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Structure and physiological activity of the motoneurons of the nematode *Ascaris*

(neurobiology/synaptic activity/electron microscopy/simple nervous system)

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ABSTRACT **The nervous system of the nematode worm *Ascaris* contains about 250 nerve cells; of these, the motoneurons consist of five segmental sets, each containing 11 cells. Morphologically, the motoneurons can be divided into seven different types. Their geometry is simple: some are unbranched, others have one branch point, and the most complex have two. There is no neuropil in the nerve cords; synapses are made by axo-axonal contact or onto short spines. These features enable us to study the anatomy and physiology of the system with a degree of completeness that would be difficult in other systems. The physiological activity of five of the motoneurons has been investigated, three being excitatory and two inhibitory. The excitatory motoneurons receive input from intersegmental interneurons. The inhibitory motoneurons do not receive input from the interneurons; instead they receive their input from the excitatory motoneurons in a circuit that can mediate reciprocal inhibition between the dorsal and the ventral musculature.**

One of the outstanding problems in neurobiology is to understand the capabilities of assemblies of neurons in terms of the properties of the cells of which they are composed. In vertebrate nervous systems this problem has been approached in situations in which small numbers of cell types are arranged in a repeating pattern (e.g., cerebellum, retina); by studying the smallest fundamental repeating unit in these systems, the logical principles by which information is transformed might be inferred.

An alternative approach is to select an invertebrate system in which there is only a small number of neurons. A number of interesting results have already emerged from the study of small assemblies of neurons in invertebrates (1–7). However, these assemblies still contain a large number of interacting components, and the geometry of the neurons in many cases is as complex as that found in vertebrates.

In this paper we will describe the motor nervous system of the large parasitic nematode *Ascaris lumbricoides*. This system has a small number of neurons with extremely simple geometry. This allows us to study the physiology and anatomy of the system with a degree of completeness that would be difficult in other systems.

The salient advantages of the *Ascaris* nervous system are threefold.

(*i*) *Cell number.* The entire nervous system contains only about 250 neurons; the motor nervous system that we will describe here is divided into five segments, each containing 11 motoneurons, and there are six nonsegmental interneurons traversing the segments. By contrast, in truly segmented animals such as the crayfish and the leech, each segmental ganglion contains several hundred cells and there are hundreds or thousands of neurons impinging upon each ganglion from other centers.

(*ii*) *Neuronal geometry.* The second, and more important, advantage of the *Ascaris* motor nervous system is that the geometry of each neuron is simple. Each one sends out a straight fiber with, at most, two branch points; some neurons are completely unbranched. The neurons have short (1–2 μm) spines where they make synapses to muscle. Although there is no neuropil, there is an abundance of synaptic contacts between these neurons in the nerve cord despite their simple shapes.

(*iii*) *Relationship to other nematodes. Caenorhabditis elegans* is another nematode that is being investigated intensively in several laboratories because its mode of reproduction and small size make it an excellent animal for genetic and electron microscopic analysis (8–11). Brenner and his colleagues have isolated many mutants that are defective in their movement and are analyzing them by various techniques (12–16). However, the small size of this worm makes it unsuitable for electrophysiology.

Because *Ascaris* is a parasitic animal with a long generation time, it is not suitable as a genetic system; however, it has the important advantage that its nerve and muscle cells are large and can be penetrated with microelectrodes. Recently, White *et al.* (11) have analyzed the morphology of the motor nervous system of *C. elegans* and have shown that the neuronal types are identical to those in the motor system of *Ascaris* (see below). It is reasonable to assume that the functions of morphologically analogous elements will be similar in the two animals, so that the different experimental approaches being followed for each animal will give complementary insights that will be directly applicable to both systems.

The nervous system of *Ascaris* consists of a series of ganglia in the head associated with the nerve ring. Two major nerve cords, the dorsal and ventral cords, originate in the nerve ring and run along the animal to the tail where there is a second, smaller set of ganglia.

In 1909, Goldschmidt (17) completed his detailed anatomical studies on the head and tail ganglia of *Ascaris*. He showed that the number of neurons in the central nervous system was small (e.g., 149 in the head ganglia) and reproducible from animal to animal.

The neuromuscular system of nematodes is unusual in that the neuromuscular contacts are made not by neurons sending processes to peripheral muscles but by specialized branches of muscle cells, called muscle arms, extending to the nerve cords. The muscle arms subdivide and interdigitate near the nerve cord, forming electrical junctions with each other (18, 19).

The muscles of *Ascaris* exhibit spontaneous activity, and the musculature is arranged in blocks of cells that have coordinated activity (20). del Castillo and his colleagues (18, 21–23) showed that the muscle cells generate spontaneous action potentials at

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or near the neuromuscular junctions. The ionic mechanisms of these action potentials have been investigated by Weisblat *et al.* (24). del Castillo *et al.* (23) also explored the role of the nervous system in controlling the muscle action potentials and concluded that the frequency of the spontaneous action potentials is modulated by excitatory or inhibitory neural input.

We have extended the anatomical work to the nerve cords that stretch between the head and tail ganglia analyzed by Goldschmidt. In this paper we show that all of the neurons with cell bodies in this region are motoneurons; on the basis of anatomical criteria, they can be divided into seven types. We have also carried out electrophysiological studies on the neuromuscular synapses made by five of the motoneuron types and show that three are excitatory and two are inhibitory.

MATERIALS AND METHODS

Methods of handling animals and histological procedures for making serial 10-μm Epon sections for light microscopy were as described (25). For electron microscopy, fixation was carried out in 2.8% glutaraldehyde/2.0% formaldehyde (from paraformaldehyde) in 0.1 M pH 7.4 phosphate buffer followed by postfixation in 2% osmium tetroxide in the same buffer. After dehydration and embedding in Epon, thin sections were made with a Porter–Blum MT2B ultramicrotome and examined in a Hitachi HS7 electron microscope.

Electrophysiological recordings were made from muscle cells with 20 to 30-megohm electrodes filled with KCl or KOAc. The preparation was continuously perfused at 37° with physiological saline (125 mM NaOAc/4 mM NaCl/24.5 mM KCl/4.9 mM $MgCl_2$/5.9 mM $CaCl_2$/10 mM Tris·maleate, pH 7.4).

Typically animals between 18 and 25 cm long were used both for the anatomical and electrophysiological experiments. Except for the two male worms that were completely serially sectioned (see below), all experiments were carried out on female worms.

RESULTS

Segmentation in the nervous system and its reproducibility

Commissures. We have found that the nervous system of *Ascaris* contains a repeating pattern of neurons. These repeating units are called "segments," even though nematodes are not normally considered by zoologists to be segmented worms. The first indication of a repeating pattern came from scanning by light microscopy of complete serial sections of four worms, two male and two female (each series comprised 16,000–18,000 10-μm sections). It was found that, at intervals, ventral cord neurons send out a branch that traverses the circumference of the animal and joins the dorsal cord [cf. Brandes (26)]. These branches, called "commissures," are buried within the thin layer of hypodermal tissue that underlies the cuticle. Some commissures traverse the left side of the animal and others, the right. There is a marked bilateral asymmetry in that, apart from the first segment, for every left-handed fiber there are six right-handed ones arranged in three pairs (Fig. 1). These seven commissural fibers make up the basic repeating unit, or segment. The boundaries of the segments have been arbitrarily chosen to be the left-handed commissures that are found in one copy per segment. There are five such segments in the body of *Ascaris*. The front of the first segment is modified in that the most anterior of the three pairs of right-handed commissures is reflected to the left, and there is also an extra right-handed commissure.

Within the five segments of females there are 36 commissures. Anterior to the first segment, there is a triplet of left-handed commissures, and posterior to the fifth segment is a single left-handed commissure, making a total of 40 commissures. The basic pattern for males is the same, but there are 4 additional left-handed commissures, making a total of 44. The basic repeating pattern has been confirmed by serially sectioning the first three or four segments of 21 additional worms. The position of the commissures is reproducible from animal to animal.

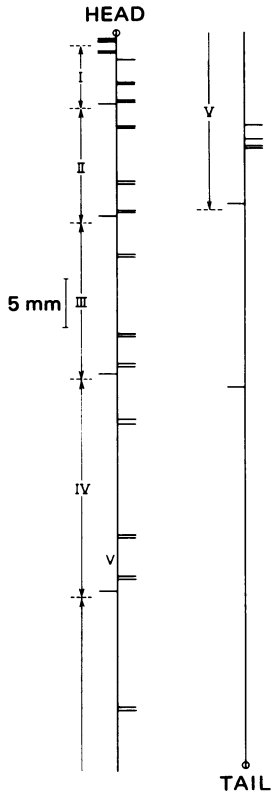

FIG. 1. Diagram of the ventral cord of female *Ascaris* showing the position and handedness of the commissures. The ventral cord stretches between the head and tail ganglia (circles). Left- or right-handed commissures leave the cord at intervals. Each line perpendicular to the cord represents a single commissural fiber. Dashed lines indicate the ends of the segments. Segments are numbered with Roman numerals. V represents the vulva.

Cell Bodies. In Fig. 2 the cell bodies found in the first three segments are shown. Because the cells are not clustered together in obvious ganglia in the body of *Ascaris*, the cell bodies have been divided into groups arbitrarily, usually with reference to the position of commissures. Once again, a basic repeating unit is found that is congruent with the segments already described. Each segment has a set of 11 cells associated with the seven commissures. The first segment is modified in that it has four additional cells.

Axon Tracing. In order to trace axons in the nerve cords of *Ascaris*, 5000–8000 10-μm serial sections were examined from each specimen.

The projections in the dorsal and ventral cords of the neuronal processes of each of the segmental neurons of the first three segments are shown in Fig. 3. No cells have been found with more than one commissure. There are no cell bodies in the dorsal cord. All but two dorsal nerve fibers arise from commissures and are branches of cells that also have processes in the ventral cord (the two fibers that do not arise from commissures originate in the nerve ring). Each commissural neuron has a cell body in the ventral cord.

The segmental neurons can be differentiated from one another by the branching pattern of their processes (Fig. 4); these patterns are relatively simple. Among the 11 cells, there are seven distinct types. Three types (DI, DE2, and DE3) are represented once in each segment, and the remaining four types (VI, DE1, V-1, and V-2) occur twice per segment. Of the four cell types that appear twice in each segment, two (DE1 and VI)

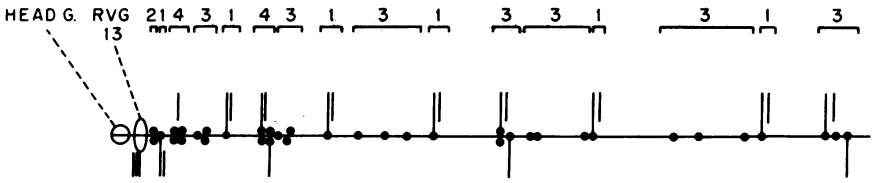

FIG. 2. Grouping of cell bodies in the ventral cord in the first three segments of female *Ascaris*. The size of the cell bodies (black circles) is much magnified in this diagram. Apart from the retrovesicular ganglion (RVG), the cells are not in fact arranged in obvious ganglionic clusters. In each pair of commissures, one arises directly from a cell body; the cell body of the other lies posterior to the commissure (see Fig. 3).

have commissures and the other two (V–1 and V–2) have processes only in the ventral cord. In each segment the right-handed commissures are arranged in two DE1/VI pairs and one DE2/DI pair; the unpaired DE3 commissure is left-handed.

In addition to the segmental neurons there are six large intersegmental axons ("through" fibers) that are confined to the ventral cord. Their cell bodies are located in the head or the tail. We have traced these axons from the head to the fourth segment, and we suspect that they extend throughout the entire length of the ventral cord. They are located deep in the nerve cord, away from the neuromuscular contact region, and appear to be the interneurons that control or modulate the segmental motoneurons.

Synaptic contacts made by the segmental neurons

We have investigated the synaptic relationship between nerve and muscle by electron microscopy. The neuromuscular synapses are made by short (1–2 μm) neuronal extensions which penetrate the hypodermal layer and come into contact with the ends of the muscle arms. As described by Rosenbluth (19), the following ultrastructural features are associated with areas of synaptic contact: (*i*) clear spherical vesicles, 40 nm in diameter, clustered immediately adjacent to the presynaptic membrane; (*ii*) dense-core vesicles, about 80–100 nm in diameter, that are more sparsely distributed; (*iii*) giant mitochondria, often closely associated with the clusters of vesicles; and (*iv*) membrane thickenings on both pre- and post-synaptic membranes. As will be described below, we have correlated the occurrences of synapses defined by these ultrastructural criteria with areas of synaptic contact detected electrophysiologically. We also observe neuron–neuron synapses in both nerve cords.

In order to examine the neuromuscular synapses of specific segmental neurons, we first identified the neurons by tracing their fibers through serial 10-μm sections in the light microscope. This is the only method that gives unambiguous identification of the neurons. Selected sections were then thin-sectioned for electron microscopy. So far we have carried out this procedure on the dorsal cords of two animals and the ventral cord of one. The results presented below involve between 4 and 14 examples of each type of synapse and in most cases are derived from neurons in the first segment.

In the dorsal cord, neuromuscular synapses were found for five of the seven segmental neurons that have commissures (types DI, DE1, DE2, and DE3; see Fig. 4). The remaining two (both type VI) had small processes that make contact with muscles but do not make neuromuscular synapses. Instead, they receive synaptic input from excitatory motoneurons. Therefore, we conclude that the dorsal processes of this class of neuron are dendrites.

In the ventral cord, the V–1 and V–2 neurons make neuromuscular synapses. Extensive neuromuscular synapses in the ventral cord also were found for the two VI neurons whose dorsal processes are dendritic. The DI neurons have synapses that are complementary to those of the VI cells: the DI neurons make neuromuscular contacts (but not neuromuscular synapses) where they receive inputs from V–1 and V–2 motoneurons in the ventral cord; they make neuromuscular synapses in the dorsal cord.

In summary, all of the segmental neurons are motoneurons since they make neuromuscular synapses in either the dorsal or ventral cord (but not both); none of the segmental neurons is an interneuron making synapses only onto other neurons.

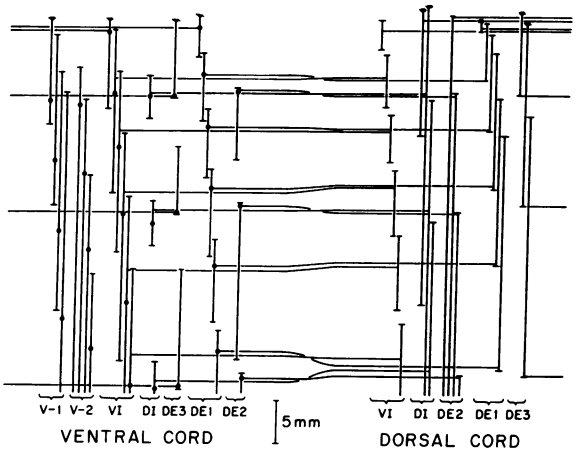

FIG. 3. Diagram of the projections of the processes of segmental neurons in the dorsal and ventral cords. The longitudinal extent and position of each process is correctly represented but the relative lateral position of neighbors is not portrayed here. The diagram is of the first three segments of an animal that has been cut open along the left lateral line and pinned out flat. The V-1 and V-2 cells in the first segment have been omitted. The top of the diagram is anterior.

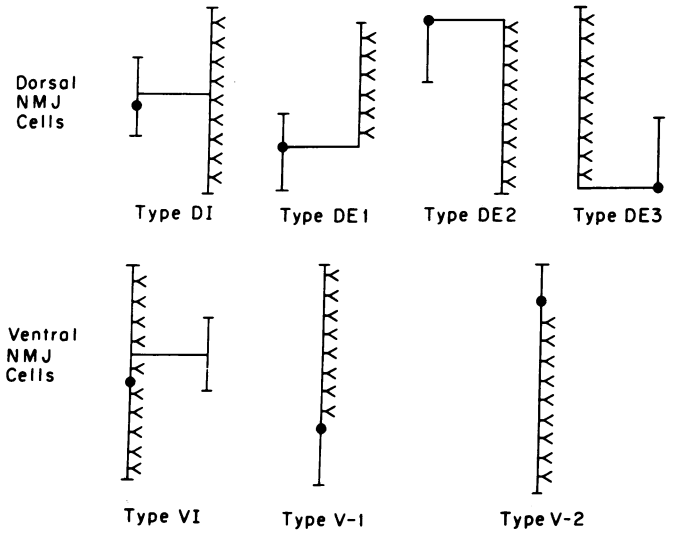

FIG. 4. Diagram of the seven types of segmental neurons. The cell body (black circle) is in the ventral cord. Forked projections represent regions where neuromuscular synapses are made. Horizontal lines represent commissures between dorsal and ventral nerve cords. Note that the commissure of the type DE3 cell is left-handed. NMJ, neuromuscular junction.

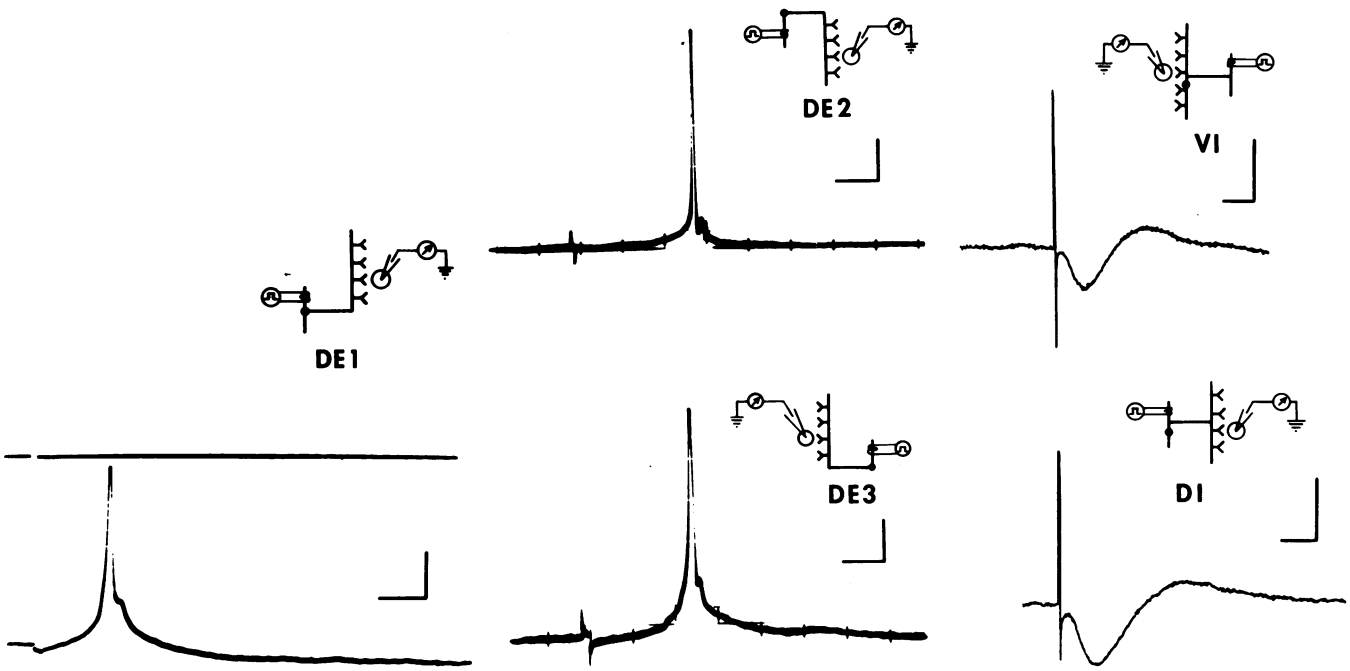

FIG. 5. Intracellular recordings from muscle cells in response to nerve stimulation to type DE1, DE2, DE3, DI, and VI neurons. Single excitatory responses are shown for type DE1, DE2, and DE3 cells. The hyperpolarizing records for type DI and VI cells are averages of 128 responses. (*Insets*) In each record these show the position of stimulating and recording electrodes. Stimulus artifacts precede each response. In the record for the DE1 cell, the top trace is a stimulus monitor. Scale for DE1, DE2, and DE3: vertical, 10 mV; horizontal, 50 msec. Scale for DI and VI: vertical 1 mV; horizontal, 100 msec.

In the ventral cord, the "through" fibers make synapses onto the DE1, DE2, DE3, V-1, and V-2 motoneurons but not onto the DI or VI cells. We surmise that the through fibers are the interneurons that control the motoneurons.

Functional classification of neurons

In parallel with the ultrastructural investigation of the neuromuscular synapses made by segmental neurons, we have carried out electrophysiological experiments to classify these synapses functionally. In these experiments the ventral cord was stimulated with a suction electrode while intracellular recordings were made from the dorsal musculature. Our knowledge of the neuroanatomy has allowed us to make preparations in which all but one commissure has been cut. In such preparations the dorsal musculature receives signals from only a single motoneuron. After each experiment we verified that the surgery was carried out correctly and identified the intact commissure by fiber tracing in serial 10-μm sections. At least three, and in most cases five, experiments were carried out on 21 of the 22 commissural neurons in the first three segments of female worms (the supernumerary neuron with the single right-handed commissure in the first segment has not yet been examined).

When single type DE1, DE2, or DE3 neurons were activated ventrally, depolarizing responses, often giving rise to overshooting action potentials, were recorded from the dorsal musculature (Fig. 5). Hyperpolarizing responses in the dorsal musculature were found when type DI neurons were stimulated in the ventral cord whereas ventral stimulation of type VI neurons gave no dorsal response. Homologous neurons from different segments showed identical responses, and members of those sets of neurons that have two copies per segment were indistinguishable. In complementary experiments on these neurons, in which their dorsal processes were stimulated and responses were recorded from the ventral muscles, hyperpolarizing responses were produced by type VI cells; no responses were evoked from types DE1, DE2, DE3, and DI neurons.

To eliminate the possibility that the responses described above were produced by direct electrical activation of the muscles, control experiments were carried out in which the single remaining commissure linking dorsal and ventral cords was cut while the evoked responses were being recorded. For each neuronal type, cutting the commissure obliterated the response.

The depolarizing responses are excitatory because they often gave rise to action potentials. The hyperpolarizations are inhibitory. In those preparations in which there were spontaneous action potentials in the muscle, stimulation of the relevant type DI or type VI neurons produced a reduction or cessation of the spontaneous activity.

Sometimes the hyperpolarizations were followed by depolarizations when type DI or VI was stimulated; the source of these depolarizations is not yet understood. They may result from the electrical properties of the muscle cells or from synaptic interactions with other neurons in the nerve cord.

For each neuron with a commissure there is output to muscle cells in only one of the nerve cords; the process in the other cord does not produce electrical signals in the muscle cells. These physiological results are completely congruent with the ultrastructural results presented above.

DISCUSSION

Structure and Function Relationships. The assignment of each of the motoneurons to one of seven different classes was carried out initially on the basis of anatomical criteria. These include the handedness of the commissure and its position relative to the cell body, the pattern of branching in the dorsal and ventral nerve cords, and the distribution of neuromuscular contacts in the two nerve cords. Cells with common sets of features recurred at intervals along the nerve cords, distributed in a series of repeating units, the segments. Some cells had two copies per segment and the others occurred once in each segment. In the three segments examined electrophysiologically,

Table 1. Neuronal types

Ascaris neuron type	Neuromuscular output region	Function in *Ascaris*	*C. elegans* neuron type (ref. 11)
DE3	Dorsal	Excitatory	DA
DE2	Dorsal	Excitatory	DB
DE1	Dorsal	Excitatory	DAS
DI	Dorsal	Inhibitory	DD
VI	Ventral	Inhibitory	VD
V-1	Ventral	Excitatory*	VA
V-2	Ventral		VB

*The function of the V-1 and V-2 neurons has not yet been elucidated directly, but stimulation of the ventral cord produced excitation of the ventral muscles, so either V-1 or V-2 neurons must be excitatory and we suspect that both classes are excitatory.

cells that had the same structure also had the same physiological activity; there was complete correlation between structure and function.

Comparison with *C. elegans*. The seven types of motoneurons in *Ascaris* are remarkably similar to the motoneurons of *C. elegans* described by White *et al.* (11). On the basis of their geometry and the location of neuromuscular synapses, equivalent cells in both animals can be identified (Table 1).

We would expect that cells with the same structure have the same function in the two animals. The functions of the seven classes of neurons are somewhat different from the predictions of White *et al.* (11). Using different techniques, L. Byerly and R. L. Russell (personal communication) have found essentially similar results in their electrophysiological experiments on *Ascaris*.

Synaptic Structure. In using anatomical techniques alone to describe the wiring diagram of a system of neurons, it is usually taken for granted that there are structural features that can be used to define the sites where synaptic transmission occurs. Recently, doubt has been cast on this assumption; it has been suggested that certain synapses, defined anatomically, may be "silent" physiologically (27, 28). In *Ascaris*, however, we have found that, where physiologically active neuromuscular transmission occurs, morphologically identified synapses between nerve and muscle exist. Conversely, where no physiologically active contacts exist, the ultrastructural correlates are absent. This gives us confidence in the anatomical criteria we used to characterize synapses in this organism.

Reciprocal Inhibition and the Control of Movement. The inhibitory motoneurons (types DI and VI) have dendrites in one nerve cord and neuromuscular synapses in the other; the dendrites receive their synaptic input from other motoneurons. The electron microscopical studies reported here have shown that the neurons that inhibit the ventral musculature (type VI neurons) receive synapses from excitatory motoneurons in the dorsal cord. The DE1, DE2, and DE3 neurons have been shown electrophysiologically to make excitatory synapses onto the muscle cells. We find electrophysiologically that their synapses onto the VI dendrites are also excitatory, so a circuit exists that can mediate reciprocal inhibition between dorsal and ventral cords solely by interactions between the motoneurons. This circuit would ensure relaxation of the ventral muscles opposite a dorsal contraction. A complementary circuit exists between ventral V-1 and V-2 cells (which we believe to be excitatory onto muscle) and the dorsal inhibitor, type DI. The same function for this circuit in *C. elegans* was predicted by White *et al.* (11).

In this animal, then, reciprocal inhibition appears to be wired at the level of the motoneurons rather than at the interneuron level as in the crayfish (2). The intersegmental "through" fibers make synapses onto the excitatory motoneurons and it is reasonable to suppose that coordination of the activity of all of the motoneurons to produce locomotory movements depends on this interneuronal input. At present it is not clear whether the interneurons act as general gating devices that switch motoneurons in all the segments on or off, or whether each of the interneurons drives specific subsets of motoneurons to produce preprogrammed movements.

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