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# Chapter 1: Elegantly

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## Abstract

With a compact nervous system and nearly complete knowledge of its connectivity, genome, and developmental lineage, *Caenorhabditis elegans* offers some open questions about the neuronal mechanisms that underlie its locomotion. The nematode undulates its rod-shaped body elegantly to move forward or backward, by propagating body bends against the direction of translocation. The pattern of muscle activity that underlies this motor program is a propagation of alternating differential activation of antagonistic muscles, essentially a simplified version of any other motor program for animal locomotion. Simplified, because of the small number of muscle cells, and the lack of limbs and tendons that translate muscle contraction. In *C. elegans* each body wall muscle cell contributes to local bending. Moreover, the musculature is controlled by a compact nervous system. With only 302 neurons in every hermaphrodite animal (385 in the male), all named and identifiable by location and morphology, it is arguably the most comprehensively described nervous system. The only organism-wide wiring diagram, painstakingly reconstructed from electron micrographs [1, 2] is accompanied by the first sequenced and annotated genome of a multicellular organism [3], and a complete developmental lineage [4, 5]. Here, we describe the

locomotor behavior and the underlying neuromuscular system and summarize findings that suggest how the locomotion circuit generates the motor pattern, as well as research approaches. Finally, we offer a framework of analogy to compare *C. elegans* with other animal locomotor networks.

## Keywords

*C. elegans*; Neurobiology; Neuroethology; Undulation; Nematode; Motoneurons; Proprioception; Connectome; Crawling; Swimming

## In the beginning

Locomotion and its underlying neuronal circuit were prominent in motivating and shaping the field of *Caenorhabditis elegans* research. When Sydney Brenner established *C. elegans* as a model animal, it was with the explicit intent to figure out how genes code for development, structure, and function of nervous systems [1, 2–6]. Although the first published paper is simply named “The genetics of *Caenorhabditis elegans*” [4], much of it is dedicated to charting out a plan for investigating the effects of mutations on nervous systems. Brenner reasoned that isolation and characterization of mutants with behavioral phenotypes must be supported by studying the intermediate between the gene and behavior, namely, the nervous system. He suggested to split the problem into two: how genes specify nervous systems; and how nervous systems work to produce behavior. Both problems require knowledge of the structure of nervous systems, published as a wiring diagram [1, 7]; an achievement that was not yet replicated for any other animal. Another requirement for this line of research is a rigorous description of changes in the most obvious and accessible behavior: locomotion. Two-thirds of the six hundred or so mutant animals described in the first study were mutants with altered locomotion, named “uncoordinated” or “unc” [4]. Originating from the field of

genetics has influenced the trajectory of discoveries in neurobiological research of *C. elegans*. Brenner's approach to first determine the "list of parts" as a requirement to understanding how they come together encouraged completeness of data collection but also early computational modeling as soon as the details were available.

## ***C. elegans* lends itself to a wide range of experimental approaches**

The nematode is transparent and its size accommodates a wide range of microscopy techniques [8]. As a self-fertilizing hermaphrodite with 3-day generation time it is very amenable to genetic and transgenic manipulation [9]. Moreover, many reagents are available at low or no cost, from plasmids (AddGene), to mutant and transgenic strains (CGC) as well as naturally occurring ones (CeNDR); and sending reagent upon request is the default practice of laboratories and research groups. *C. elegans* provided the first sequenced and annotated genome of a multicellular organism [10], as well as a complete developmental lineage [11, 12]. Much of the established knowledge is organized in community-supported resources that include WormBase, for accurate, current, accessible information concerning the genetics, genomics, and biology [13]; WormAtlas, for structural anatomy [14]; and WormBook, a comprehensive, open-access collection of original, peer-reviewed chapters and protocols (WormMethods). The animals are easy and inexpensive to maintain and do not require specific permits. Most generated strains can be deeply frozen to be thawed and used after many years [15], reducing the burden and risk of maintaining important strains.

**The organism-wide connectivity dataset (connectome)** of *C. elegans* is so far the only such dataset published. The anatomical connections were manually and painstakingly reconstructed from electron micrographs over 15 years; a process that involved timely developments in electron microscopy techniques and even

premature attempts in pioneering the laser scanning confocal microscope [16] and adapting computerized reconstruction [1]. The relatively small nervous system and detailed knowledge of anatomy allowed the reconstruction of most neurons, chemical synapses, and gap junctions. In total 5958 chemical synapses (1207 of which are neuromuscular junctions) and 1106 gap junctions were identified in the adult nematode [7, 17, 18]. This achievement, unmatched for any other nervous system during the 34 years that have passed, attracted wide attention from both computational and experimental neuroscientists. However, it has two compounding flaws. Most of the dataset, including a portion of the ventral and dorsal nerve cords, was reconstructed in only a single animal [7, 17, 19], leaving the variability of connectivity among animals unknown. Moreover, the dataset focuses on the head and tail ganglia, and the nerve cords that contain the locomotor circuit were reconstructed only halfway along the body, and the data for the region posterior to the vulva are still incomplete. Connectivity data are partial or missing for 39 of the 302 hermaphrodite neurons, including 28 of the 75 locomotor motoneurons [17, 18, 20]. Several publications, notably about network properties, have ignored this gap in data, shedding doubt on their conclusions. To overcome the gap in connectivity data Haspel and O'Donovan [19, 21] extrapolated and predicted the missing connections [18] after demonstrating that data for the anterior section exhibit repeating patterns when they are mapped according to muscle innervations [19, 21]. Until new connectivity data are collected, the extrapolated connectivity is our best approximation.

**Electrophysiology** and specifically patch clamp recording have been adapted to *C. elegans* neurons and muscle cells [22, 23]. In the locomotion system, muscle cells have been recorded electrophysiologically [24, 25], and in two studies also motoneurons [26, 27]. The protocols for patch clamp methods were developed for sensory neurons [23] with cell bodies that are much larger (10–15  $\mu\text{m}$  compared to 1–2  $\mu\text{m}$  for motoneurons), and where recording from immobilized animals is more relevant. The cellular properties of all the motoneurons are unknown and they are assumed to be bistable

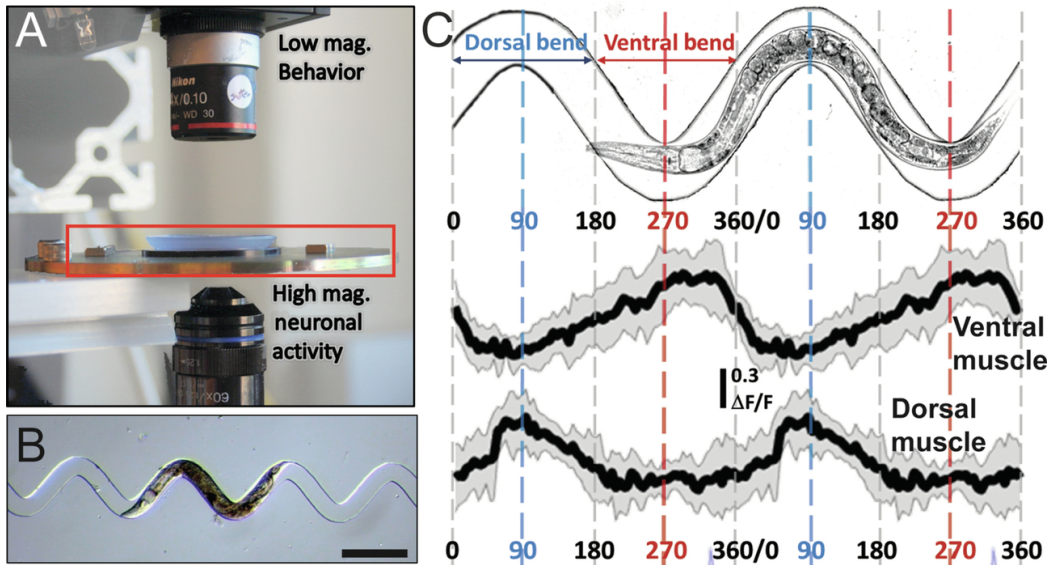
[26] or passive integrators, with graduated synapses. They could, however, also be pacemakers, or as other neurons in *C. elegans* were found, generate action potentials [28], or other regenerative and nonlinear dynamics [29].

**Laser ablation** was first developed in *C. elegans* and demonstrated in 1980 when Sulston and White used it to study cell-cell interaction in postembryonic stages [30]. The opportunity for testing the necessity of specific identifiable neurons in intact animals was clear and it was soon used to ablate neurons of the circuit for touch sensitivity that was inferred from the wiring diagram, including interneurons and some of the motoneurons of the locomotion network [31]. Modern laser ablation platforms and the widespread use of fluorescent proteins allow ablation of cells (by aiming at cell bodies or nuclei), very accurate and localized injuries to axons and dendrites, or targeting of particular intracellular structures [32–34]. The first laser axotomy and regeneration were also demonstrated in *C. elegans* [35]. When commissural neurites of inhibitory D motoneurons were disconnected, animals exhibited “shrinking” that recovered after 24 h in correlation to morphological regeneration and reconnection of the severed neurite [35]. Regeneration was observed in about 30% of disconnected neurons and might depend on the site of injury as well as neuronal identity, developmental stage at the time of injury, and the amount of energy delivered by the laser [36].

*C. elegans* has been a testbed for cutting-edge technologies that combine microscopy and transgenic manipulation at least since the development of transgenic GFP [37] because of size and transparency, short generation time, and established methods for generating transgenic animals. It was the first animal for in vivo demonstration of scanning laser confocal microscopy [16], laser axotomy [34], as well as genetically encoded calcium sensors [38–41], optogenetic tools [42, 43], and other transgenic tools such as GRASP, in which split-GFP reconstructs across synaptic gaps to map synaptic connections [44], correlating EM and light microscopy [45], expansion microscopy [46] or sonogenetic activation of neurons with ultrasound [47].

**Genetically encoded calcium indicators (GECIs)** are by far the most common tool used to record neuronal and muscular activity in *C. elegans*. The size, transparency, and relatively slow behavior, together with a short generation time and established protocols for transgenic expression, make GECIs an ideal method for this purpose. Muscle activity was recorded in freely moving animals and correlated to body bends [48]; and the neuronal activity of all six classes of cholinergic motoneurons has been measured in restricted, tethered, and freely behaving animals with genetically encoded calcium-sensitive protein indicators [49–53]. Recently, neuronal activity was recorded from both GABAergic motoneuron classes during restricted locomotion [54]. The advantages of noninvasive recording of neuronal and muscular activity during uninterrupted behavior for the study of the neural basis of locomotion are obvious. Indeed, our knowledge about the function of the locomotion circuit has benefited greatly from this method. Yet, the collected data are not easily comparable because researchers have been using a variety of sensors, microscopy systems, and scientific approaches. Moreover, there is no clear acceptable method of demonstrating coordinated activity, nor is there a common frame of reference. We suggest that the perimotor locations of neurons and muscle cells [19] and the locomotion cycle could serve as the axes of such a frame of reference (Fig. 1C). The main limit of calcium imaging is that free calcium levels are an indirect measure of neuronal activity and membrane potential. Hyperpolarizing voltage changes might not affect calcium signal and the sensor dynamic range and signal-to-noise ratio limit its resolution of a small depolarization. The changes in membrane potential of locomotion motoneurons are still unknown and the common assumption is that they do not generate action potentials but might exhibit bistability. Even large events such as the bursts of action potentials in muscle cells were not temporally resolved with calcium imaging and were first found with patch electrodes [55, 56]. Finally, to our knowledge there is one published case of a GECI subtly but measurably changing behavior, in this case an olfactory threshold for positive chemotaxis [57].





**FIG. 1** Opposing microscope objectives and microfluidic channels allow synchronous recording of cellular activity and behavior. Microscope setup design for activity imaging varies and depends on the scientific question. (A) Behavior and calcium imaging can be captured simultaneously with opposing low-magnification and high-magnification-high-NA objectives, respectively. (B) Microfluidic devices provide a controlled environment and conveniently fit in the microscopic field of view. For example, waveform channels restrict undulatory locomotion to a behaviorally relevant path that spatially fixes the locomotion cycle. Scale bar = 0.5 mm. (C) Calcium imaging is usually plotted as fraction of mean or initial fluorescence intensity against time, or as this example against the locomotion phase. Maximal dorsal bend was arbitrarily designated as 90 degrees, and maximal ventral bend 270 degrees; *solid lines* and *shaded areas* are mean and standard deviation, scale bar = 0.3  $\Delta F/F_{\text{mean}}$ . (Credit: Gal Haspel.)

**Simultaneous imaging of cellular activity and behavior** is useful for many neuroethological studies and specifically for locomotion. In *C. elegans* it has been mostly used to assign a behavioral context (direction of locomotion, turns, and pauses) rather than record the locomotor phase or frequency. In most cases, two opposing objective lenses are set around the animal (Fig. 1A), high numerical aperture (NA) for calcium imaging against low magnification to record behavior [58, 59], or position an image sensor at the image plane of the condenser, using it as a low magnification objective [51]. When the sample is on a motorized microscope stage, real-time tracking can be calculated from either objective, allowing in some cases recording of almost 100 neurons in the head ganglions in an animal freely crawling on an agarose surface [60, 61]. Other studies [51] choose to restrict or tether the animal to minimize requirements for tracking or other experimental constraints (Fig. 1B and C). Another approach is to image the head area at high resolution and deduce the animal behavior from neck translational and curve movement [62].

**Behavior analysis** has rapidly evolved in the last decade with technical improvement and availability of cameras, computation, and data storage, allowing development of different automated trackers [63–65] replacing or augmenting heuristic classifications. In turn, the collection of high-resolution data encouraged development of analysis software to extract features and quantify parameters for crawling [66–68], swimming [69], and intermediate patterns [70]. Body shape data of sufficiently high spatial and temporal resolution showed that the space of shapes adopted by *C. elegans* is low dimensional, revealing an underlying simplicity of seemingly complex locomotion dynamics [71, 72]. The quantitative description of behavior arising from the building blocks spanning the space of observed body postures, termed *eigenworms*[72], provided a framework for unbiased scoring of previously undetectable phenotypes [73, 74]. Other approaches include using local linear models within windows that are determined adaptively from data [75], scale-invariant feature transform [76], and relying on machine vision to score generation, propagation, and decay of individual body bends as fundamental primitives of undulatory locomotion



[77]. For experimental convenience, all published tracking results have been recorded in two-dimensional space, on top of agar surface, in a microfluidic device, or a dorsoventral plane of swimming. Future investigations of locomotion in three dimensions will no doubt expand the known behavior repertoire.

**Optogenetics** uses light to control certain parameters of a cell. Optogenetic actuators are proteins that modify the activity of the cell in which they are expressed when that cell is exposed to a particular wavelength of light [78]. Following earlier optogenetic tools (e.g., ChARge [79]; Haspel Grass Fellowship, 2003, unpublished), the first widely adopted activator has been channelrhodopsin 2 (ChR2), a blue-light activated cation channel found in algae [80, 81]. Its first use in intact behaving animals was quickly demonstrated in *C. elegans* [43]. The first optogenetic inactivator, halorhodopsin (Halo, NpHR), a yellow-light activated chloride pump found in archaea was also first described in vivo in *C. elegans* as its simultaneous use with ChR2 was demonstrated in the first publication [82]. Many other optogenetic tools are now available for *C. elegans* [42, 83]. Optogenetic tools were used to test hypotheses about the neural basis of locomotion. Finally, optogenetic ablation of cells can be induced by the release of reactive oxygen species (e.g., miniSOG, killerRed [84, 85]).

**Microfluidics** methods match very well with *C. elegans* size and mode of locomotion. The temporal and spatial control over flow rate, reagent concentration, mass and heat transfer, along with versatile design were adapted to carry out a variety of experiments that were otherwise impossible or very technically tasking [86]. There are many examples of using microfluidics for *C. elegans* research from behavioral analysis under precise stimulation, long-term observation, and imaging, including genetic screens, optogenetic studies, and laser ablation. Most microfluidic devices are made by soft lithography replica molding of the silicone elastomer, polydimethylsiloxane (PDMS) that is optically transparent, gas permeable, nontoxic, and inert (Fig. 1B). The design usually includes 1–100  $\mu\text{m}$  deep channels, chambers, or pillars. For the study of locomotion devices were used for well-defined structural

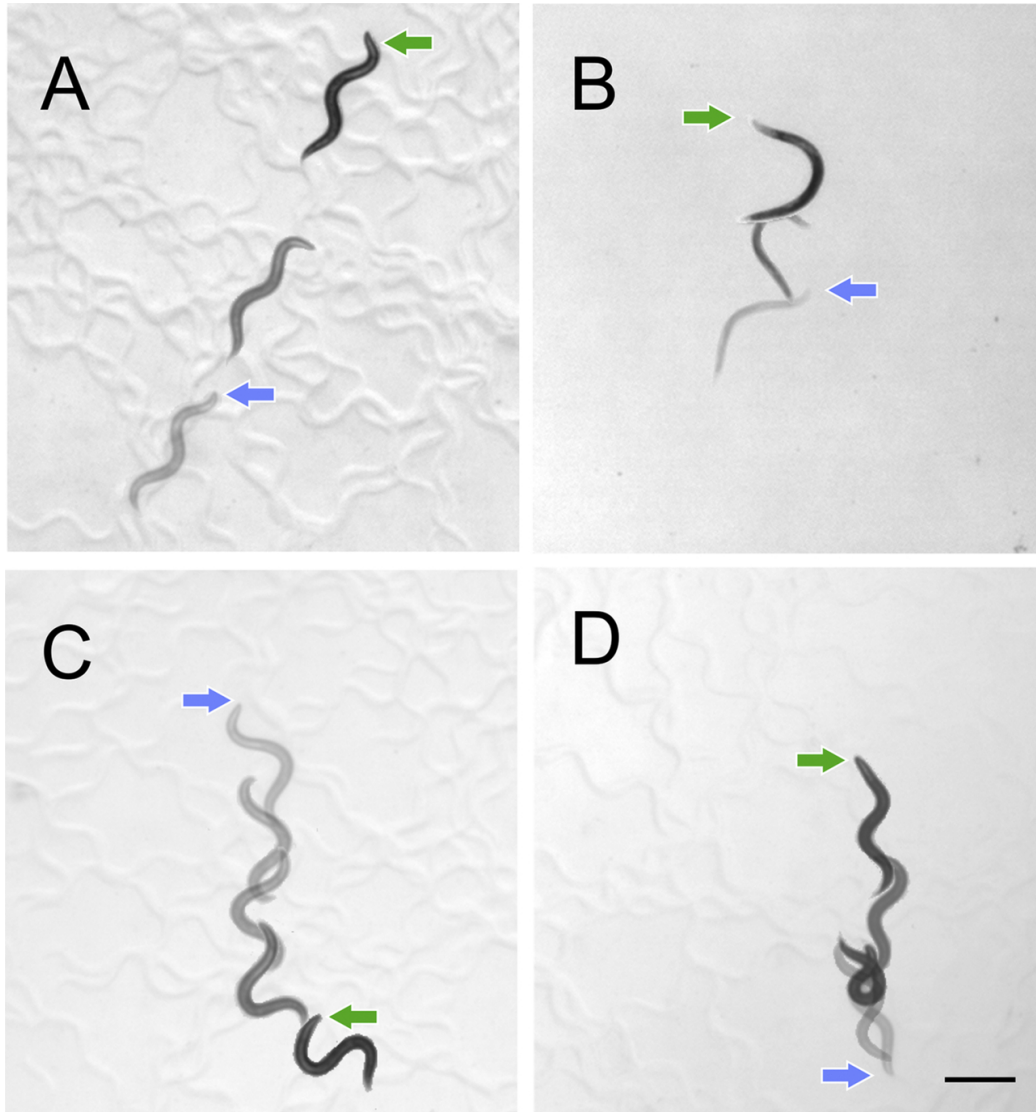
surroundings and for measurement of forces. The “artificial dirt” device consists of arranged pillars at different spacing to mimic dirt that nematodes encounter in nature, made of PDMS [87] or agar [88]. Locomotion has also been studied in waveform channels fabricated with different amplitudes and wavelengths (Fig. 1B) [54, 87]. Microfabrication was also used to measure the forces exerted by crawling animals as they deflect micropillars or attached to cantilevers on the device [89–92].

**Computational models** of *C. elegans* locomotion have been instrumental in formulating hypotheses for mechanisms that underlie locomotion and testing their feasibility. The models span a range from purely neural [93–95] to purely mechanical [96, 97] and from minimal circuits incorporating only forward motoneurons [98] to detailed ones incorporating most known circuit elements [19, 94, 99, 100]. Computational approaches were used to address questions about network connectivity, neural dynamics, behavioral aspects, neurophysiology, mechanisms of rhythm generation, biophysical properties of neurons, muscles, and material properties, as well as neuromodulation and extrasynaptic communication. Some insights are mentioned later, but a thorough survey is beyond the scope of this chapter. For a detailed discussion of computational models from a systems approach see recent reviews [101, 102]. A major ambitious challenge is to integrate models and simulations at many levels to produce a highly detailed in silico model of the nematode in its environment. Efforts such as OpenWorm [103] and Si-elegans [104] aim to combine current models in a modular hierarchy to examine emergent properties. Ideally, pluralistic design will allow users to swap existing parts with user hypothesis or data-driven model, or to control different elements for in silico experiments to generate predictions [105, 106]. Computational models demonstrate the possible given a set of assumptions. Therefore, it is not surprising that all published models for *C. elegans* locomotion produce an approximation of the undulatory motor output, because this is a preliminary requirement. It is important to evaluate computational insight by their assumptions regarding cellular properties, synaptic parameters, and connectivity. For example, using the connectivity

dataset that is only partially reconstructed for the locomotion circuit or its annotated update [7, 17] can be misleading. Instead, the extrapolated connectivity is a much better choice [18, 19].

## Locomotion behavior

Undulatory locomotion is relatively simple and likely ancestral. Nematodes generate thrust by propagating dorsoventral body bends along the body against the direction of locomotion [107–110]. When the nematode is presented with high mechanical loads, such as those on the surface of an agar gel or in liquid 10,000-fold more viscous than water, the wavelength of undulation is less than a single body length, which produces a typical S shape (Fig. 2A and C), alternating at approximately 0.4 Hz [53, 70, 111–113], with slightly higher frequency and lower amplitude for forward than backward. When the animal swims in water or liquid of comparable viscosity, the propagated wavelength of undulation is almost twice the body length, which produces C-shape conformations (Fig. 2B) at a frequency of about 1.5 Hz with slightly higher frequency for forward than backward [53, 70, 111–113]. Although swimming has been loosely referred to as “thrashing,” it is a directional behavior that enables orientation, such as in chemotaxis assays [112]. Varying the mechanical load imposed by the environment between these two extremes reveals a gradual transition of the corresponding wavelengths and frequencies [70, 111, 114]. This continuum, as opposed to discrete gaits, suggests that a single motor program shaped by physical forces and proprioceptive feedback underlies locomotion [115]. Nevertheless, the two gait extremes were found to be pharmacologically and genetically discrete: dopamine induces crawling in a low-viscosity environment, whereas serotonin induces swimming in shallow liquid [113]. Furthermore, animals occasionally pump their feeding organ (the pharynx) during crawling behavior but not swimming [113]. Therefore, it is likely that biogenic amines mediate the perception of the mechanical properties of the environment to modulate proprioception, as well as directly modulating the locomotor program.



**FIG. 2** *C. elegans* moves in an undulatory pattern, propagating bends along its body against the direction of movement. The wavelength and amplitude of the locomotion cycle depend mainly on the resistance of the environment, and they are shorter for viscous fluids or high surface tension. Forward crawling on an agar plate (A) produces shorter wavelengths (less than body length) and smaller amplitudes than swimming in liquid (B). Less frequent behaviors, such as reversals (C) and turns (D), are also stereotypic during crawling and swimming (not shown). *Blue*

*arrows* indicate the head position at the beginning of a sequence and the *green arrows* indicate the head position of the same animal at the end. The sequence lengths for A–D are 12, 1, 10, and 14 s, respectively; note that undulation frequency is higher for swimming. Scale bar = 0.5 mm.

(Credit: Lan Deng and Gal Haspel.)

The locomotion behavior is richer than bidirectional undulations. *C. elegans* can gradually change its heading by biasing head and neck undulations [116], or induce larger changes in direction by increasing a single curve until it touches or crosses over a more posterior portion of the body (Fig. 2D), in a so-called Omega- or Delta-turn, respectively [117, 118]. These turns are usually associated with an aversive response but were also demonstrated to contribute to positive chemotaxis.

In this chapter, we concentrate on the generation of forward- and backward-directed undulatory locomotion. We focus on the adult hermaphrodite animal, because the vast majority of research on the locomotion of *C. elegans* was performed on that life stage; we briefly discuss the first (of four) larval stage that uses an even smaller nervous system.

## Body mechanics

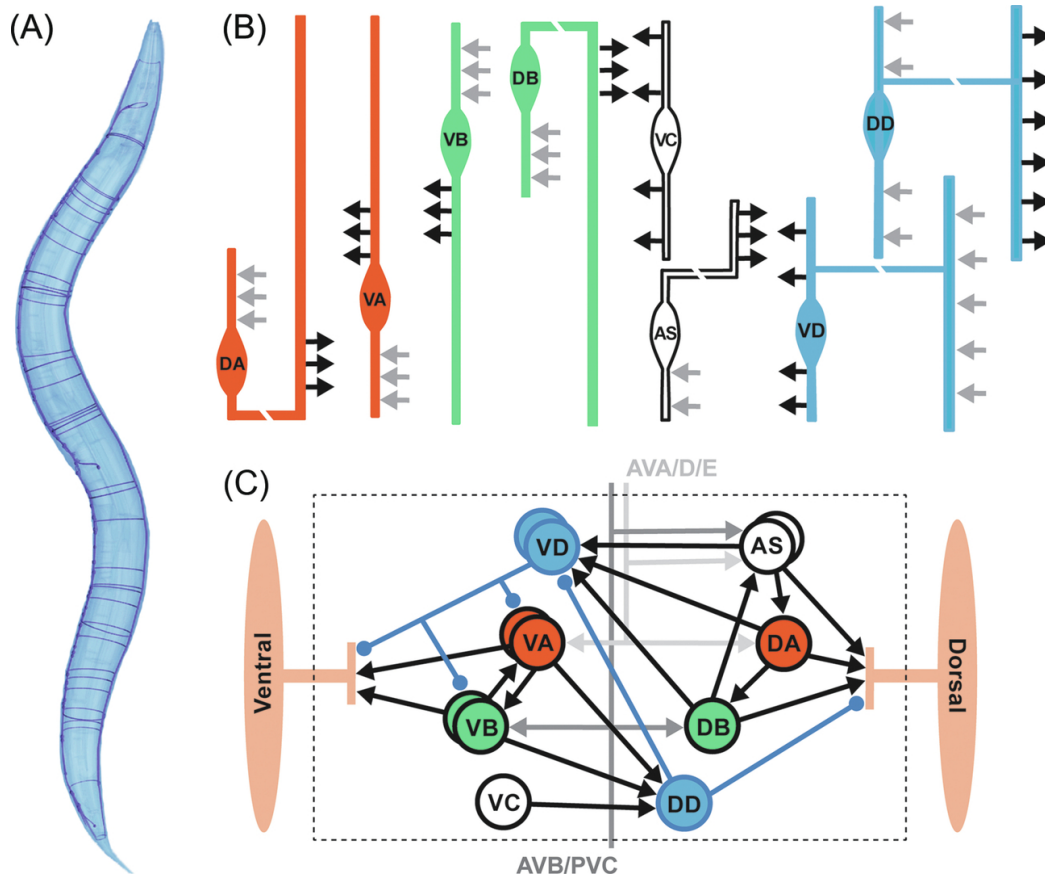
Similar to other nematodes, the body of *C. elegans* is a slim unsegmented cylinder tapered at both ends and with a wispy tail [110]. The adult hermaphrodite is about 50  $\mu\text{m}$  in diameter and 1 mm long and at this size, it is smaller than the capillary length of the water-air interface, which is approximately 2 mm; when in fluid, *C. elegans* swims in a low Reynolds-number regime [119], in which the viscous forces are greater than the inertial forces [108]. Whether crawling or swimming under these conditions, the animal stops moving almost instantly once it ceases to produce force. Measurements of mechanical parameters were made with different techniques and under variable conditions and in some cases range



several orders of magnitudes. The stiffness and the elastic modulus of the static body of a wild-type adult animal were found to be approximately 0.60 N/m and in the range of 100–200 kPa, respectively [111, 119–121]. Hydrostatic pressure contributes modestly to stiffness. Puncturing the cuticle decreases the body stiffness by about 18% [122], and the animal continues undulating after being punctured (our observation). Manipulating the contraction of muscles pharmacologically or optogenetically has suggested that the resting muscle tone is a major contributor to the resting body stiffness [121]. The coordinated action of body wall muscles during swimming delivers propulsive force on the order of nanoNewtons [89, 119] and exerted forces on the order of a few to tens of microNewtons [89–92].

## The locomotion circuit

The locomotion circuit is composed of 10 descending premotor interneurons, 75 cholinergic and GABAergic motoneurons, divided by their morphology into 8 classes that innervate 95 muscle cells arranged along the body (Fig. 3). Altogether 180 cells decode descending control to body movement. In this functionally compressed nervous system each neuron might take multiple roles and might serve different roles in different behavioral contexts. In *C. elegans*, all the elements that compose the locomotion system and their connectivity are known [14, 17, 19–21, 123], as well as some neural mechanisms, yet the production of coordinated muscle activity remains unresolved.



**FIG. 3** The musculature and nervous system are compact and stereotypic. (A) Cell bodies of all the locomotor motoneurons are located in the ventral nerve cord (left, head is upwards) and some send commissures to the dorsal nerve cord (right). Only the ventral cord motoneurons are shown (*dark blue*) for clarity. (B) Eight classes of motoneurons are delineated by their morphology, connectivity (*black arrows*: presynaptic area, *gray arrow*: postsynaptic area), neurotransmitter and location of their neuromuscular junctions. All cell bodies are located in the ventral cord and while ventral motoneurons (VA, VB, VC, and VD) innervate ventral muscle cells; dorsal motoneurons (AS, DA, DB, and DD) innervate dorsal muscle cells. Six classes are cholinergic, while VD and DD (*light blue*) are GABAergic. (C)

Six functional segments along the ventral cord consist of about 11 interconnected motoneurons each (one segment is shown). Descending input (*gray arrows*) from premotor interneurons consists of cholinergic synapses and gap junctions to A, B, and AS motoneurons. Most gap junctions among motoneurons (not shown for simplicity) connect between sequential motoneurons of the same class DD, VD, DB, and VB, and between AS and DA motoneurons. (Credit: Gal Haspel and Daphne Soares. (A) Adapted from WormBase Virtual Worm Project (Lee RYN, Howe KL, Harris TW, Arnaboldi V, Cain S, Chan J, et al. WormBase 2017: molting into a new stage. Nucleic Acids Res 2018;46(D1):D869–74).)

**Descending input** Five pairs of interneurons are sometimes referred to as “command neurons” but are more accurately termed premotor-interneurons, because they do not satisfy Kupfermann and Weiss three-part definition of the former term [124], as detailed here. These are four bilaterally symmetric interneuron pairs (namely, AVA, AVB, AVD, with cell bodies in the head ganglia, and PVC with cell bodies in the tail ganglia) with large-diameter unbranched axons that run the entire length of the ventral nerve cord and provide input to the ventral cord motoneurons. A fifth pair, AVE, with cell bodies in the head ganglia has axons that span only the anterior cord. The premotor interneurons are by far the main source of input to the excitatory motoneurons through cholinergic synapses and gap junctions [7, 18]. They are divided into two pairs that are associated with forward locomotion (left and right, AVB and PVC), and three pairs that are associated with backward (AVA, AVD, and AVE; [31, 125–127]). They are active without oscillations during locomotion only in the associated direction [49, 50, 128] and inactivation or ablation of one pool reduces initiation of locomotion in the associated direction [31, 50, 125]. When all premotor interneurons are ablated, animals propagate body bends, although they are slow, uncoordinated [127], and at times propagate simultaneously in both

directions [50, 129]. Ablation of all premotor interneurons and either A or B motoneurons (see below) produced slow locomotion in forward or backward direction, respectively [129], suggesting that the motoneuronal network is sufficient to produce an alternating and propagating motor pattern and that interneuronal input selects between the two programs. In addition to promoting reversal through cholinergic synapses when active, the AVA interneurons were demonstrated to reduce the probability of spontaneous reversal while inactive (during forward locomotion) through gap junctions with motoneurons [50]. The premotor interneurons are likely bistable [130], each pool coactive, and for the most part backward and forward pools are mutually exclusive by reciprocal inhibition [60–62, 130]. They are connected to each other topologically with high efficiency, creating a so-called Rich Club [131] that is connected to many other neurons. Accordingly, their activity reflects global neural dynamics [62, 132]. A few other neurons have sparse input to the motoneurons, and other sensory and interneurons affect locomotion [133, 134] but do not make direct synaptic contact with the locomotion motoneurons. Finally, neuromodulation through monoamines and neuropeptides affects the dynamics of locomotion [135–138].

**A ventral nerve cord** that runs along the body, connecting the head and tail ganglia, includes the axons of the premotor interneurons, as well as all the cell bodies and some synapses of locomotor motoneurons (Fig. 3A). The motoneuronal morphology is relatively simple, with a single unbranched dendrite, receiving input from premotor interneurons and from other motoneurons, and a single unbranched axon (Fig. 3B). Four motoneuron classes are ventral, with a ventral axon that is presynaptic to ventral muscle cells and other motoneurons; the other four are dorsal, connected with a commissural neurite and a dorsal axon to dorsal muscle cells and other motoneurons (Fig. 3B and C) [123, 139]. Cholinergic and GABAergic neuromuscular junctions [24] occur *en passant* on the axon of the motoneuron [140].

**Cholinergic excitatory A and B motoneurons** each include a ventral and a dorsal class: 12 VA, 9 DA, and 11 VB, 7 DB (Fig. 3B and

C). Ventral motoneurons excite ventral muscle cells and dorsal inhibitory motoneurons; dorsal motoneurons excite dorsal muscle cells and ventral inhibitory motoneurons. Past the neuromuscular junction area, motoneurons of these classes exhibit long neurites that lack synaptic connection and extend toward the head for A, or the tail for B motoneurons (Fig. 3B). The A and B classes receive segregated inputs from premotor interneurons such that A motoneurons receive input from interneurons associated with backward, while B motoneurons receive input from those associated with forward locomotion. Accordingly, A motoneurons are active during backward and B during forward locomotion [50, 51], and those behaviors are specifically absent when these classes are ablated [31, 125]. VA motoneurons were demonstrated to be bistable [26] and spontaneously switch between stable membrane potentials at an interval of 30–50 s in dissected preparations [26, 129], suggesting that they may act as pacemakers.

**Cholinergic excitatory AS and VC motoneurons** were mostly overlooked as part of the locomotion circuit [141]. Eleven AS are morphologically similar to DA (cell bodies in the ventral nerve cord, commissures, and dorsal neuromuscular junctions) but lack the long dorsal-anterior neurite (Fig. 3B) [123]; like DA they excite dorsal muscle cells and ventral VD (Fig. 3C). They are connected with chemical and electrical synapses to all forward and backward premotor interneurons [7, 19], and are accordingly active at the frequency of undulations during locomotion in both directions [52]. Optogenetic ablation or inactivation of AS did not prevent locomotion but induced a ventral bias, lower speed, and increased curvature; locomotion only stopped when AS were strongly hyperpolarized, probably due to disinhibition via VD and hyperpolarization by gap junction of other motoneurons. Optogenetic activation of AS similarly induces a subtle decrease in speed and increase in curvature, but with a dorsal bias [52]. The subtle effects of activation and inactivation were unexpected because AS represent almost half of the excitatory dorsal neurons and provide almost half of the excitatory dorsal neuromuscular junctions [20]. Of the six motoneurons of the VC class, VC4 and VC5 are



involved in egg laying and have neuromuscular junctions to vulva muscle. The anterior VC motoneurons 1–3 have very few neuromuscular junctions to ventral muscle (Fig. 3B); and instead are interconnected with gap junctions and are connected with chemical synapses to DD and VD motoneurons (Fig. 3C) [19]. Their function in the locomotion circuit, as well as the connectivity of the most posterior VC6, are unknown.

**GABAergic inhibitory D motoneurons** are shaped like the letter H; six DD motoneurons each has a ventral dendrite and a dorsal axon and thirteen VD motoneurons each has a dorsal dendrite and a ventral axon, connected by a commissural neurite (Fig. 3B). They are not innervated by interneurons; instead, they only receive inputs from other motoneurons [7, 19, 142]. Each VD motoneuron receives input from the dorsal cholinergic motoneurons that innervate the opposing muscle cells, while DD motoneurons receive input from the ventral cholinergic motoneurons. In addition to their synaptic output at neuromuscular junctions, DD and VD motoneurons also reciprocally innervate the opposing DD and VD, whereas VD motoneurons also innervate the local VA and VB (Fig. 3C). Based on their morphology and connectivity, they have been suggested to provide dorsoventral cross-inhibition to the antagonistic muscle of their presynaptic motoneurons, also supported by the timing of their inactivity [54]. Accordingly, nematodes that are defective in GABAergic transmission (so-called *shrinker* mutants) respond to a noxious stimulus with coactivation instead of alternation of antagonistic dorsoventral muscle, producing a shrinking response instead of a coordinated escape [54, 143]. Slow undulatory locomotion is not abolished in these mutant animals, however, and they propagate dorsoventral bends [143]. Ablation of multiple DD and VD motoneurons demonstrated their role in dorsoventral cross-inhibition when it replicated the shrinking phenotype of GABA transmission mutants in response to head touch [143]. Laser ablation of either VD or DD motoneurons induces a bending bias toward the ablated side causing the animal to move in circles [137]; while optogenetic activation or inactivation of DD motoneurons also induces ventral or dorsal bending, respectively [137]. Another

suggested role is to provide an inhibitory reset to allow higher locomotion frequency through inhibition of ventral motoneurons [54, 98]. The GABAergic motoneurons seem then to participate in some but not all modes of locomotion, possibly to regulate direction and speed.

**The neck and head** musculature is innervated by 38 motoneurons of 11 classes, namely, RIM, RIV, RMF, RMG, and RMH, each bilaterally symmetric; RME, SMB, SMD, and URA, with four members each; and IL1 and RMD neurons with six members each [7]. The motor programs of head and neck provide some thrust and most of the steering during locomotion, but the neural mechanism is only known for the regulation of dorsoventral curvature. Cholinergic motoneurons SMD are proprioceptive and supply positive feedback to drive dorsoventral neck bending [116]. They also regulate GABAergic RME head motoneurons that are active in correlation with and limit the curvature of head bending by inhibiting SMD [144]. In addition, extrasynaptic cholinergic feedback from SMD to RME provides gain control to set head bending amplitude [144, 145]. A recent model integrates the head and body circuits in the physical constraints of the body and environment [99]. Yet, these circuits seem to be independent as animals keep oscillating their heads and slowly move after ablation of all body motoneurons [129].

**Musculature** The body wall muscle cells are excitable actuators, connected through thin processes called muscle arms to synapses from excitatory and inhibitory motoneurons. They are rhomboid in shape and are obliquely striated. As in other nematodes, the muscles are anchored to the cuticle along their entire length (not only at their ends) to distribute contractile forces [107, 146–148]. Ninety-five body wall muscle cells are staggered in two rows in each of four quadrants along the anterior-posterior axis. The muscle cell bodies are electrically coupled by high conductance (300 pS) gap junctions to muscle in the other row of the same quadrant and with low conductance (75 pS) on muscle arms to muscle in the contralateral quadrant [149] to synchronize muscle activity [150]. Thrust is produced by dorsoventral bending of the entire body, whereas steering is achieved by differential activation of the 20 anterior

muscle cells in the head and neck. Accordingly, the head and neck quadrants of muscles are independently innervated by head motoneurons (with cell bodies in the nerve ring ganglion) and can turn freely relative to the anterior-posterior axis of the body [146]. In contrast, along the rest of the body, the muscles from the two subdorsal quadrants send muscle arms into the dorsal cord, whereas those from the two subventral quadrants send arms into the ventral nerve cord [123, 139]. This pattern of innervation is consistent with the observation that the vast majority of locomotion patterns involve the generation and propagation of dorsoventral bends. Cholinergic and GABAergic neuromuscular junctions [24, 151] occur *en passant* on the axon of the motoneuron inside the ventral or dorsal nerve cord or on the nerve ring, through the thin arms that protrude from the muscle main mass [146]. The neuromuscular junctions are graded with stochastic tonic release of neurotransmitter that is up- or downregulated in response to hyperpolarization or depolarization, and postsynaptic evoked responses that correlate to the size of the depolarization [25]. Further, while cholinergic neuromuscular junctions exhibit considerable and quick depression, GABAergic synapses exhibit facilitation under high-frequency stimulation, followed by slow depression; the neuromuscular junction provides a shifting integration of analog inputs [25]. Calcium-mediated action potentials are induced by postsynaptic current bursts that are mostly mediated by a persistent current through activation of nicotinic receptors [152]. Based on their morphology and function, and the extreme difference in volume between muscle arms and muscle cells, muscle arms probably also propagate action potentials to depolarize the muscular membrane. Muscle cells are also activated by an intrinsic, homeostatically regulated mechanism, as suggested when muscle activity seems to resume in the absence of cholinergic input: muscular action potential frequency recovers within seconds of a pharmacological block of a cholinergic input, a GABAergic input, or both [55]. Secondly, animals keep their current posture rather than assume a straight one when cholinergic motoneurons are acutely hyperpolarized [53]. The pattern of muscle activation is surprisingly consistent across locomotion patterns spanning a threefold change in

wavelength in different viscosity and mutants with longer or shorter bodies; the peak muscle activation occurs inside the curve at approximately an eighth of a cycle ahead of peak local curvature [48].

**Motoneuronal feedback** could be transmitted through the gap junctions with premotor interneurons (AS and A with AVA, B with AVB). These connections are usually considered part of descending control but for AS [52] and A motoneurons [50], the gap junctions were demonstrated to change the activity of AVA and affect behavior. The gap junctions between AVA and A motoneurons are antidromically rectifying and amplify synaptic release by depolarizing the interneuron [27]. Motoneuronal feedback that regulates interneuronal activity was recently reported in several animal models, including leech [153], fruit fly [154], zebrafish [155], and mouse [156, 157].

**Proprioception** is involved in *C. elegans* locomotion but the underlying molecular, cellular, and network mechanisms are unknown. During locomotion *C. elegans* responds to its environment in a manner that suggests proprioception. Most notably during locomotion in media of higher viscosity the undulatory wavelength and the frequency of undulations both decrease in a manner that cannot be explained by feedforward and physical interactions alone [70, 111, 115]. During forward locomotion signals related to body curvature are mediated by B motoneurons to induce muscle contraction about 100–200  $\mu\text{m}$  posterior to an ipsilateral body bend [53]. The molecular and cellular basis of this mechanosensation is still unknown. It is also unknown whether the long undifferentiated processes, typical of A and B motoneuron classes (Fig. 3B), are mechanosensitive, a morphology-based hypothesis first suggested by Byerly and Russell (personal communications cited by White et al. 1986). The projection of the processes is counterintuitive if they were to mediate proprioceptive propagation; they would relay information about the former and not the upcoming propagating bend [93]. Yet, computational models demonstrated that propagation can be driven with proprioception from either direction [115]. Furthermore, it was not demonstrated that A or B motoneuron

activity is directly affected by the mechanical stresses generated during movement, or whether a complementary proprioceptive signal is mediated anteriorly during backward locomotion by A motoneurons. The fourfold symmetric SMD head motoneurons are proprioceptive as proposed based upon their contralateral morphology and reciprocal connectivity [7]. The lateral pair of dorsal SMDD and ventral SMDV are activated by dorsal and ventral bending, respectively, and are activated in an antiphase manner during forward locomotion [116]. Expression of two TRPC channels is necessary for proprioception in SMDD, while the molecular mechanism is unknown for SMDV.

Finally, two mechanosensory neurons, PVD [158, 159] and DVA [160–162], are considered proprioceptive because their ablation affects locomotion and posture. They probably integrate overall body curvature rather than local bending because their dendrites span the whole animal.

## Rhythm and pattern generation

Central pattern generators (CPGs) underlie locomotion in all animals studied to date. Yet their existence, location, neuronal mechanism, and role in *C. elegans* locomotion are still to be determined. It is also possible that feedforward oscillations underlie the motor program for one direction or frequency of locomotion but are not necessary for others. Undulatory frequencies were not observed in the premotor interneurons, and directional undulation persists if they and either A or B motoneurons are ablated [129]. Hence, if CPGs do exist they could be located in some or all motoneuron classes, in muscle cells, or in networks of motoneurons [141]. Rhythm generation by motoneurons is found in the crustacean stomatogastric ganglion and was recently suggested for the vertebrate spinal cord [157]. A spontaneous switch between bistable states with an interval of about 30 s was recorded in a dissected preparation of DA5 motoneuron (only) [26], and from muscle cells at a similar time scale [27, 129]. In another dissected preparation, bursting and sporadic rhythmic muscular postsynaptic currents and

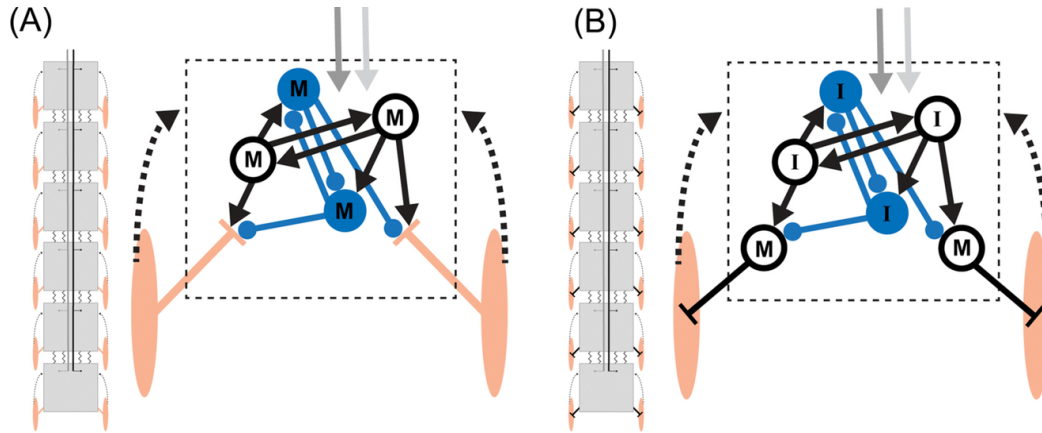


action potentials were reported [163]. The activity is mediated by a P/Q/N-high-voltage-activated calcium channel UNC-2 [129], and could be evoked by optogenetic stimulation of premotor interneurons. A sodium leak channel (NCA-1) seems to be involved by depolarizing premotor interneurons and motoneurons [163], but is not necessary for bursting to occur [129]. After optogenetic ablation of all premotor interneurons and B motoneurons, with optogenetic activation of A motoneurons, the bursts prolonged to 3 s and their frequency to 90 s interval [129]. Importantly, calcium imaging of A motoneurons in some intact glue-immobilized animals produces activity of varying amplitude and frequency at about 50 s interval that persist in mutants that are defective for synaptic release and can be increased or decreased in gain-of-function or loss-of-function mutations of the calcium channel [129]. Hence, at least A motoneurons are pacemakers at about two orders of magnitude slower than the behavior when isolated from proprioceptive feedback and other circuit neurons. It is unclear whether other classes of motoneurons are pacemakers and whether fictive locomotive patterns of propagating alternation are produced in the absence of proprioceptive feedback. By disconnecting the ventral nerve cord at different locations Fouad et al. [164] demonstrated that the undulatory rhythm for forward locomotion can be generated independently at different locations along the body. In an extreme case, when the nerve cord was completely severed, two different frequencies were generated, a phenomenon that was abolished by ablating B motoneurons or the forward group of premotor interneurons [129]. They did not, however, demonstrate that it is a feedforward pattern, generated without sensory feedback that carries timing information, as required to infer a central pattern generator. In a computational model, Olivares et al. [165] suggest a ring network oscillator composed of the pair of AS, DA, and DB motoneurons in every functional segment along the body. In this model, AS neurons, which are tonically active, excite DA that in turn excites DB that closes the loop by inhibiting AS (Fig. 3C). Inhibition of AS sequentially stops excitation of DA and DB, and then AS to complete the rhythmic cycle. However, ablation and inactivation

experiments of AS [52], and DB [129] motoneurons do not support the necessity of this neuronal loop for locomotion.

## Analogy to other systems and framework of comparison

Because of its small size, the locomotor circuit might seem at first glance to be different from other studied animals. Notably (and similar to the crustacean stomatogastric network), it is composed only of motoneurons, with descending interneurons serving as input from head or tail ganglia. Similar to cephalochordata such as *Amphioxus* [166], the body wall muscle of *C. elegans*, and other nematodes send thin processes (40 nm in diameter), named muscle-arms into the nerve cord where they form *en passant* synapses with motoneurons [123, 146]. Yet, unlike chordates and similar to other invertebrates, each nematode muscle cell is innervated by at least four and up to eight cholinergic and GABAergic motoneurons. These two peculiarities (namely, muscle arms and multiple innervations) can encumber comparison to other locomotion systems. Instead, we suggest another functional framework of comparison to the vertebrate spinal cord for example (Fig. 4). In this framework, nematode locomotion interneurons are analogous to descending interneurons (e.g., corticospinal or reticulospinal); nematode motoneurons are analogous to spinal interneurons, integrating sensory and descending inputs, while generating and coordinating motor programs via their interconnectivity; and nematode muscle arms are analogous to spinal motoneurons. Finally, each nematode muscle cell can be considered a motor unit. Accordingly, some *C. elegans* motoneurons are dedicated to a direction of locomotion [51], while the muscle arms serve as the final common path [167].



**FIG. 4** Analogy to other motor circuits is based on muscle arm function analogous to motoneurons. (A) The *C. elegans* locomotion circuit (simplified diagram) is functionally segmented (insert) and layered such that descending input modulates the activity of interconnected motoneurons that in turn synaptically converge with excitation and inhibition onto muscle arms that integrate synaptic input and relay a motor program to the muscle. (B) We suggest that muscle arms are functionally analogous to motoneurons in most other locomotion systems (very simplified diagram). Accordingly, *C. elegans* ventral cord motoneurons (M) are analogous to local locomotor interneurons (I) that integrate descending input (*gray arrows*) and sensory feedback (*dashed arrows*) and interact to generate the locomotor program. (Credit: Gal Haspel and Daphne Soares.)

## Locomotion of the first-stage larva

Only three motoneuron classes (of eight in the adult, Fig. 3) are present in the first of four larval stages (L1): two cholinergic classes that innervate dorsal muscle (DA and DB) and one GABAergic class, DD that at this stage innervates ventral muscles. DD motoneurons

rearrange their neuromuscular junctions at the L1 to L2 molt to innervate dorsal muscle when six more classes differentiate [168]. Laser ablation of DB motoneurons in L1 impairs forward locomotion, leaving backward intact; ablation of DA impairs backward but not forward; and ablation of DD impairs locomotion in both directions [31]. With only three classes of motoneurons L1 crawling and swimming presents a conundrum: how can dorsal cholinergic (presumably excitatory) and ventral GABAergic (presumably inhibitory) neuromuscular junctions produce a ventral body bend? A hypothesis that GABA neuromuscular junctions might be excitatory early in development (as described for other systems [169]) to contract ventral muscle was refuted because although exogenous muscimol depolarizes muscles to induce contraction, it appears that activation of GABA<sub>A</sub> receptors by synaptic release induces shunting inhibition [170]. Furthermore, mutant L1 larva that do not synthesize GABA still undulate without this neurotransmitter and exhibits ventral coiling [170]. We suggest three possible mechanisms for ventral contraction. First, sublateral cords motoneurons: the three SAB motoneurons innervate anterior ventral body wall muscle in the L1 larva [7] and could potentially account for the ventral activation needed for its locomotion. Second, hypertonic ventral muscle: an imbalance in baseline activity causes the dorsal muscle to be relaxed until activated by DA and DB and the ventral muscle to be contracted until it is inhibited by DD. Finally, structural ventral spring: all three motoneuron classes contribute to a dorsal bend (by exciting dorsal muscles or inhibiting ventral ones) against a passive ventral bend for the animal to assume all bending angles.

## **Completeness and compactness, maps and hope**

*C. elegans* is delivering on the promise to be a model animal in which the parts can be exhaustively described. Explorers of this system arrive equipped with maps and hope. Maps for genes, cellular

development, neurons, and connections offer a completeness of description; while hope for understanding emerges from the compactness of the system with only hundreds of neurons and thousands of connections and a reliable and simple to describe locomotion behavior that grounds hypotheses to reality. Because of its geneticist founding fathers, *C. elegans* has a different trajectory than most model animals for the neurobiology of locomotion; many of the field's established transgenic, genetic, genomic, and imaging methods and the knowledge they gather are exciting breakthroughs for some of the classic models; on the other hand, neurophysiological and network mechanistic explanations that have been known for decades for other animals are now being discovered.

## About the authors

**Gal Haspel.** My BSc degree (1996) at the Ben-Gurion University (Israel) was followed by a PhD in Neuroscience and Life Sciences (2003) at the same university under the mentorship of Frederic Libersat, on the behavior-changing sting of the parasitoid wasp *Ampulex compressa* and its effect on motor output of its cockroach prey. During my graduate school I took the Neurobiology course at the Marine Biological Laboratory (2001), where during a 12-h period I heard a lecture on the emerging technology of genetically encoded calcium indicators from the late Roger Tsien, followed by an introductory lecture about the neurobiology of *C. elegans* from Anne Hart. Combining the two was obvious. I began studying the neurobiology of *C. elegans* during a summer of independent research as a Grass Fellow (2003) also at the MBL. Before the word optogenetics was coined, I expressed three opsin-related proteins (a system named chARGE) in nematode neurons and muscle and tested the induced effect of light on behavior. I joined the laboratory of Anne Hart at Harvard Medical School in Boston (USA) where I was the locomotion-guy in a worm lab and then joined the laboratory of Michael O'Donovan at the National Institutes of Health (USA) where I was the worm-guy in a locomotion lab. I was fortunate to have learned immeasurably from my three official mentors and to



have spent time learning, researching, and teaching at the MBL. Since 2013, in my laboratory in the New Jersey Institute of Technology we study the connectivity, activity, and recovery from injury of the locomotion circuit in *C. elegans*, using a combination of technologies and approaches that are reflected in this chapter.

**Lan Deng.** I received a BSc in Life Science and Biotechnology from the China Pharmaceutical University, and a PhD from the Biological Science program at the New Jersey Institute of Technology and Rutgers University Newark. I joined the Haspel Laboratory to study the locomotor system of *C. elegans*, to understand how neurons work together to alternate antagonistic muscles. My research focuses on the inhibitory GABAergic motoneurons, and I studied how these motoneurons contribute to the rapid undulatory locomotion.

**Maria Belen Harreguy.** I obtained two BSc degrees in Biotechnology and in Biotechnology Engineering from ORT University in Montevideo, Uruguay, and an MSc in Biotechnology from the University of South Florida with the support of a Fulbright Scholarship. I am a PhD candidate in the Federated Department of Biological Sciences at Rutgers University Newark and the New Jersey Institute of Technology, mentored by Drs Tracy Tran and Gal Haspel. The focus of my research is to understand the role of the semaphorin pathway in both mice and *C. elegans* in response to dendritic injury that I induce with laser microsurgery. I hope that my research will inspire and underlie treatments for spinal cord injury.

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# The Neural Control of Movement

Model Systems and Tools to Study Locomotor Function



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