time, I should be able to silence all the neurons in wild-type L1 without potentially affecting the developmental programs, and assess muscle activities by calcium imaging. In addition, to test if there is room for further reduction of neurotransmission in synaptic transmission mutants, I used HisCl1 in these mutants and measured muscle activities.

2.4.5.4.1 Pan-neuronal inhibition in wild-type

By expressing HisCl1 under Prgef-1, all neurons can be silenced after exposure to histamine. Strikingly, in wild-type L1 larvae, body wall muscle cells on both dorsal and ventral sides went silent after histamine treatment (Fig. 2.14A). The larvae can be recovered from histamine
treatment by moving onto regular plates, indicating that the larvae were not in the process of apoptosis.

This result is dramatically different from the muscle activities found in *unc-13(s69)* and *unc-18(sks1)*, in which overall muscle activities are high. This indicates that, body wall muscle cells are probably not myogenically active, and that dorsal and ventral body wall muscle cells are not intrinsically different. It also implies that there is room left for further inhibition of nervous system in synaptic transmission mutants.

2.4.5.4.2 Pan-neuronal chemical inhibition in neurotransmission mutants silences body wall muscle activities

To test if muscle activities can be reduced in *unc-13(s69)*, I expressed HisCl1 under Prgef-1 in *unc-13(s69)*, and recorded muscle calcium levels after histamine treatment. Indeed, pan-neuronal inhibition by HisCl1 silenced body wall muscle activities in *unc-13(s69)*, in direct contrast to control group (Fig. 2.14B). Hence, the robust activities observed in synaptic transmission mutants are probably not caused by myogenic activities, but rather by trace level of neurotransmission.

Together, these results consolidate the conclusion that body wall muscle activities are not myogenic, and that dorsal and ventral body wall muscle cells are not intrinsically different.
Figure 2.14 Chemogenetic inhibition of all neurons silences body wall muscle activities

A) Muscle activities upon panneuronal chemical inhibition in wild-type larva. The strain used here is ZM10464. Upper: Muscle cell dynamics on the dorsal and ventral sides in one larva, and the angle change for the same recording in control group. Bottom: Muscle cell dynamics on the dorsal and ventral sides in one larva, and the angle change for the same recording in histamine-treated group.

B) Muscle activities upon panneuronal chemical inhibition in *unc-13(s69)*. The strain used here is ZM10541. Left: Overlapped traces for average dorsal and ventral activities from all recordings,
with mean values displayed in darker color and thicker lines. Histamine-treated larvae are shown in orange, control in grey. Right: Comparison between mean dorsal and ventral activities for control and histamine-treated unc-13(s69) larvae. Dorsal larvae \( n \geq 9 \), **\( p = 0.0023 \), median of differences -0.6398 (Mann-Whitney U test). Ventral larvae *\( p = 0.0465 \), median of differences -0.2368 (Mann-Whitney U test). No difference is found between dorsal and ventral activities within histamine-treated group. \( n = 11 \), \( p = 0.0537 \), median of differences -0.08566 (Wilcoxon matched-pairs sign rank test).

2.4.5.5 Optogenetic pan-neuronal inhibition silences body wall muscles

HisCl1 induced silencing is acute compared with genetic mutants. However, it is still at the time span of 10-20 minutes. Cellular activity change is a much faster process. Because all-optical interrogation is possible in *C. elegans* L1 larva as described in 2.4.4, acute inhibition with much higher temporal resolution becomes feasible. This method will allow immediate measurement of muscle activity change upon pan-neuronal inhibition as well as activation, and further verify the origin of body wall muscle activities in *C. elegans* L1 larvae.

I tested how body wall muscle cells respond to the inhibition of all neurons by expressing GtACR2 under Prgef-1. With all-optical interrogation, I recorded how body wall muscle activities change upon pan-neuronal inhibition. Consistent with the results from chemical-genetic approach, optogenetic inhibition of all neurons caused silencing of both dorsal and ventral body wall muscles (Fig. 2.15A).

Together, it is concluded that body wall muscle activities are not myogenic, and that there is no difference between dorsal and ventral body wall muscle cells in their intrinsic excitabilities.

2.4.5.6 Optogenetic pan-neuronal activation excites body wall muscles

With results from pan-neuronal inhibition, it is natural to expect both dorsal and ventral body wall muscle cells to be excited upon pan-neuronal activation. If this is the case, then body wall muscle cells in *C. elegans* L1 larva are indeed neurogenic.
2.4.5.6.1 Pan-neuronal activation in wild-type

To test how body wall muscle cells respond to pan-neuronal activation, I expressed Chrimson in all neurons under *Prgef-1*, and recorded body wall muscle activities with the all-optical interrogation protocol. Counterintuitively however, activation of all neurons only activated dorsal side, but inhibited ventral side (Fig. 2.15B).

Why was ventral body wall muscle inhibited upon pan-neuronal activation? DD motor neurons innervate the ventral side, and they are GABAergic. It is likely that upon pan-neuronal activation, the presumably inhibitory GABA released from DD motor neurons overwrote the excitatory neurotransmitters and inhibited the ventral side. Thus, it is important to abolish GABA synthesis to see if the ventral body wall muscle can be activated during pan-neuronal activation.

2.4.5.6.2 Pan-neuronal activation in glutamic acid decarboxylase mutant *unc-25(e156)*

To remove GABA in the L1 larva, I used glutamic acid decarboxylase null mutant *unc-25(e156)* (Jin et al., 1999). This mutant lacks GABA and maintains normal synaptic development (Jin et al., 1999). Pan-neuronal activation is expected to elicit ventral body wall muscle activity in *unc-25(e156)* if body wall muscle activities are neurogenic.

I examined the body wall muscle response to pan-neuronal activation in the absence of GABA by expressing Chrimson under *Prgef-1* in *unc-25(e156)*. Indeed, upon activation, both dorsal and ventral body wall muscles were excited in *unc-25(e156)* (Fig. 2.15C). This result shows that neurons are sufficient to activate body wall muscles.

All together, it is concluded that body wall muscle cell activities are neurogenic, and that there is no difference between dorsal and ventral body wall muscle cells for their intrinsic activities in *C. elegans* L1 larvae.
Figure 2.15 Neurons are necessary and sufficient for body wall muscle activities with optogenetic manipulations

A) Muscle activities change upon panneuronal optogenetic inhibition in wild-type. The strain used here is ZM10579. Each column represents muscle activity changes in dorsal and ventral sides upon optogenetic stimulation. The order of columns is dorsal muscle from control, dorsal muscle from ATR-fed larvae, ventral muscle from control, and ventral muscle from ATR-fed larvae. The traces are GCaMP/RFP signals averaged along anterior-posterior segments for the body wall of each larvae and then normalized to the value on the first frame. The same layout will be repeated throughout the rest of the thesis for all-optical interrogation experiments. Top panels: Overlapped traces of dorsal and ventral muscle activities upon panneuronal inhibition. Mean of all traces are shown in darker color and thicker lines. Dashed lines indicate 1. Middle panels: Individual traces of dorsal and ventral muscle activities upon panneuronal inhibition. Bottom panels: Comparison between muscle activities at the start and end of the panneuronal inhibition. Control group larvae n = 6, stimulations n = 18, dorsal p = 0.4951, median of differences -0.03045, ventral p = 0.3247, median of differences 0.05169. ATR-fed group larvae n = 12, stimulations n = 26, dorsal ****p < 0.0001, dorsal median of differences -0.3291, ventral ****p < 0.0001, ventral median of differences -0.3114. (Wilcoxon matched-pairs sign rank test for all comparisons).

B) Muscle activities change upon panneuronal optogenetic activation in wild-type. The strain used here is ZM9648. The layout is the same as in E). Control group larvae n = 9, stimulations n = 17, dorsal p = 0.0569, dorsal median of differences -0.03582, ventral p = 0.4038, ventral median of differences -0.01868. ATR-fed group larvae n = 10, stimulations n = 18, dorsal ****p < 0.001, dorsal median of differences 0.6733, ventral ****p < 0.0001, ventral median of differences -0.2257. (Wilcoxon matched-pairs sign rank test for all comparisons).

C) Muscle activities change upon panneuronal optogenetic activation in unc-25(e156). The strain used here is ZM9660. The layout is the same as in E). Control group larvae n = 9, stimulations n = 14, dorsal p = 0.7609, dorsal median of differences 0.008630, ventral p = 0.0580, ventral median of differences 0.04153. ATR-fed group larvae n = 11, stimulations n = 21, dorsal ****p < 0.0001, dorsal median of differences 0.8520, ventral ****p < 0.0001, ventral median of differences 0.7199. (Wilcoxon matched-pairs sign rank test for all comparisons).


2.4.6 Non-motor cholinergic neurons are required for ventral muscle activities in L1 larvae

The next immediate question is which type of neurons generate body wall muscle activities. Cholinergic neurons are the primary excitatory neurons in *C. elegans* ([Rand, 2007](#)). It is reasonable to suspect cholinergic neurons to be activating body wall muscles. Hence, I first examined whether cholinergic neurons as a whole activate body wall muscles, and then studied the role of those cholinergic neurons that innervate body wall muscles in L1 larva.

Provided that cholinergic DA and DB motor neurons innervate dorsal side (Fig. 2.1), and correlate with dorsal bending in both directional movements at L1 stage (Fig. 2.8), it is expected that they contribute to dorsal body wall muscle activities. I manipulated their activities and recorded subsequent body wall muscle activity changes.

Cholinergic sublateral motor neurons innervate both dorsal and ventral body wall muscles (Fig. 2.2D), but only correlate with forward locomotion (Fig. 2.10). Although it is difficult to explain ventral muscle activity during backward locomotion if sub-later motor neurons are the sole underlying source of excitability, I examined their causative role in body wall muscle activities with all-optical interrogation.

2.4.6.1 Cholinergic neurons are necessary for body wall muscle activities

To examine if cholinergic neurons as a whole are necessary for the body wall muscles, I expressed GtACR2 under *Punc-17* ([Alfonso et al., 1993](#)), and used the all-optical interrogation protocol. Upon inhibition of all cholinergic neurons, both dorsal and ventral body wall muscles were silenced, recapitulating the effect of pan-neuronal inhibition (Fig. 2.16A). This result suggests that cholinergic neurons are necessary for body wall muscle activities.

2.4.6.2 Cholinergic neurons are sufficient to for body wall muscle activities

Whether cholinergic neurons are sufficient to activate body wall muscle activities on both sides remains to be seen. As in the pan-neuronal manipulation experiments, I examined the effect of cholinergic neurons on body wall muscles in wild-type and *unc-25(e156)*.
2.4.6.2.1 Activation of cholinergic neurons in wild-type

To test whether cholinergic neurons all together can activate body wall muscles, ChR2 was expressed under Punc-17 (Alfonso et al., 1993; Nagel et al., 2005), and used the all-optical interrogation protocol in wild-type L1 larvae. However, similar to pan-neuronal activation in wild-type, activation of cholinergic neurons only stimulated the dorsal side but inhibited ventral side (Fig. 2.16B).

Because DA and DB motor neurons are cholinergic, activation of all cholinergic neurons should also activate their down targets, DD motor neurons. DD motor neurons could potentially mask the excitatory effect of cholinergic neurons by releasing GABA onto ventral side.

2.4.6.2.2 Activation of cholinergic neurons in unc-25(e156)

With the potential masking effect of GABA from DD motor neurons, I again used unc-25(e156) to abolish GABA synthesis. In this mutant, activation of cholinergic neurons stimulated both dorsal and ventral muscles, recapitulating the effects of pan-neuronal activation in unc-25(e156) (Fig. 2.16C). This result indicates that cholinergic neurons are sufficient to activate both dorsal and ventral muscles.

Altogether, cholinergic neurons are both necessary and sufficient for body wall muscle excitability.
A. **WT; Cholinergic inhibition**

- **Dorsal Control**
- **Dorsal ATR**
- **Ventral Control**
- **Ventral ATR**

Muscle activity (F/F₀)

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B. **WT; Cholinergic activation**

- **Dorsal Control**
- **Dorsal ATR**
- **Ventral Control**
- **Ventral ATR**

Muscle activity (F/F₀)

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C. **unc-25(e156); Cholinergic activation**

- **Dorsal Control**
- **Dorsal ATR**
- **Ventral Control**
- **Ventral ATR**

Muscle activity (F/F₀)

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**Figure 2.16 Cholinergic neurons are necessary and sufficient to body wall muscle activities in optogenetic manipulations**

A) Muscle activities change upon optogenetic inhibition of all cholinergic neurons in wild-type. The strain used here is ZM10575. The layout is the same as in Fig. 2.4, hereinafter inclusive. Control group larvae n = 3, stimulations n = 16, dorsal p = 0.8999, dorsal median of differences -0.03233, ventral p = 0.4332, ventral median of differences -0.02208. ATR-fed group larvae n = 5, stimulations n = 27, dorsal ****p < 0.0001, dorsal median of differences -0.2252, ventral ****p < 0.0001, ventral median of differences -0.2485. (Wilcoxon matched-pairs sign rank test for all comparisons).

B) Muscle activities change upon optogenetic activation of all cholinergic neurons in wild-type. The strain used here is ZM9429. Control group larvae n = 7, stimulations n = 15, dorsal p = 0.6788, dorsal median of differences -0.03453, ventral p = 0.3894, ventral median of differences -0.01967. ATR-fed group larvae n = 10, stimulations n = 19, dorsal ****p < 0.0001, dorsal median of differences 0.4603, ventral ****p < 0.0001, ventral median of differences -0.1888. (Wilcoxon matched-pairs sign rank test for all comparisons).

C) Muscle activities change upon optogenetic activation of all cholinergic neurons in unc-25(e156). The strain used here is ZM9573. Control group larvae n = 6, stimulations n = 18, dorsal p = 0.7989, dorsal median of differences -0.008189, ventral p = 0.6705, ventral median of differences 0.004484. ATR-fed group larvae n = 10, stimulations n = 30, dorsal ****p < 0.0001, dorsal median of differences 0.5742, ventral ****p < 0.0001, ventral median of differences 0.5241. (Wilcoxon matched-pairs sign rank test for all comparisons).

**2.4.6.3 Cholinergic motor neurons are not necessary for ventral muscle activity**

DA and DB motor neurons are cholinergic. They are expected to activate dorsal muscle cells. By expressing opsin in DA and DB motor neurons, dorsal muscle cells should be activated. However, I noticed that under Pacr-2(s), very faint fluorescent signals can be detected in body wall muscle cells at least during L1 stage. Therefore, if the opsin is only driven under Pacr-2(s),
muscle cells will certainly be optogenetically manipulated regardless of the input from DA and DB motor neurons.

To specifically restrict opsin into DA and DB motor neurons, I utilized intersectional Cre-loxP system. By inserting the $loxp::EBFP::STOP::loxp$ cassette between the promoter and opsin gene, opsin will not be expressed unless Cre recombinase is present in the same cell and cut the $loxp::EBFP::STOP$ cassette out of the sequence (Hubbard, 2014; Schmitt et al., 2012). Specifically, I used two plasmids, $Pacr-2(s)::loxp::EBFP::STOP::loxp::opsin::wCherry$ and $Punc-17::Cre$. Because $Pacr-2(s)$ is expressed in cholinergic DA and DB motor neurons plus body wall muscle cells, whereas $Punc-17$ is expressed in cholinergic neurons but not muscle cells (Alfonso et al., 1993), this combination of Cre-loxP system should allow opsin to be expressed only in DA and DB motor neurons. wCherry is used to visualize the specificity of the opsin expression.

I used Chrimson to activate, and GtACR2 to inhibit DA and DB motor neurons respectively. Due to an alternative open reading frame after loxP sequence inadvertently induced by Nhe1 cutting site, Chrimson construct expression is very low, and therefore can barely be detected by fluorescence imaging. But GtACR2 construct expression is high enough to be detected. Indeed, $Pacr-2(s)::loxp::EBFP::STOP::loxp::GtACR2::wCherry$ and $Punc-17::Cre$ allows expression of opsin in DA and DB motor neurons specifically. Therefore, this experimental paradigm can be used to specifically manipulate DA and DB motor neurons with all-optical interrogation protocol.

Inhibition of DA and DB motor neurons would reveal the necessity of these neurons in the body wall muscle activities. By expressing GtACR2 with Cre-loxP system as described above, DA and DB motor neurons are inhibited in the all-optical interrogation protocol. Remarkably, upon inhibition of DA and DB motor neurons, only dorsal body wall muscle cells were deactivated, whereas ventral body wall muscle cells were disinhibited (Fig. 2.17A). This result suggests that indeed, dorsal muscle activities require input from DA and DB motor neurons, but ventral muscle activities do not. The fact that ventral body wall muscle was disinhibited makes sense since DD motor neurons were deactivated upon DA and DB inhibition, and therefore their inhibitory effect on the ventral side was lifted.
Therefore, unknown cholinergic neurons should be contributing to the ventral body wall excitability, perhaps SIAs and SIBs.

2.4.6.4 Inhibition of sublateral motor neurons reduces dorsal but not ventral body wall muscle activities

To examine the role of sublateral motor neurons in body wall muscle excitability, I expressed GtACR2 in the SIA and SIB sublateral motor neurons using the same Cre-loxP system as described in 2.4.6.3. The promoter that is reported to be relatively specific to SIA and SIB sublateral motor neurons is Pceh-24 (Harfe and Fire, 1998; Kennerdell et al., 2009). It is also expressed in SMD, vulval muscles and m8 pharyngeal muscle (Harfe and Fire, 1998; Kennerdell et al., 2009). However, as I noticed in calcium imaging of SIA and SIB motor neurons in 2.4.3.4, there is significant expression of Pceh-24 in body wall muscle cells. Therefore, to restrict opsins in sublateral motor neurons, I used Cre-loxP system again as described above. Specifically, two plasmids Pceh-24::loxP::EBFP::STOP::loxP::opsin::wCherry and Punc-17::Cre were utilized to avoid muscle expression.

Upon inhibition, dorsal muscle was deactivated but not ventral muscle, which is similar to inhibition of DA and DB motor neurons (Fig. 2.17A). As such, SIA and SIB sublateral motor neurons are not necessary for ventral body wall muscle excitability. This is not surprising, because SIA and SIB sub-lateral motor neurons do not have activities during backward movement, and they should not be the sole source of ventral muscle excitability.

But it remains to be elucidated as to whether DA, DB, SIA, SIB together are necessary for the ventral muscle excitability.

2.4.6.5 Inhibition of A-, B-type and sublateral motor neurons reduces dorsal but not ventral body wall muscle activities

Combining Pacr-2(s):loxP::EBFP::STOP::loxP::GtACR2::wCherry, Pceh-24::loxP::EBFP::STOP::loxP::GtACR2::wCherry and Punc-17::Cre, all cholinergic motor neurons that innervate body wall muscles can be silenced. Upon inhibition of DA, DB, SIA, SIB,
however, dorsal but not ventral body wall muscle was deactivated (Fig. 2.17B). This result confirmed that DA, DB, SIA, and SIB are not necessary for the ventral body wall muscle excitability.

Altogether, it is concluded that there are unknown cholinergic neurons other than DAs, DBs, SIAs, and SIBs that provide the excitability for ventral body wall muscle cells.
Figure 2.17 Cholinergic motor neurons are not necessary for ventral body wall muscle activity

A) Muscle activities change upon optogenetic inhibition of DAs and DBs, or SIAs and SIBs separately in wild-type. The strains used here are ZM10458 and ZM10715. All are ATR-fed groups. \textit{Left:} Inhibition of DAs and DBs, larvae n = 12, stimulations n = 35, dorsal ****p < 0.0001, dorsal median of differences -0.1913, ventral *p = 0.0107, ventral median of differences 0.08643. \textit{Right:} Inhibition of SIAs and SIBs, larvae n = 8, stimulations n = 22, dorsal ****p < 0.0001, dorsal median of differences -0.4206, ventral ****p < 0.0001, ventral median of differences 0.2671. (Wilcoxon matched-pairs sign rank test for all comparisons).

B) Muscle activities change upon optogenetic inhibition of DAs, DBs, SIAs, SIBs together in wild-type. The strain used here is ZM10580. Control group larvae n = 11, stimulations n = 27, dorsal p = 0.9341, dorsal median of differences -0.05464, ventral p = 0.6964, ventral median of differences -0.001322. ATR-fed group larvae n = 12, stimulations n = 33, dorsal ****p < 0.0001, dorsal median of differences -0.4492, ventral *p = 0.0370, ventral median of differences 0.1488. (Wilcoxon matched-pairs sign rank test for all comparisons).

2.4.6.6 Activation of A- and B-type cholinergic motor neurons activates dorsal muscle in wild-type

To examine whether DA and DB motor neurons are sufficient for body wall muscle activities, I expressed Chrimson with the Cre-loxP system as described above. In wild-type, upon activation of DA and DB motor neurons, the dorsal body wall muscle cells were activated, whereas the ventral body wall muscle cells were inhibited (Fig. 2.18A). Thus, DA and DB motor neurons are excitatory to dorsal muscle cells as expected. Because DD motor neurons should also be subsequently stimulated in this case, GABA synthesis need to be abolished to reveal any other potential excitatory effect from DA and DB motor neurons.
2.4.6.7 Activation of A- and B-type cholinergic motor neurons activates dorsal and ventral muscles in *unc-25(e156)*

Using *unc-25(e156)*, GABA is absent from the larva. Strikingly, activation of DA and DB motor neurons stimulated both dorsal and ventral body wall muscles in *unc-25(e156)* (Fig. 2.18B). Since there are no presynaptic structures on the ventral side for DA and DB motor neurons, it is unexpected that DA and DB motor neurons can also induce ventral body wall muscle excitability.

Can ephaptic coupling (Anastassiou et al., 2011; Katz and Schmitt, 1940) explain this result? Membrane depolarization of the long processes of DA and DB on the ventral nerve cord might directly stimulate adjacent ventral muscle cells through extracellular fields. Nonetheless, based on their activity profiles during locomoting, DA and DB motor neurons do not correlate with ventral muscle contraction at all (see 2.4.3). And if ephaptic coupling is working, then when one DA or DB motor neuron is excited, muscle cells on both dorsal and ventral sides of this neuron will be stimulated. Ergo, in *unc-25(e156)* mutant which does not have GABA, muscle cells on dorsal and ventral sides in the same segment would synchronize their activities, creating a peristaltic wave along the anterior-posterior axis. This is clearly in contrast to the pattern of *unc-25(e156)* mutant muscle activities (see 2.4.9.1). As such, it is highly unlikely to explain the effect with ephaptic coupling.

By examining the details of the muscle activity dynamics after activation of DA and DB motor neurons, I noticed that the ventral side appeared to be stimulated after the dorsal side (Fig. 2.18C). This latency could suggest a secondary effect in the optogenetic manipulation: optogenetic activation of all DA and DB motor neurons might indirectly activate the source for ventral muscle activity.
Figure 2.18 Optogenetic activation of DA and DB motor neurons can also stimulate ventral body wall muscle

A) Muscle activities change upon optogenetic activation of DAs and DBs in wild-type. The strain used here is ZM10339. Control group larvae n = 7, stimulations n = 21, dorsal p = 0.1470, dorsal median of differences -0.009942, ventral p = 0.1193, ventral median of differences -0.01567. ATR-fed group larvae n = 12, stimulations n = 33, dorsal ****p < 0.0001, dorsal median of differences 0.6280, ventral ***p = 0.0007, ventral median of differences -0.1136. (Wilcoxon matched-pairs sign rank test for all comparisons).

B) Muscle activities change upon optogenetic activation of DAs and DBs in unc-25(e156). The strain used here is ZM10340. Control group larvae n = 9, stimulations n = 20, dorsal p = 0.1650, dorsal median of differences -0.04709, ventral p = 0.3118, ventral median of differences -0.1087. ATR-fed group larvae n = 14, stimulations n = 35, dorsal ****p < 0.0001, dorsal median of differences 1.029, ventral ****p < 0.0001, ventral median of differences 1.549. (Wilcoxon matched-pairs sign rank test for all comparisons).

C) Time lag between dorsal and ventral muscle activity rise upon DAs and DBs activation in unc-25(e156). The strain used here is ZM10340. Left: Individual traces of each epoch of stimulation. The point at half maximum is marked by a dot on each line. Right: Comparison between full width at half maximum (FWHM) for dorsal and ventral activities of all stimulations. Larvae n = 14. Stimulations n = 35, ****p<0.0001, median of differences 0.9444 (Wilcoxon matched-pairs sign rank test).

2.4.6.8 Restoring synaptic transmission in A- and B-type motor neurons in unc-18(sks1) results in higher dorsal muscle activity

To test if the output from DA and DB motor neurons is excitatory to both dorsal and ventral sides under physiological condition, I restored the UNC-18 expression in DA and DB motor neurons in unc-18(sks1) mutant. The transgenic larvae should exhibit balanced dorsal and ventral muscle activities, if DA and DB motor neurons are excitatory to both sides.
However, the transgenic larvae showed stronger dorsal body wall muscle activity than that on the ventral side (Fig. 2.6H). This result suggests asymmetric output from DA and DB to the body wall muscles.

Figure 2.19 DA and DB motor neurons preferentially activate dorsal muscle upon UNC-18 rescue in unc-18(sks1) mutant

A) Muscle activities in unc-18(sks1) with UNC-18 expression rescued in DAs and DBs. The strain used here is ZM9997. Overlapped traces for dorsal and ventral body wall muscle activities averaged along anterior-posterior axis of all recordings with mean values in a darker color and thicker line.

B) The strain used here is ZM9997. Comparison between dorsal and ventral body wall muscle activities averaged for each recording. n = 24, ****p<0.0001, median of differences -1.084 (Wilcoxon matched-pairs sign rank test).
2.4.7 Other mechanisms ruled out as sources for ventral muscle activation

2.4.7.1 Head mesodermal cell is not required for body wall muscle activities

Head mesodermal cell (HMC) innervates dorsal and ventral muscles in the anterior region by gap junction (Fig. 2.1, Fig. 2.2). It is possible for DAs and DBs to activate the dorsal body wall muscle, and then the dorsal side passes the excitability to the ventral side via HMC. Because muscle cells are also electrically coupled, the anterior excitability might be propagated to the rest of the posterior ventral muscle cells. If this is the case, disrupting HMC should prevent ventral muscle cells from activating.

Laser ablation has been extensively used in *C. elegans* to study the function of cells (Avery and Horvitz, 1989; Bargmann and Avery, 1995; Bargmann et al., 1993; Bargmann and Horvitz, 1991; Chalfie et al., 1985; Desai and Horvitz, 1989; Fang-Yen et al., 2012; Gabel et al., 2007; Gray et al., 2005; Sommer and Sternberg, 1994; Sulston and White, 1980; Tsalik and Hobert, 2003; Ward et al., 2008). I utilized this method to ablate HMC cells 1hr post hatching, and recorded the muscle activities approximately 4hr later with a strain that expresses GFP marker in HMC. The ablation was confirmed by the loss of HMC fluorescent signal in L4 stage (Fig. 2.20A). Both dorsal and ventral muscle activities were present and did not show difference in levels after HMC ablation (Fig. 2.20B). No apparent behavioral abnormality was found after HMC ablation in L1 larvae.

Therefore, HMC is not required for the ventral muscle excitability.

2.4.7.2 Proprioception is not essential for symmetric body wall muscle activities

Muscle cells can respond to stretch and be proprioceptive (Proske and Gandevia, 2012). Can ventral body wall muscle in L1 *C. elegans* be proprioceptive? If ventral body wall muscle is proprioceptive, then dorsal muscle contraction caused by DA and DB motor neurons could activate ventral muscle by stretch or tension without any other cellular intermediate. If this is the
case, deformation on the dorsal side should evoke ventral muscle excitability, and loss of dorsal muscle contraction should prevent ventral activation.

2.4.7.2.1 Deformation on dorsal body wall does not induce ventral body wall muscle activities

To directly test if stretch or tension change on the dorsal muscle can cause ventral muscle excitability, dorsal muscle deformation is induced. I poked the dorsal body wall muscle with pointy pipette and recorded muscle activities simultaneously. To reduce the muscle contraction from the larva itself, the experiment was carried out in *unc-18(sks1)*.

However, dorsal muscle deformation induced by pointy pipette poking did not elicit ventral muscle excitation (Fig. 2.20C), indicating that stretch or tension is not sufficient to elicit ventral muscle activities.

2.4.7.2.2 Muscle activities of *unc-54(e190)* remain balanced

I examined the muscle activity pattern in *unc-54(e190)*, a mutant that lacks functional myosin II (*Park and Horvitz, 1986*). The loss of myosin II prevents muscle contraction, and therefore should dramatically abolish the stretch or tension signal to the ventral muscle. The mutant L1 larva is paralyzed, with no visible contraction on the body wall muscle.

But activity levels of ventral muscle were no different from dorsal muscle in *unc-54(e190)* (Fig. 2.20D). This indicates that stretch or tension fluctuations are not necessary for the ventral muscle excitabilities. Together, these data suggest that proprioception is not required for ventral muscle excitability.
Figure 2.20 Neither HMC nor proprioception is required for ventral body wall muscle excitability

A) HMC is ablated by laser. The strain used here is ZM9541. Top: Absence of HMC fluorescent signal in laser-ablated larva at L4 stage. Right: Presence of HMC fluorescent signal in control larva at L4 stage. The positions of HMC are marked by arrowheads.

B) Muscle activities for HMC-ablated larvae. The strain used here is ZM9541. Left: Overlapped traces for dorsal and ventral body wall muscle activities averaged along anterior-posterior axis of all recordings with mean values in a darker color and thicker line. Right: Comparison between
dorsal and ventral body wall muscle activities averaged for each recording. \( n = 9 \), \( p = 0.5703 \), median of differences 0.03039 (Wilcoxon matched-pairs sign rank test).

C) Muscle activity dynamics upon dorsal side deformation in \textit{unc-18(sks1)}. The strain used here is ZM9527. \textit{Left}: Time-lapse images of muscle GCaMP and RFP for an epoch of glass pipette-induced dorsal deformation on a larva of \textit{unc-18(sks1)}. A glass pipette tip is very close to the larvae at time zero, and then in contact with the dorsal side of the larva, and then retracted from the larva. \textit{Right}: Activity changes of dorsal and ventral muscles, and angle changes for the same epoch of recording on the left. The mechanically-induced dorsal deformation is reflected in the angle raster plot and circled in dashed line. The same region is also circled on the muscle activity raster plots for dorsal and ventral sides.

D) Muscle activities for \textit{unc-54(e190)}. The strain used here is ZM9711. \textit{Left}: Overlapped traces for dorsal and ventral body wall muscle activities averaged along anterior-posterior axis of all recordings with mean values in a darker color and thicker line. \textit{Right}: Comparison between dorsal and ventral body wall muscle activities averaged for each recording. \( n = 12 \), \( p = 0.0522 \), median of differences -0.3649 (Wilcoxon matched-pairs sign rank test).
2.4.8 GABAergic motor neurons inhibit ventral muscles by synaptic transmission and dorsal muscles by extra-synaptic transmission via cholinergic motor neurons

GABAergic motor neurons DDs innervate ventral muscle cells in L1. They receive inputs from cholinergic DAs and DBs on the dorsal side. Because their activities correlate with ventral relaxation, it is less likely but not impossible that they can cause ventral muscle excitability.

GABA itself is excitatory to enteric muscles through cationic GABA receptor EXP-1 in adult C. elegans (Beg and Jorgensen, 2003; McIntire et al., 1993; Thomas, 1990). Can GABA be excitatory to body wall muscle cells in L1 C. elegans? A previous work used behavior readouts to infer the inhibitory role of GABA, but did not definitively rule out the possibility of GABA being excitatory to body wall muscles in L1 C. elegans (Han et al., 2015).

There are neurons in C. elegans that express multiple neurotransmitters (Duerr et al., 2001; Serrano-Saiz et al., 2017). Can DD motor neurons also co-express other excitatory neurotransmitters and release them in locations different from site of the release for GABA?

With these potential mechanisms contributing to ventral muscle excitabilities in L1 C. elegans, I addressed them with behavioral genetics, calcium imaging, and all-optical interrogation.

2.4.8.1 GABA is inhibitory to muscle activities

By mutating the GABA synthase UNC-25, the overall synthesis of GABA is blocked while neurogenesis remains normal (Jin et al., 1999). The null mutant unc-25(e156) (Jin et al., 1999) is utilized to assess the role of GABA in body wall muscle excitability.

unc-49 encodes a chloride channel that is gated by GABA (Schofield et al., 1987). UNC-49 is the receptor that mediates inhibitory effect of GABA on body wall muscles (Bamber et al., 1999; Bamber et al., 2005). UNC-49 is expressed only on the ventral but not dorsal body wall muscle in L1 (Bamber et al., 2003; Gally and Bessereau, 2003). The null mutant unc-49(e407) (Bamber et al., 1999) is utilized to assess the role of GABA in body wall muscle excitability.

Because both mutations are genome-wide, these assays cannot directly address the role of GABA from DD motor neurons, but only the overall function of GABA to body wall muscles.
2.4.8.1.1 Behavioral change of *unc-25(e156)* and *unc-49(e407)* in low-viscosity liquid

Using the same PDMS well as in the wild-type liquid thrashing assay, L1 larvae of *unc-25(e156)* and *unc-49(e407)* were subjected to M9 liquid. Their body posture distribution was counted manually. Both *unc-25(e156)* and *unc-49(e407)* showed obvious preference to ventral bending, which implies that GABA signaling is inhibitory to ventral body wall muscle (Fig. 2.21A).

2.4.8.1.2 Muscle activities change of *unc-25(e156)* and *unc-49(e407)* on agar pad

By introducing GABA signaling mutations into calcium imaging allele, I was able to record body wall muscle activities in *unc-25(e156)* and *unc-49(e407)* respectively. Compared to dorsal muscle, both mutants showed spatially broader pattern of muscle activities along the ventral side (Fig. 2.21B). This result indicates that in the absence of GABA signaling, inhibitory signal is lifted; therefore, the ‘should-be’ inhibited muscles are still active, forming a continuum of muscle activity pattern along the ventral side.

Together, both behavioral and muscle activity readouts suggest that overall, GABA is inhibitory to ventral body wall muscle. This is consistent with the previous study (Han et al., 2015). But it remains to be elucidated whether GABAergic DD motor neurons are indeed inhibitory to ventral body wall muscle. The remaining sections of 2.4.8 will be focusing on this question.
Figure 2.21 GABA is inhibitory to body wall muscles in L1 larvae

A) Preference of dorsal and ventral bending in liquid thrashing assay for *unc-25(e156)*, *unc-49(e407)*. The strains used here are CB156 and CB407. Preference for *unc-25(e156)*, n = 10, **p = 0.0020, median of differences 0.2133. Preference for *unc-49(e407)*, n = 6, *p = 0.0313, median of differences 0.3372.

B) Muscle activity patterns in *unc-25(e156)*, *unc-49(e407)*. The strains used here are ZM9172 and ZM10451. *Left panels:* Angle change is on the left, and muscle activities are on the right for dorsal and ventral sides. Head is oriented on top, tail bottom. The boundary between head and body wall is marked by a dashed line in each plot. *Right panels:* Correlation between muscle activity patterns with angle patterns in *unc-25(e156)*, *unc-49(e407)*. Correlation for *unc-25(e156)*, n = 12, **p = 0.0034, median of differences -0.1500. Correlation for *unc-49(e407)*, n = 11, **p = 0.0068, median of differences -0.1856.
2.4.8.2 Activation of D-type motor neurons silences both dorsal and ventral muscles in wild-type

To directly probe the functional role of DD motor neurons to body wall muscles, I used the same all-optical interrogation methodology to activate DD motor neurons and record body wall muscle activity change. It is reasonable to speculate that DD motor neurons regulate only ventral muscle, because they only innervate ventral side (Fig. 2.1). Chrimson was specifically expressed in DD motor neurons under the promoter Pttr-39 (Petersen et al., 2011) and used to activate them. To my surprise, optogenetic activation of DDs inhibited not only ventral but also dorsal muscle cells (Fig. 2.22A).

This result is in accord with the previous data in 2.4.8.1 that GABA is inhibitory to ventral body wall muscle. But it is not immediately clear why the dorsal muscle cells are also inhibited upon DD motor neuron activation, since there is no synapse between DD motor neurons and dorsal muscle cells. This question will be addressed in the following sections.

2.4.8.3 Inhibition of D-type motor neurons increases both dorsal and ventral muscle activities in wild-type

As opposed to activation, by expressing Archaerhodopsin under the promoter Pttr-39, DD motor neurons are inhibited optogenetically. Interestingly, body wall muscle cells on both dorsal and ventral muscle sides are disinhibited upon inhibition DD motor neurons (Fig. 2.22B).

Given that activation of DD motor neurons led to inhibition of ventral body wall muscle, it is not surprising that inhibition would cause otherwise. But the fact that dorsal muscle is also disinhibited is as puzzling as the dorsal muscle response in 2.4.8.2.

2.4.8.4 Activation of D-type motor neurons does not affect body wall muscle activities in unc-25(e156)

With the conclusion that GABA being inhibitory to body wall muscle cells in L1 larvae, another possibility needs to be addressed: DD motor neurons might contain more than one
neurotransmitter type in L1 larvae. If this is the case, GABA can mask the potential effects from other neurotransmitters in experiments above.

To examine this possibility, I used mutant *unc-25(e156)* that lacks GABA (Jin et al., 1999). If there are other unknown excitatory neurotransmitters from DD motor neurons, activation of DD motor neurons in *unc-25(e156)* should activate ventral muscle.

However, no apparent changes were found in muscle activities upon activation DDs (Fig. 2.22C). This result suggests that DDs only release GABA as their functional neurotransmitter onto body wall muscle cells.

Therefore, DDs are inhibitory to body wall muscles via GABA release on the ventral side.
**Figure 2.22 DD motor neurons are inhibitory to dorsal and ventral body wall muscles via GABA signaling**

A) Muscle activities change upon optogenetic activation of DDs in wild-type. The strain used here is ZM9551. The layout is the same as in Fig. 2.4, hereinafter inclusive. Control group larvae n = 7, stimulations n = 13, dorsal p = 0.5300, dorsal median of differences 0.009468, ventral p = 0.5759, ventral median of differences 0.008537. ATR-fed group larvae n = 10, stimulations n = 18, dorsal ****p < 0.0001, dorsal median of differences -0.3107, ventral ****p < 0.0001, ventral median of differences -0.3693. (Wilcoxon matched-pairs sign rank test for all comparisons).

B) Muscle activities change upon optogenetic inhibition of DDs in wild-type. The strain used here is ZM9313. Control group larvae n = 5, stimulations n = 10, dorsal p = 0.2754, dorsal median of differences -0.01106, ventral p = 0.1934, ventral median of differences -0.04058. ATR-fed group larvae n = 10, stimulations n = 19, dorsal ****p < 0.0001, dorsal median of differences 0.2121, ventral ****p < 0.0001, ventral median of differences 0.4171. (Wilcoxon matched-pairs sign rank test for all comparisons).

C) Muscle activities change upon optogenetic activation of DDs in *unc-25(e156)*. The strain used here is ZM10176. Control group larvae n = 5, stimulations n = 15, dorsal p = 0.4543, dorsal median of differences -0.04067, ventral *p = 0.0151, ventral median of differences -0.05336. ATR-fed group larvae n = 12, stimulations n = 34, dorsal p = 0.9865, dorsal median of differences -0.02089, ventral p = 0.5318, ventral median of differences -0.04229. (Wilcoxon matched-pairs sign rank test for all comparisons).

**2.4.8.5 Activation of D-type motor neurons inhibits A- and B-type motor neurons**

It was proposed by optogenetic behavioral experiments that A- and B-type motor neurons might receive negative feedback from D-type motor neurons in adults (*Schultheis et al.*, 2011). To examine if DD motor neurons inhibit DA and DB motor neurons, I expressed Chrimson in DD motor neurons by *Pptr-39*, and GCaMP in DA and DB motor neurons by *Pacr-2(s).*
Interestingly, with all-optical interrogation protocol, DA and DB motor neurons were indeed inhibited upon activation of DD motor neurons (Fig. 2.23A). Compared to responses of DA and DB motor neurons upon muscle relaxation, the inhibitory effect exerted from DD motor neurons is more stable. This result suggests that DD motor neurons can negatively feedback onto the DA and DB motor neurons.

2.4.8.6 Muscle relaxation partially inhibits A- and B-type motor neurons

It remains to be elucidated as to what caused dorsal body wall muscle inhibition upon activation of DD motor neurons. DB motor neurons are proprioceptive (Wen et al., 2012). DA motor neurons are probably proprioceptive as well based on calcium imaging data from 2.4.3.1. Therefore, it is possible that in experiments of 2.4.8.2, DD motor neurons inhibit the dorsal side by simply relaxing the ventral side and subsequently inhibiting DA and DB motor neurons proprioceptively.

If this is the case, null mutation of GABA receptors on the ventral body wall muscle would prevent inhibition of both ventral and dorsal muscle cells upon activation of DD motor neurons, because relaxation of the ventral body wall muscle must first take place before DA and DB motor neurons are inhibited proprioceptively. In *unc-49* null mutant, ventral body wall muscle cells can no longer be inhibited by GABA.

Furthermore, forced relaxation of body wall muscles should stably inhibit activities of DA and DB motor neurons. Directly inhibiting the muscle cells would induce the relaxation effect in the activation of DD motor neurons. If the hypothesis is correct, then DA and DB motor neurons would be inhibited upon muscle relaxation.

By expressing GtARC2 in body wall muscle cells, I artificially relaxed the body wall muscles and recorded the changes of DA and DB motor neurons. Although some DA and DB motor neurons showed reduced activities, a significant portion of them failed to be inhibited upon this manipulation (Fig. 2.23B). This result suggests that relaxation of muscle cells is not the major driving force to inhibit DA and DB motor neurons.
2.4.8.7 Activation of D-type motor neurons partially inhibits A- and B-type motor neurons in GABA ionotropic receptor mutant unc-49(e407)

Since proprioception change is not stably inhibiting DA and DB motor neurons, the more likely mechanism to DD motor neurons to inhibit DA and DB motor neurons is via GABA spillover. If this is the case, then disrupting GABA receptors on the ventral body wall muscle should not abolish the inhibitory effect from DDs onto DAs and DBs. Therefore, I tested if DDs can still inhibit DAs and DBs in unc-49(e407) mutant, which eliminates the only known excitatory GABA receptor expressed by ventral body wall muscle cells (Gally and Bessereau, 2003).

DD motor neurons partially inhibited DAs and DBs in unc-49(e407) (Fig. 2.23C). The inhibitory effect was slightly stronger than that of muscle relaxation. This result suggests that without first relaxing the muscles, DDs can still act on the DAs and DBs, although at a lower efficiency.

2.4.8.8 Activation of D-type motor neurons partially inhibits A- and B-type motor neurons in GABA metabotropic receptor mutant gbb-2(tm1165)

In hippocampal slice, it was shown that GABA can act on GABA_B receptors in neighboring terminals by spillover (Isaacson et al., 1993; Scanziani, 2000). There are metabotropic GABA_B receptors expressed on DA and DB motor neurons, GBB-1 and GBB-2 (Dittman and Kaplan, 2008). It was proposed by that GBB-1 and GBB-2 might mediate the negative feedback from D-type motor neurons to A- and B-type motor neurons in adult C. elegans (Dittman and Kaplan, 2008; Schultheis et al., 2011). However, direct evidence for this notion is lacking.

I directly tested if the negative feedback from DD motor neurons to DA and DB motor neurons are disrupted without GBB-1 and GBB-2 by all-optical interrogation. Because GBB-1 and GBB-2 forms functional heterodimers, mutation of either gene would disrupt the function of the receptor (Dittman and Kaplan, 2008).

In null mutant gbb-2(tm1165), the inhibitory effect from DD motor neurons to DA and DB motor neurons was partially disrupted (Fig. 2.23D). This result suggests that GBB-1/GBB-2 complex participates in the inhibition from DD onto DA and DB motor neurons.
Figure 2.23 DD motor neurons silence DA and DB motor neurons primarily through GABA signaling

A) DAs and DBs activities change upon optogenetic activation of DDs in wild-types. Please note that this is not a measurement of muscle activity as shown in other figures, but neuronal activity. The strains used here is ZM10393. ATR-fed larvae n = 4, stimulations for all DAs and DBs n = 28, ****p < 0.0001, median of differences -0.3133.

B) DAs and DBs activities change upon optogenetic inhibition of muscles in wild-types. The strain used here is ZM10585. ATR-fed larvae n = 9, stimulations for all DAs and DBs n = 209, ****p < 0.0001, median of differences -0.1613.

C) DAs and DBs activities change upon optogenetic activation of DDs in unc-49(e407). The strain used here is ZM10440. ATR-fed larvae n = 14, stimulations for all DAs and DBs n = 246, ****p < 0.0001, median of differences -0.2027.

D) DAs and DBs activities change upon optogenetic activation of DDs in gbb-2(tm1165). The strain used here is ZM10673. ATR-fed larvae n = 6, stimulations for all DAs and DBs n = 137, ****p < 0.0001, median of differences -0.1630.
2.4.8.9 Activation of D-type motor neurons preferentially inhibits dorsal muscle in *unc-49(e407)*

UNC-49 serves as the inhibitory GABA receptor on *C. elegans* body wall muscles (Bamber et al., 1999; Bamber et al., 2005). It is known that the expression of UNC-49 is restricted to the ventral body wall muscle in L1 larval stage (Gally and Bessereau, 2003). If GABA is released onto DA and DB motor neurons by extra-synaptic transmission, activation of DD motor neurons in *unc-49* null mutant should still inhibit dorsal muscle cells. But if proprioception is underlying the inhibition of dorsal muscle caused by DD motor neurons, then dorsal muscle cells should not be inhibited in *unc-49* null mutant upon activation of DD motor neurons.

Indeed, the dorsal body wall muscle activity dropped upon activation of DD motor neurons in *unc-49(e407)* (Fig. 2.24A-B). The lack of inhibitory GABA receptors failed to block dorsal muscle inhibition caused by DD motor neurons. Interestingly, for the ventral body wall muscle, its activity also decreased. It might be because the underlying cellular mechanisms for ventral muscle excitability are also inhibited by GABA released from DD (GBB-1/2 are in fact expressed in many neurons, including premotor interneurons which will later be shown to provide excitability to ventral body wall muscles in L1 (Yemini et al., 2019)). This result indicates that the initiation of ventral muscle relaxation is probably not required for dorsal muscle inhibition upon DD motor neurons.

2.4.8.10 Activation of D-type motor neurons predominantly inhibits ventral muscle activities in *gbb-2(tm1165)*

It remains to be seen what the responses are for the body wall muscles upon activation of DD motor neurons in *gbb-2(tm1165)*. If the extra-synaptic transmission is underlying the inhibitory effect from DDs onto the dorsal side, then a much weaker dorsal inhibition should be found when activating DDs in *gbb-2(tm1165)*.

Indeed, activation DD motor neurons in *gbb-2(tm1165)* strongly inhibited the ventral side, and only slightly inhibited the dorsal side (Fig. 2.24C-D). The difference between the inhibitory effect on dorsal and ventral sides is significant. Compared to the same stimulation in wild-type
(Fig. 2.22A), this result shows that disruption of GBB-1/GBB-2 receptor complex largely prevented the inhibitory effect from DDs onto the dorsal side.

Together, it is concluded that GABA is inhibitory not excitatory to body wall muscles; DD motor neurons do not harbor other excitatory neurotransmitters that can elicit ventral body wall muscle excitability; DD motor neurons are inhibitory to the ventral side by directly inhibiting ventral muscle; DD motor neurons are inhibitory to the dorsal side by inhibiting DAs and DBs through extra-synaptic transmission of GABA via GBB-1/GBB-2 complex, as well as proprioceptive changes of muscle cells, with extra-synaptic transmission being the primary factor.

Figure 2.24 DD motor neurons silence dorsal body wall muscles primarily through GABA signaling

A) Muscle activities change upon optogenetic activation of DDs in *unc-49(e407)* shown in normalized GCaMP/RFP signals. The strain used here is ZM10441. Control group larvae n = 22, stimulations n = 37, dorsal p = 0.3777, dorsal median of differences -0.03230, ventral p =
0.7429, ventral median of differences -0.01344. ATR-fed group larvae n = 20, stimulations n = 51, dorsal ****p < 0.0001, dorsal median of differences -0.2965, ventral ****p < 0.0001, ventral median of differences -0.1749. (Wilcoxon matched-pairs sign rank test for all comparisons).

B) Muscle activities at start and end of stimulations in shown as GCaMP/RFP signals for control and ATR-fed groups separately. Control group larvae n = 22, stimulations n = 37, p > 0.9999, mean rank difference -0.1892, p > 0.9999, mean rank difference 2.243, p > 0.9999, mean rank difference 0.3514, p > 0.9999, mean rank difference -2.081. ATR-fed group larvae n = 20, stimulations n = 51, p > 0.9999, mean rank difference -0.05882, ****p < 0.0001, mean rank difference -52.22, **p = 0.0037, mean rank difference -40.02, p > 0.9999, mean rank difference 12.14 (Kruskal-Wallis test, Dunn’s multiple comparison test for all comparisons).

C) Muscle activities change upon optogenetic activation of DDs in gbb-2(tm1165) shown in normalized GCaMP/RFP signals. The strain used here is ZM10410. Control group larvae n = 6, stimulations n = 18, dorsal p = 0.6397, dorsal median of differences 0.02901, ventral p = 0.5509, ventral median of differences -0.05438. ATR-fed group larvae n = 7, stimulations n = 39, dorsal ****p < 0.0001, dorsal median of differences -0.1343, ventral ****p < 0.0001, ventral median of differences -0.4396. (Wilcoxon matched-pairs sign rank test for all comparisons).

D): Muscle activities at start and end of stimulations in shown as GCaMP/RFP signals for control and ATR-fed groups separately. Control group larvae n = 6, stimulations n = 18, p > 0.9999, mean rank difference -3.111, p > 0.9999, mean rank difference 2.778, p > 0.9999, mean rank difference -6.333, p = 0.4786, mean rank difference -12.22. ATR-fed group larvae n = 7, stimulations n = 39, p = 0.8763, mean rank difference 14.87, p = 0.0706, mean rank difference -25.77, ****p < 0.0001, mean rank difference -83.10, ***p < 0.0002, mean rank difference -42.46. (Kruskal-Wallis test, Dunn’s multiple comparison test for all comparisons).
2.4.9 **Cholinergic premotor interneurons activate ventral muscles via extra-synaptic transmission**

Since D-type motor neurons can act on A- and B-type motor neuron by extra-synaptic transmission, is it possible that certain neurons also act on the ventral body wall muscle cells by similar fashion? If such neurons exist, they should be excitatory and physically in proximity with ventral body wall muscle cells. The premotor interneurons AVA, AVD, AVE, AVB and PVC can satisfy these criteria.

2.4.9.1 **Proximity between cholinergic premotor interneurons and ventral body wall muscles**

Premotor interneurons AVA, AVD, AVE, AVB, and PVC are all cholinergic (Pereira et al., 2015). They send out long processes along the ventral nerve cord. These processes are axons where AVA, AVD, AVE synapse onto DA motor neurons, and AVB, PVC synapse onto DB motor neurons. The synapses between premotor interneurons and motor neurons are in close proximity to ventral body wall muscle (Fig. 2.25A).

With such organization, it is not impossible for acetylcholine released from premotor interneurons to be diffused onto extracellular space on the ventral nerve cord, and act on nearby ventral body wall muscle cells.

2.4.9.2 **Cholinergic premotor interneurons activities correlate with locomotion in both directions**

It is known that in adults, AVA, AVD, and AVE premotor interneurons direct backward locomotion, whereas AVB and PVC direct forward locomotion (Chalfie et al., 1985). But it remains to be seen whether these neurons together can cover both directional movements.

By expressing GCaMP under Ptwk-40(s) (Hung et al., in preparation), calcium signals in AVA, AVE and AVB premotor interneurons (plus an additional neuron in the tail DVA) can be visualized. Using the same setup as described in 2.4.3, activities of these neurons were recorded
during movements. The same MATLAB program used in 2.4.3 was employed to analyze activities of premotor interneurons.

By correlating calcium signals with directional movements, it was shown that AVA and AVE increased their activities during backward locomotion, whereas AVB were active during forward locomotion (Fig. 2.25B). Although the peaks of the GCaMP signals are normally reached with a delay after the onset of the directional movement, the initial rise or fall of GCaMP signals are in agreement with the behavioral changes. This is consistent with the delay observed in GCaMP dynamics (Chen et al., 2013).

Therefore, premotor interneurons correlate with both directional movements by respective neurons under physiological conditions. The prerequisite for premotor interneurons to provide stimulation to ventral body wall muscle cells in both forward and backward locomotion is met.

**2.4.9.3 Cholinergic premotor interneurons activation causes directional movements**

With correlations established, it remains to be seen if premotor interneurons can cause directional movements. I optogenetically activated AVA and AVB separately, and analyzed the velocity changes under these conditions. Consistent with their correlations, activation of AVA triggered backward locomotion, whereas stimulation of AVB induced forward locomotion (Fig. 2.25C).
**Figure 2.25 Premotor interneurons are candidate cells providing ventral body wall activity**

A) Anatomy of all premotor interneurons. The strain used here is N2 wild-type. The order of neurons displayed are AVA, AVD, AVE, AVB, and PVC. Each panel consists of two viewing angles, with the orthogonal view at the top, side view bottom. Side view is zoomed in. Muscle cells are in yellow, interneurons in green. Presynaptic sites are red and visible from the side view. Postsynaptic sites are teal. The white arrows indicate the locations of presynaptic sites. With the exception of AVE which terminates its process at around 1/3 of the body wall, all other premotor interneurons extend their processes throughout the ventral body wall muscle ([Credit: Witvliet, Mulcahy, et al.](#)).

B) Correlation between activities of premotor interneurons and directional movements. The strain used here is ZM10281. *Top:* Traces of AVA and AVB during locomotion from the same recording, with backward locomotion highlighted in grey. *Middle panels:* Activities of AVA and AVB pooled in forward and backward locomotion separately. The traces are aligned by the initial change of directional movements. *Bottom panels:* Comparison between activities in forward and backward locomotion for AVA and AVB respectively. For AVA, n = 19, ****p < 0.0001, median of differences 0.3234. For AVB, n = 19, ***p = 0.0006, median of differences -0.06087 (Wilcoxon matched-pairs sign rank test).

C) Velocity changes upon optogenetic stimulations of premotor interneurons. The strains used here are ZM10206 and ZM7419. *Top panels:* sample velocity profiles for larvae treated with four epochs of optogenetics stimulations for AVA and AVB activations respectively. Upper panels show ATR-fed larvae, lower control. *Bottom panels:* Comparisons between average velocities during non-stimulation and stimulation phases for AVA and AVB activation respectively. AVA activation control group n = 12, p = 0.2334, median of differences -3.286μm/s. AVA activation ATR-fed group n = 15, ****p < 0.0001, median of differences -39.62 μm/s. AVB activation control group n = 9, p = 0.4961, median of differences -7.060 μm/s. AVB activation ATR group n = 15, ****p < 0.0001, median of differences 42.50 μm/s.
2.4.9.4 Activation of cholinergic premotor interneurons activates ventral muscle

To test if premotor interneurons can indeed cause ventral muscle excitation, Chrimson or ChR2 was expressed in AVA or AVB. To avoid the masking effect of GABA from DD motor neurons, *unc-25(e156)* mutant was used. I activated AVA and AVB in *unc-25(e156)* and monitored subsequent muscle activity changes respectively.

2.4.9.4.1 Activation of AVA stimulates ventral body wall muscle cells

To specifically express Chrimson in AVA, the same intersectional Cre-loxP system as described 2.4.6.3 in was utilized. Because *Prig-3* is expressed in AVA plus a number of other neurons ([Schwarz et al., 2009](#)), two plasmids *Prig-3::loxP::EBFP::STOP::loxP::Chrimson::wCherry* and *Ptwk-40(s)::Cre* were co-injected in order to create specific expression for AVA. Caution should be exercised here as additional neurons, DVA and a couple of pharyngeal neurons, are also labelled.

Strikingly, upon AVA activation in *unc-25(e156)*, the ventral body wall muscle cells were stimulated (Fig. 2.26A). The pattern of ventral body wall muscle activity was continuous along the anterior-posterior axis. The dorsal body wall muscle activity was also slightly increased, but its segmented activity pattern was in contrast from the continuum of activity on the ventral side. This difference is critical, as DA motor neurons only become activated when they are positioned around the dorsal bent regions (see 2.4.3.1). Because the total number of dorsal grooves remain roughly unchanged during the stimulation, the average activity level on the dorsal side only increased slightly. This result suggests that AVA could activate ventral body wall muscle cells.

2.4.9.4.2 Activation of AVB stimulates ventral body wall muscle cells

To specifically express ChR2 in AVB, *Psra-11* is used ([Altun-Gultekin et al., 2001](#)). Although AIY is also labelled by this promoter, the only neuron labelled by *Psra-11* that extends the processes along the ventral nerve cord is AVB.
Reminiscent of AVA, activation of AVB in *unc-25(e156)* also led to ventral muscle excitation in a continuous pattern, and slight increase for dorsal muscle in a segmented pattern (Fig. 2.26B). Ventral muscle excitation here is unlikely caused by motor neurons because the patterns of activity are decoupled between dorsal and ventral muscles.

Together, both activation of AVA and AVB premotor interneurons can stimulate ventral body wall muscles along the anterior-posterior axis.

### 2.4.9.5 Inhibition of cholinergic premotor interneurons inhibits overall muscle activity

With the sufficiency established, it remains to be seen whether the cholinergic premotor interneurons are necessary for the ventral body wall muscle activities. Using cell-specific expression of GtACR2 in AVA and AVB with Cre-loxP system, I was able to specifically inhibit AVA and AVB.

Remarkably, upon AVA and AVB inhibition, both dorsal and ventral body wall muscle cells lost their activities (Fig. 2.26C). This result suggests that AVA and AVB are necessary for ventral body wall muscle activities, and since DAs and DBs are downstream to AVA and AVB, they are also required for dorsal muscle activities.
Figure 2.26 Premotor interneurons are necessary and sufficient for ventral body wall muscle activity in optogenetic manipulations

A) Muscle activities change upon optogenetic activation of AVA in *unc-25(e156)*. The strain used here is ZM10311. The layout is the same as in Fig. 2.4, hereinafter inclusive. Control group larvae n = 17, stimulations n = 51, dorsal p = 0.4426, dorsal median of differences -0.03122, ventral p = 0.8416, ventral median of differences -0.02823. ATR-fed group larvae n = 12, stimulations n = 43, dorsal ****p < 0.0001, dorsal median of differences 0.2645, ventral ****p < 0.0001, ventral median of differences 0.3963. (Wilcoxon matched-pairs sign rank test for all comparisons).

B) Muscle activities change upon optogenetic activation of AVB in *unc-25(e156)*. The strain used here is ZM10174. Control group larvae n = 10, stimulations n = 44, dorsal ***p = 0.0002, dorsal median of differences -0.04421, ventral **p = 0.0079, ventral median of differences -0.08847. ATR-fed group larvae n = 16, stimulations n = 56, dorsal ***p = 0.003, dorsal median of differences 0.04255, ventral ****p < 0.0001, ventral median of differences 0.4437. (Wilcoxon matched-pairs sign rank test for all comparisons).

C) Muscle activities change upon optogenetic inhibition of AVA, AVB in wild-type. The strain used here is ZM10552. Control group larvae n = 6, stimulations n = 28, dorsal p = 0.7966, dorsal median of differences -0.02450, ventral p = 0.9375, ventral median of differences 0.003995. ATR-fed group larvae n = 13, stimulations n = 49, dorsal ****p < 0.0001, dorsal median of differences -0.2317, ventral ****p < 0.0001, ventral median of differences -0.1892. (Wilcoxon matched-pairs sign rank test for all comparisons).

2.4.9.6 Ablation of cholinergic premotor interneurons preferentially reduce ventral muscle activity

To further verify that cholinergic premotor interneurons are necessary for ventral muscle activities, I used light-induced cell ablation with miniSOG protein to ablate them and record muscle activities afterwards (*Qi et al., 2012*). miniSOG protein is expressed in all cholinergic premotor interneurons with *Pnmr-1* and *Plgc-55* (*Kawano et al., 2011*).
2.4.9.6.1 Muscle output is dorsally biased after premotor interneuron ablation in wild-type

After miniSOG-induced ablation of premotor interneurons, the wild-type L1 larvae were coiled to the dorsal side on the plate. After subjecting them to M9 liquid, these larvae preferred thrashing on the dorsal side.

Interestingly, the muscle activities in L1 larvae with premotor interneurons ablation showed strong activities on the dorsal side, and dramatic reduction of the ventral muscle activity (Fig. 2.27A). This is consistent with the behavioral assay where strong dorsal bend posture was found in premotor interneurons ablate L1 larvae (Fig. 2.27B).

However, the strong dorsal side activity might be responsible for the loss of ventral muscle activity. It is reported that the excitatory motor neurons harbor intrinsic activities, and these activities are manifested only after ablation of premotor interneurons (Gao et al., 2018). This effect can explain why the dorsal muscle activities remain robust after ablation of premotor interneurons in L1 larvae, because DA and DB motor neurons innervate the dorsal side, and become highly active after ablation of premotor interneurons. But if this is the case, then DD motor neurons should also be activated in these larvae, since DD motor neurons are downstream of DA and DB motor neurons. If DD motor neurons are active, then the ventral side should be silenced even if there are other excitatory inputs. Therefore, I cannot conclude simply from this result that the premotor interneurons are providing excitability to the ventral body wall muscle in L1 larvae.

2.4.9.6.2 Muscle activities change after premotor interneurons ablation on agar pad

To preclude the interference of DD motor neurons, I used unc-25(e156) again. With no GABA, DD motor neurons do not exert any effect on the body wall muscles (see 2.4.8.4).

Before ablation of premotor interneurons, unc-25(e156) L1 larvae prefer thrashing to the ventral side (Fig. 2.27D). Surprisingly, after ablation of premotor interneurons in unc-25(e156), L1 larvae coiled to the dorsal side on plate and preferred to thrash to the dorsal side in M9 liquid (Fig. 2.27D).
The muscle activities after ablation of premotor interneurons in *unc-25(e156)* were consistent with the behavioral assay. The dorsal side still showed robust activity, whereas the ventral side had significantly reduced activity (Fig. 2.27C).

Therefore, even in the absence of GABA, the ventral muscle activity is significantly reduced after ablation of premotor interneurons in L1 larvae. Together, these results suggest that premotor interneurons are required for the ventral muscle excitability.

2.4.9.7 Null mutation of acetylcholinesterases leads to anterior-posterior expansion of ventral muscle activities

How do premotor interneurons stimulate ventral muscle without direct NMJs or gap junctions? I considered the possibility of extra-synaptic neurotransmission between premotor interneurons and ventral muscle cells. If extra-synaptic neurotransmission is underlying ventral muscle excitability, disrupting hydrolysis of neurotransmitters should lead to their enhanced diffusion along the surface of the muscular membrane. This post-synaptic potentiation (Hartzell et al., 1975; Magleby and Terrar, 1975) should result in spatially expanded muscle activity pattern along the anterior-posterior axis.

All premotor interneurons are cholinergic (Pereira et al., 2015). Four genes, namely *ace-1* to *ace-4*, encode acetylcholinesterases in *C. elegans* (Combes et al., 2003; Culotti et al., 1981; Johnson et al., 1981). Among them, *ace-1* and *ace-2* are the major catalytic activity contributors, *ace-3* is localized only on the dorsal muscle, and *ace-4* has no catalytic activity (Combes et al., 2003; Culotti et al., 1981; Johnson et al., 1981). Double null mutation of *ace-1;ace-2* should eliminate acetylcholine hydrolysis activities on ventral muscle, but leave ACE-3 on the dorsal muscle. As such, it is expected that ventral muscle activity should be more continuous along the anterior-posterior axis than the dorsal muscle in *ace-1;ace-2* null mutant L1 larvae.

To test this hypothesis, I examined the muscle activity patterns for double null mutant *ace-1(p1000);ace-2(g72)* (Culotti et al., 1981; Johnson et al., 1981). Indeed, *ace-1(p1000);ace-2(g72)* displayed more expanded activity pattern on ventral muscle compared to dorsal muscle in L1 larvae, similar to *unc-25(e156)* and *unc-49(e407)* (Fig. 2.27E-F). This indicates the acetylcholine build-up in the extracellular matrix is diffusing further away without acetylcholinesterases and acts on distant ventral muscle cells.
These results all together suggest the role of extra-synaptic neurotransmission from cholinergic premotor interneurons underlying ventral muscle excitability in L1 *C. elegans*.

2.4.9.8 Visualization of acetylcholine dynamics near body wall muscle cell surface

To definitively show the spill-over of acetylcholine, I tested the recently-developed *in vivo* fluorescent acetylcholine indicators GACCh2.0 and GACCh4.3 (*Jing et al., 2018*). I also tested another fluorescent acetylcholine indicator iAChSnFR (Borden *et al.*, in preparation). Unfortunately, after several rounds of improvement, no reliable fluorescent signal from acetylcholine can be detected with any of these indicators in *C. elegans* (data not shown).
Figure 2.27 Premotor interneurons are required for ventral body wall muscle activity by acetylcholine extrasynaptic transmission

A) Muscle activities after premotor interneurons ablation in wild-type. The strain used here is ZM7465. Angle change is on the left, and muscle activities are on the right for dorsal and ventral sides. Head is oriented on top, tail bottom. The boundary between head and body wall is marked by a dashed line in each plot.

B) Preference of dorsal and ventral bending on plate assay for premotor interneurons-ablated wild-type larvae. The strain used here is ZM7971. Premotor interneurons-ablated larvae are transferred onto NGM medium by an M9 droplet, and manually scored for the time spent in dorsal, neutral, and ventral postures. n = 10, **p = 0.0039, median of differences -0.2139 (Wilcoxon matched-pairs sign rank test for all comparisons, because the only comparison is between dorsal and ventral bending proportions).

C) Muscle activities after premotor interneurons ablation in unc-25(e156). The strain used here is ZM10484. Top panels: Angle change is on the left, and muscle activities are on the right for dorsal and ventral sides. Head is oriented on top, tail bottom. The boundary between head and body wall is marked by a dashed line in each plot. Bottom right panels: Overlapped traces for average dorsal and ventral activities from all recordings, with mean values displayed in darker color and thicker lines. Premotor interneurons-ablated larvae are shown in orange, control in grey. Bottom left: Comparison between dorsal and ventral body wall muscle activities averaged for each recording for premotor interneurons ablated larvae. n = 10, **p = 0.0020, median of differences -0.1360. (Wilcoxon matched-pairs sign rank test).

D) Preference of dorsal and ventral bending in liquid thrashing assay for premotor interneurons-ablated unc-25(e156) larvae. The strain used here is ZM10484. Top: Bending distribution for control group. n = 10, **p = 0.0020, median of differences 0.2133. Same data as in Fig. 2.7A upper panel. Bottom: Bending distribution for premotor interneurons-ablated group. n = 11, ***p = 0.0010, median of differences -0.7533 (Wilcoxon matched-pairs sign rank test for all comparisons, because the only comparison is between dorsal and ventral bending proportions).

E) Muscle activity patterns in ace-1(p1000);ace-2(g72). The strain used here is ZM10312. Angle change is on the left, and muscle activities are on the right for dorsal and ventral sides. Head is
oriented on top, tail bottom. The boundary between head and body wall is marked by a dashed line in each plot.

F) Correlation between muscle activity patterns with angle patterns in ace-1(p1000);ace-2(g72). The strain used here is ZM10312. n = 17, ***p = 0.0008, median of differences -0.1946.
2.4.10 Model for L1 larvae functional motor circuit

With all data presented, the motor circuit of L1 larval *C. elegans* thus consists of synaptic and extra-synaptic connections from motor neurons DAs, DBs, DDs, sublateral motor neurons SIAs, SIBs, and premotor interneurons to muscle cells (Fig. 2.28).

DAs, DBs, DDs, and premotor interneurons together function in both directional movements, whereas SIAs and SIBs only weakly associate with forward locomotion. The asymmetric wiring of motor neurons is largely compensated by the extra-synaptic transmissions. Premotor interneurons provide the excitability to the ventral body wall muscles by extra-synaptic transmission of acetylcholine in both forward and backward movements. DDs negatively feedback onto DAs and DBs by extra-synaptic transmission of GABA via metabotropic receptors GBB-1/2 and proprioceptive coupling, resulting in indirect inhibition on the dorsal body wall muscles.

Those active DAs or DBs cause segmented dorsal muscle activity pattern, and subsequently stimulate their downstream DDs, resulting in cross-inhibition of the same segment on the ventral side through ionotrophic receptor UNC-49. This cross-inhibition intercalates the continuous ventral muscle activity pattern caused by the long processes of premotor interneurons, and thus forms a complementary muscle activity pattern on the ventral body wall muscle.

This work provides insights into how diverging neural circuits can converge at functional level, and identifies the direct regulation of muscle activities by premotor interneurons via extrasynaptic transmission. The similar methodology should shed light on the 'invisible' wiring diagrams in other working neural networks.
Figure 2.28 *C. elegans* L1 larvae functional motor circuit generates balanced output

The asymmetric motor circuit of DA-DB-DD is balanced by extrasynaptic transmission from cholinergic premotor interneurons and GABAergic DD motor neurons. Together, they also generate the complementary patterns on dorsal and ventral sides. In the larva depiction, muscles are labelled in yellow, excitatory premotor interneurons (eINs) in red, excitatory motor neurons (DA, DB) in green, inhibitory motor neurons (DD) in blue. The non-active DA-DB-DD unit is labeled in grey. Muscle activities are shown in red on the muscle. The inlet shows detailed connectivities for one DA-DB-DD unit together with premotor interneurons, following the style in Figure 1.2C.
3 Discussions and future perspectives

3.1 Discussions

3.1.1 Divergent neural circuits converge at the output level

By combining a simple all-optical interrogation method with neurophysiology and genetics, we unraveled the unexpected dual role of premotor interneurons, the importance of extra-synaptic transmission of acetylcholine and GABA, and thoroughly characterized previously elusive motor circuit in vivo at L1 larval *C. elegans*. Previous electrophysiology studies showed that motor circuit wiring diagrams differ between the leech and lamprey, nudibranch molluscs *Melibe leonina* and *Dendronotus iris*, but the leech and lamprey exhibit similar sinusoidal swimming pattern, and *M. leonina* and *D. iris* share resembling flexions ([Mullins et al., 2011]; [Sakurai and Katz, 2017]). However, from a developmental perspective, studies on how juvenile animals adapt to an underdeveloped motor circuit while preserving the stereotyped locomotor patterns are lacking. Our study revealed the mechanistic details of how anatomically different neural circuits achieve similar functional output in an intact and developing animal system. I demonstrated the adaptability and inclusiveness of functional neural circuits.

3.1.2 Connectome is a blueprint that can guide the neural circuit study

In this work, I relied on the connectomics data to identify candidate cells that contribute to the body wall muscle activities. Many connectivities are experimentally validated at the functional level. For example, DD motor neurons are post synaptic to cholinergic DA and DB motor neurons. Bidirectional manipulations resulted in opposite DD activity changes, which are predicted by the connectivities. It reiterates the importance of understanding the structure to study the function in biological systems. But the connectivity is not the whole picture. Whether the connectivity is excitatory or inhibitory, and how the network operates and dynamically changes in live animals under different circumstances require functional studies.

In other species, the complete connectome is still on its way. Partial reconstructions however do exist. An elegant example is the study of neurons mediating startle response in *Drosophila* ([Ohyama et al., 2015]). The authors reconstructed a network of neurons that control the startle response in *Drosophila* larva. They then combined optogenetics and electrophysiology to probe
how this neural circuit operates at functional level. Their study identified the subpopulations of neurons at work in different scenarios. This result again illustrates the unmatched significance of connectome in neural circuit studies, but at the same time points to the necessity of functional studies that completes our understanding of the network.

3.1.3 Extra-synaptic transmission plays a critical role in neuron-neuron, neuron-muscle communications

Another layer of functional neural circuit is the non-synaptic connectivity, which is not visible from the connectome. It is well-established that the monoamines and neuropeptides can diffuse to distance target neurons. It is still debatable as to what extent the extra-synaptic transmission of fast neurotransmitters plays its role in neural circuits. Previous studies suggest that the fast neurotransmitters are quickly digested or transported back, and therefore cannot diffuse too far from the synaptic sites.

In this study, not only neurons are communicating between themselves through extra-synaptic transmission of fast neurotransmitters, but also neurons and muscle cells. The extra-synaptic transmission is so important in this case that it balances an asymmetric neural circuit, and results in symmetric output.

With a complete connectome, *C. elegans* is perhaps the most ideal model to study extra-synaptic transmission, because by reasoning, if a connectivity is experimentally demonstrated but not shown in the connectome, then this connectivity should be extra-synaptic. A number of studies in *C. elegans* were carried out in this fashion, and largely relied on the correlation of neuronal activities in mutants. Unfortunately, the definitive proofs would require causality as well as the visualization of neurotransmitter dynamics. With the all-optical interrogation protocol developed in my work, it would be much easier to probe neural circuits with causal relationship. The recent advances in fluorescent indicators for neurotransmitters will ultimately address the role of extra-synaptic transmissions.
3.1.4 Excitatory premotor interneurons assume dual roles by bypassing the motor neuron layer and directly regulate muscle cells

This study also revealed a surprising role for premotor interneurons: motor neurons. These interneurons can directly regulate muscle cells. This is consistent with the compressed layers of neurons in *C. elegans* (Wen et al., 2018; Wen et al., 2012; Zhen and Samuel, 2015). Additionally, cell economy is achieved by this mechanism.

In higher animals, mesencephalic locomotor region and nuclei in the brainstem send descending command to the central pattern generators in the spinal cord to regulate the initiation, termination, speed, gait, direction of the locomotion (Bouvier et al., 2015; Caggiano et al., 2018; Capelli et al., 2017; Cregg et al., 2019; Shik and Orlovsky, 1976; Shik et al., 1966). With a simplistic functional motor circuit, L1 *C. elegans* couples the initiation and termination of dorsal and ventral muscles by the dual role of descending premotor interneurons. These premotor interneurons also innervate the central pattern generators, i.e. motor neurons in *C. elegans*. Such resemblance in the logic of motor circuit is striking. It will be interesting to see if this phenomenon is conserved in other animal systems at different developmental stages.

3.1.5 Inhibitory motor neurons also have dual roles

The surprising result that DDs not only inhibit ventral but also dorsal body wall muscles is highly interesting. The apparent asymmetry for the wiring of DDs is symmetrized by a combination of synaptic and extrasynaptic transmissions. With dyadic synaptic connections, DAs and DBs can multitask by assuming the role motor neurons (by innervating dorsal muscles) and interneurons (by innervating DDs). DDs can also multitask by innervating ventral muscles and extrasynaptically inhibit DAs and DBs. Therefore, the inhibitory DDs are both motor neurons and interneurons.

The multitasking capacities for L1 DAs, DBs, DDs, and premotor interneurons again show the compressed layers in *C. elegans* (Zhen and Samuel, 2015).
3.1.6 GABA is inhibitory to body wall muscles

Studies from Michael Koelle’s lab showed that by pharmacologic manipulation, GABA might be excitatory to body wall muscles for newly-hatched L1 larvae in *C. elegans* (Han et al., 2015). From this thesis, I did not observe excitatory effects from GABA or DD motor neurons by behavior, calcium imaging, or all-optical interrogations. Possibility exists that because I used 5-7hr post-hatching L1 larvae throughout the experiments, I missed the early stage when GABA was excitatory to body wall muscles. Yet, in early L1s, the same work showed that GABA was inhibitory by optogenetic behavioral experiments (Han et al., 2015). Therefore, it is highly likely that GABA is inhibitory to body wall muscles throughout L1 stage. The pharmacological manipulations in Han *et al.* might have non-specific targets.

3.1.7 Asymmetry of molecular mechanisms

The symmetric dorsal/ventral output in L1 larvae is a result of synaptic and extrasynaptic transmissions. This is enabled in part by the asymmetric expression of ionotropic GABA receptor UNC-49 and acetylcholinesterase ACE-3 in L1 stage. It appears that the asymmetric expression of these molecules is only required during the juvenile stages.

It is remarkable that UNC-49 is only restricted to the ventral body wall muscle during L1 stage, and then becomes symmetrically expressed on both dorsal and ventral sides (Gally and Bessereau, 2003). This layout allows GABA to only function directly on the ventral muscle. ACE-3 is only expressed on the dorsal body wall muscles during juvenile stages, and then becomes almost invisible in adult stage (Combes et al., 2003). The dorsal ACE-3 could restrict the diffusion of acetylcholine to local releasing sites, since DAs and DBs directly innervate the dorsal muscle. Without the ventral counterpart of ACE-3, acetylcholine could diffuse further on the ventral side. And this absence of ACE-3 facilitates the extrasynaptic transmission of acetylcholine from premotor interneurons on the ventral side. Together, the asymmetric expression of these molecules compensate the asymmetric wiring diagrams specific to L1 stage.
3.1.8 DAs and DBs are unlikely to directly activate ventral body wall muscles in L1 larvae

From Fig. 2.6, it was found that optogenetic activation of DAs and DBs stimulated both dorsal and ventral body wall muscles in *unc-25(e156)* (Fig. 2.6G). Can DAs and DBs activate ventral body wall muscles directly? Volume transmissions of acetylcholine from the long ventral processes of DAs and DBs (Fig. 2.1I-K) may account for this. Also, as already discussed in the 2.4.6.7, ephaptic coupling which is a direct interaction of electrical fields between excitable cells could be possible as well. Membrane depolarization of the long processes of DA and DB on the ventral nerve cord might directly stimulate adjacent ventral muscle cells through extracellular fields.

Several lines of evidence are not in favor of the above hypotheses. Detailed analyses showed that the ventral body wall muscle was activated with a significant delay (Fig. 2.6I) and that without optogenetic manipulation, DAs and DBs preferentially activated the dorsal side (Fig. 2.6H). These results suggest that the ventral muscle could be indirectly activated in the all-optical interrogation experiments.

Additionally, from calcium imaging experiments, the activities of DAs and DBs always correlate with dorsal contraction but not ventral contraction (Fig. 2.3A-D). If DAs and DBs can directly activate the ventral muscle, then they should also correlate with ventral contraction.

Further, in *unc-25(e156)* mutants, muscle activity patterns on dorsal and ventral sides are different (Fig. 2.8B). The ventral muscle activities are very continuous, whereas the dorsal muscle activities are segmented (Fig. 2.8B). If DAs and DBs can directly activate the ventral muscle, one active DA or DB cell can simultaneously stimulate its connected dorsal and ventral muscle cells in the same body segment in *unc-25(e156)*, because the dorsal and ventral processes of DAs and DBs are in very much aligned along the D/V axis (Fig. 2.1I-K). This would create a peristaltic and symmetrical muscle activity waves along the anterior-posterior axis in *unc-25(e156)*, which is clearly not the case.

Additionally, if DAs and DBs directly activate the ventral muscle, their downstream DDs would cancel out this effect by inhibiting the same ventral segment that DAs or DBs activate in the wild-type L1 larvae. This would result in not ventral muscle activity.
Further, inhibition of DAs and DBs could not inhibit the ventral body wall muscles (Fig. 2.6D).

But what if DAs, DBs, and premotor interneurons all activate the ventral muscle? If this is the case, then after ablation of premotor interneurons in \textit{unc-25(e156)}, the ventral body wall muscle would be active and share the same muscle activity pattern as in the dorsal muscle. Compared with the data in Fig. 2.9I, it is clearly not supported.

With these, I would rule out the possibility that DAs and DBs can directly activate ventral body wall muscles. A possible pathway for the indirect activation of ventral body wall muscle in Fig. 2.6G is: DAs/DBs activation $\rightarrow$ dorsal muscle activation $\rightarrow$ mechanosensitive sensory neurons activation $\rightarrow$ premotor interneurons activation $\rightarrow$ ventral body wall muscle activation (since there is no GABA from DDs).

\subsection*{3.1.9 Larvae hatch as soon as they possess mobility}

Why does \textit{L1 C. elegans} use this seemingly unorthodox strategy for movement? One possibility is that, the embryos need to hatch as soon as they are able to move, so that they can find food to grow. To develop into a symmetric motor circuit, just like \textit{L2 C. elegans}, 12 VA, 11 VB, and 13 VD motor neurons have to be born, differentiate and form synapses with premotor interneurons. Cell division, differentiation and synapse formation all consume energy and take time. If the minimum number of cells for mobility is reached, then the embryos should immediately hatch to forage. Besides cell economy, hatching at the earliest possible time also reduces the risk of being eaten by predator nematodes.

Another reason might be that at \textit{L1} larval stage, the size of the animal is small. The compact structure of \textit{L1} allows “fast” neurotransmitters to reach extrasynaptic targets upon release. As the body size grows, the distance for the neurotransmitters to reach the target might increase accordingly. Therefore, additional motor neurons are born at later larval stage to “amplify” the signal from premotor interneurons.

As a result, \textit{L1 C. elegans}, as soon as the minimally required cellular structures formed, combine synaptic with extra-synaptic transmission to move in a sinusoidal wave-like form.
3.2 Future perspectives

3.2.1 All-optical electrophysiology will enable high-throughput neural circuit mapping with unparalleled temporal resolution

Calcium dynamics are essentially an approximation of cellular activities. Calcium is involved in highly diversified signaling pathways that are unrelated to cellular activities. Calcium dynamics is also a low-band pass filter that filters out high-frequency cellular activities. The ultimate readout for the cellular activity is membrane potential. Membrane potential fluctuations are much faster than calcium dynamics. Recent advances in voltage imaging bear the potential of bringing new approaches to probe the neural circuits all-optically. Genetically-encoded voltage indicators (GEVIs) such as ArcLight, Arch(D95N), QuasAr1, ASAP1, Archon1, alter their emission upon electric potential change, and thus can be used to indicate membrane potential (Hochbaum et al., 2014; Jin et al., 2012; Kralj et al., 2011; Piatkevich et al., 2018; St-Pierre et al., 2014). The limitation of these indicators is the relatively low signal-to-noise ratio compared with GECIs.

Similar to all-optical interrogation with GECIs, red-shifted or infrared-shifted GEVIs such as QuasAr2-Citrine and paQuasAr3 are used in combination with opsins to probe the neural circuits in recent reports (Adam et al., 2019; Fan et al., 2018). The difference in excitation spectra between GEVIs and opsins allows independent excitation of these proteins, which enables all-optical interrogation of neural circuits with temporal resolution similar to electrophysiology. However, it remains to be seen if these GEVIs can continue to be improved in fluorescence yields.

All-optical electrophysiology could be used to probe the neural circuits with dramatically improved temporal resolution. Frequency of membrane potential fluctuations, which might carry information, can also be reported with high-fidelity in defined neuronal populations in vivo. This approach will eventually unveil the mechanisms of neural circuits operation across species.
3.2.2 Acetylcholine fluorescent indicators will definitively characterize the acetylcholine dynamics on the ventral muscle cell surface

The fluorescent indicators for neurotransmitters can allow the visualization of neurotransmitters. Nonetheless, for all the existing fluorescent indicators of acetylcholine, GACH2.0 and GACH4.3 (Jing et al., 2018), and iAChSnFR (Borden et al., unpub.), I did not detect convincing fluorescent signal dynamics near the muscle cell surface.

Novel fluorescent indicators with high fluorescence yields will eventually solve this issue. Before that, I can only rely on neural circuit perturbations in combination with genetics to deduce the role of extra-synaptic transmission. How long can fast neurotransmitters diffuse before diluted below the threshold for their receptors? How does the diffusion pattern change in different body postures, across developmental stages? How reproducible is each extra-synaptic releasing event? Does the distribution of acetylcholinesterase change during locomotion, and if so, how does the extra-synaptic transmission change accordingly? Is the pattern of expression for receptors optimal for the extra-synaptic transmission dynamics? These are the questions to be unraveled when a high signal-to-noise ratio fluorescent indicator for fast neurotransmitters is developed.

3.2.3 Extrasynaptic transmission at later stages

It remains to be seen if extrasynaptic transmission of “fast” neurotransmitters plays an important role in C. elegans beyond L1. Is it just in L1 larvae that extrasynaptic transmission of “fast” neurotransmitters so important? What happens to the ventral muscle activity if VAs, VBs, and VDs are not functioning in adult C. elegans? Can premotor interneurons continue to activate ventral muscles by extrasynaptic transmission in this case?

With the finding that metabotropic receptors for acetylcholine and GABA are widely expressed in the nervous system (Yemini et al., 2019), it is anticipated that more extrasynaptic transmission pathways for acetylcholine and GABA be identified in the future.
3.2.4 Extra-synaptic transmissions across species

The role of extra-synaptic transmissions of fast neurotransmitters in the nervous system largely remains unexplored in many species. Does extra-synaptic transmission of fast neurotransmitters play an essential role in neurotransmission of other species? Does the volume and tortuosity of extracellular matrix affect the extra-synaptic transmissions differentially in different species? How does extra-synaptic transmission affect neural computations? Can I incorporate extra-synaptic transmission in artificial neural networks?

3.2.5 Locomotion control

With the missing link for ventral excitation and dorsal inhibition identified in L1 C. elegans, many questions regarding locomotion control still remain to be unanswered. Do other neurotransmitters participate in regulating muscle activities? Do they use similar mechanisms? What initiates forward and backward locomotion, and what drives the transition between forward and backward locomotion for the worm? What terminates the worm locomotion? What regulates the speed, orientation, undulation frequency, amplitude during worm locomotion?

Answers to these questions would significant enhance our understanding of the nervous systems and motor control.


References


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