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Neuromuscular junctions in the nematode *C. elegans*

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The neuromuscular junction serves widely as a model synapse for both the study of synaptic development and synaptic transmission. We are now attempting to understand the molecular events that underlie these processes. One approach to the study of the neuromuscular junction is to analyse mutants of the nematode Caenorhabditis elegans. *We review the motor circuit in the worm responsible for locomotion, the development of the* C. elegans *neuromuscular junction, and the gene products required for the functioning of nematode synapses. This genetic approach has both identified novel components of the neuromuscular junction and has ascertained the in-vivo roles of biochemically-defined components that regulate neuromuscular transmission and development.*

Key words: development of neuromuscular junction / neurotransmission / genetic analysis / nematode / *Caenorhabditis elegans* / synapse / exocytosis

A PROMISING SYSTEM for the study of neuromuscular junctions is the non-parasitic soil nematode *Caenorhabditis elegans* (Figure 1A). *Caenorhabditis elegans* possesses a remarkably simple nervous system: there are only 302 neurons but these cells comprise greater than 30% of the somatic cells in this nematode.[1] The majority of these neurons are contained in the head ganglia (Figure 1B). Most cells in these ganglia send processes into the nerve ring surrounding the pharynx. Processes in the nerve ring form a dense plexus of *en passant* synapses which comprises the major neuropil in the animal. Other neuronal cell bodies are located in the ventral cord and in several minor ganglia in the tail. The synaptic connectivity of the nervous system has been determined from serial electron micrographs[2] and has enabled researchers to begin to understand the motor circuit of this tiny organism.

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Locomotory circuit

Interneurons that control backward or forward locomotion called command neurons extend axonal processes into the ventral nerve cord. These form gap junctions or chemical synapses to cholinergic motor neurons situated along the cord in close apposition to the nerve bundle (Figure 1B, 2A).[3,4] The motor neurons are also specialized for forward (B-type neurons) or backward (A-type neurons) locomotion. For example, the command neuron AVB is required for forward locomotion[5] (Figure 3). It forms gap junctions to the motor neurons responsible for forward locomotion, the B-type neurons, DB and VB.[4] The command neurons set the direction of locomotion but are probably not involved in creating the sinusoidal posture or in directing the wave of contraction down the length of the animal; these properties are probably defined locally by interactions among the motor neurons and the muscles.

The motor neurons synapse to the ventral or dorsal body muscles. Forty-two of the ventral cord motor neurons form neuromuscular junctions to the ventral muscles, and 33 send commissures circumferentially to the dorsal side of the animal. These commissures extend axons along the dorsal nerve cord and form neuromuscular junctions to the dorsal muscles. The muscles form longitudinal tracts along either side of the dorsal and ventral nerve cords and send processes called muscle arms to the motor neurons in the cords (Figure 2A,B). The neuromuscular junctions are formed *en passant* at the outer edge of the nerve bundles and are distinguished by remarkably-thickened presynaptic specializations (Figure 2C). By contrast, the postsynaptic specialization does not contain junctional folds nor is there a conspicuous postsynaptic density, although a darkened region on the muscle membrane is detectable.

Electrophysiological recording from these neuromuscular junctions are not yet possible due to the small size of *C. elegans* — an adult is only one millimeter in length. For this reason electrophysiological studies have been conducted on the larger nematode *Ascaris*, which can reach 35 cm. Surprisingly, these two nematodes have the same number

and organization of motor neurons in the ventral cord. Thus, models of *C. elegans* locomotory behavior have relied heavily on the physiological recordings performed in *Ascaris*.[7,8]

At rest or while moving the worm has a sinusoidal posture and the formation of these bends is controlled by the local motor circuitry. Unlike skeletal muscles in vertebrates, the body muscles in the nematode receive both excitatory input from cholinergic motor neurons and inhibitory input from GABAergic motor neurons (Figure 3). These GABAergic motor neurons receive input at the cholinergic neuromuscular junctions (see Figure 2C) and inhibit contralateral muscles so that antagonistic muscles are relaxed and a bend will form.[9] The inhibition from the GABAergic motor neurons seems to be most important for resetting posture, for example when the animal is reversing direction or is initiating rapid movement, when the phase of excitation along the body axis is being set up. But once an animal is moving, lack of GABA input does not interfere with wave propagation, it only reduces the amplitude of the wave. These local excitatory and inhibitory interactions are thus able to form a single bend in the animal, however, it is not clear which neurons determine the sinusoidal posture, that is, the alternating contractions and relaxations along the whole length of the body. It has been suggested that the long undifferentiated processes on the cholinergic motor neurons may act as stretch receptors or receive feedback from the muscles and thus provide information about the state of contraction along the body.[2]

How the wave of muscle contraction is propagated anteriorly or posteriorly is even more mysterious. At some level it depends on the neuronal circuit shown

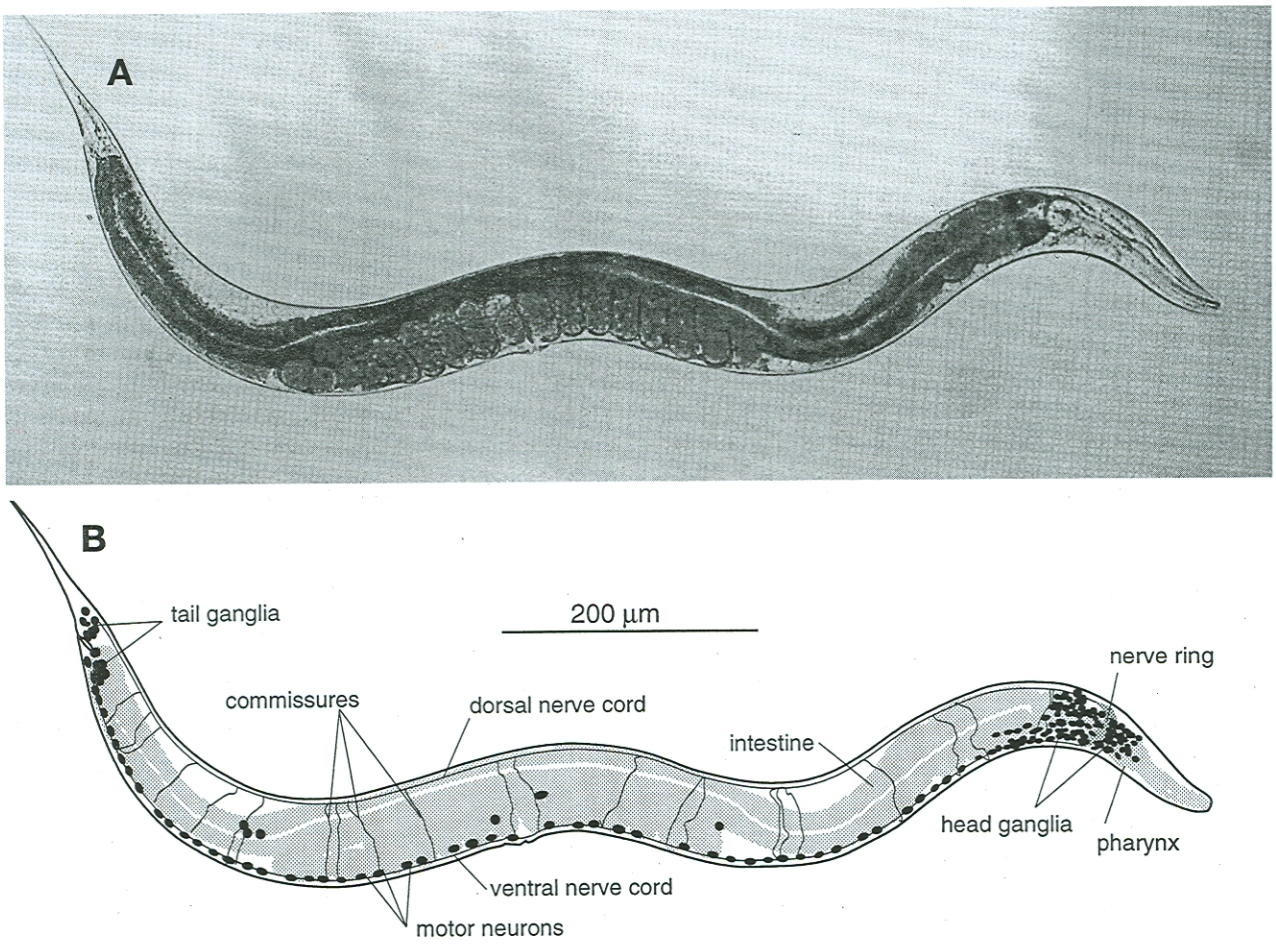

Figure 1. A *Caenorhabditis elegans* adult. (Photo: G. Beitel). (B) A diagram of the adult nervous system.

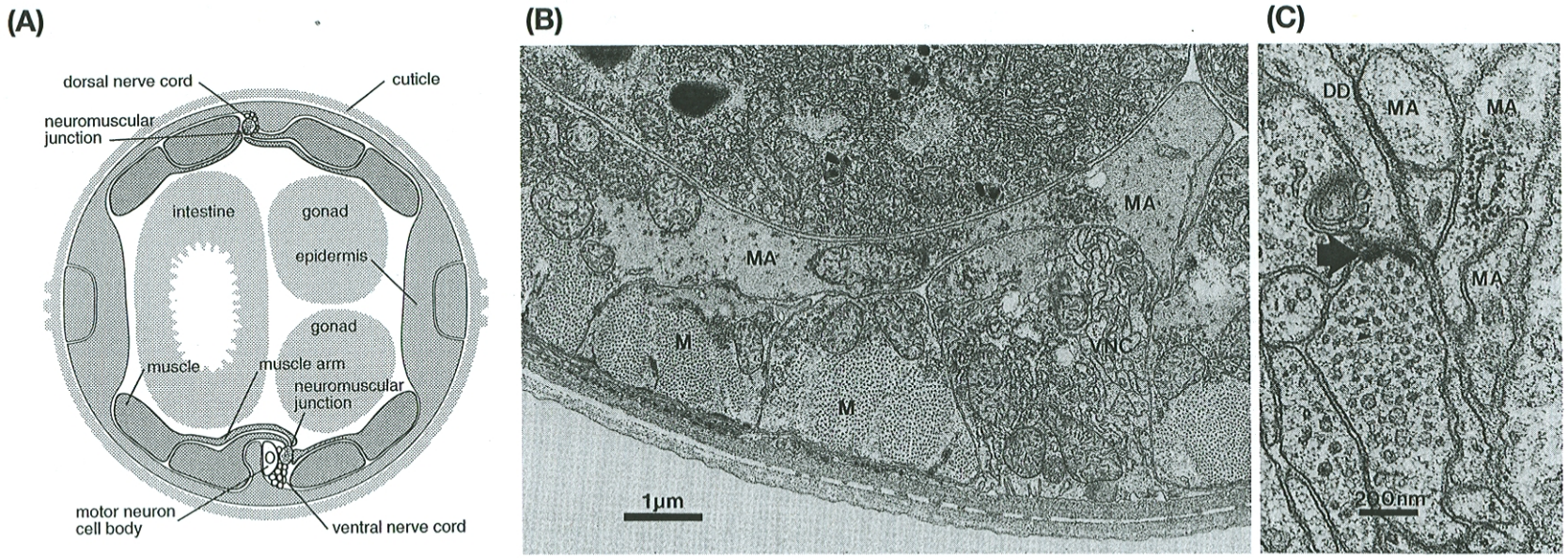

Figure 2. (A) Diagram of the cross-section of an adult worm. (B) Electron micrograph of the ventral nerve cord and adjacent muscles. Note the muscle arm (MA) extending to the ventral nerve cord (VNC). M, muscle. (C) Electron micrograph of a neuromuscular junction. Note that the GABAergic motor neuron DD has extended a process to intercept input from the ventral cholinergic motor neuron. Large arrow, active zone; arrowheads, synaptic vesicles. (Photos: E. Hartwieg).

in Figure 3, since activation of the forward or backward motor neurons by the command interneurons is required for locomotion. However, such diagrams can be deceiving in that they emphasize the neuronal input; it has been suggested that the muscles may be capable of propagating contractions on their own and that the nervous system acts only to coordinate the ventral and dorsal sides of the animal.[4]

The action of modulatory neurotransmitters such as peptides and the aminergic transmitters is only beginning to be explored. In *Ascaris*, a large number of peptides have been isolated and some of these can cause striking changes in the physiology of the motor neurons.[7] A subset of the cholinergic motor neurons in *C. elegans* express peptides related to FMRFamide, suggesting these peptides may also play a role in

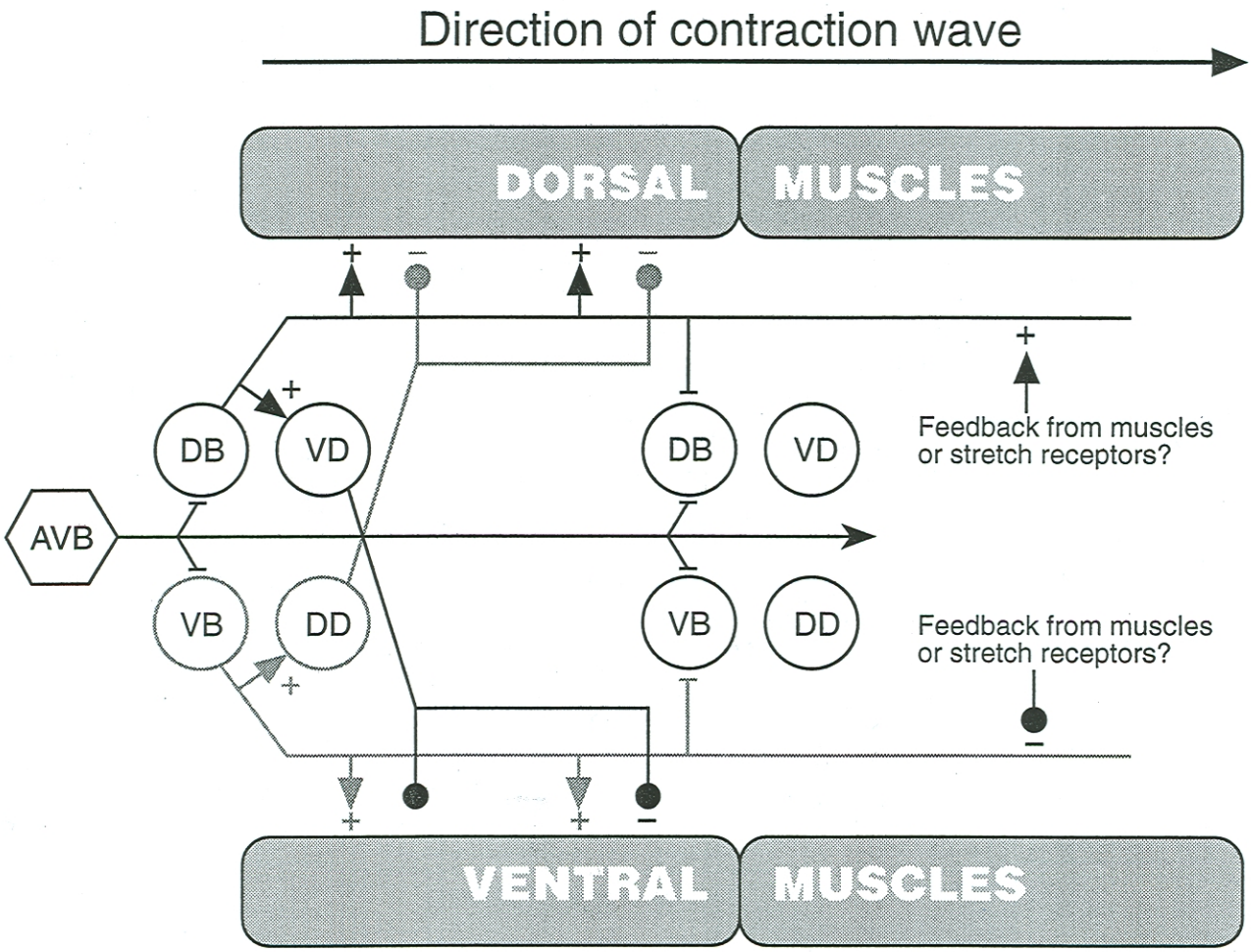

Figure 3. Diagram of the forward locomotory circuit. AVB is a forward command interneuron. The motor neuron circuit is repeated along the length of the body, and each repetitive unit contains motor neurons from each morphological class. Motor neurons are subdivided by whether they function in forward or backward locomotion and whether they innervate the ventral or dorsal muscles. For example, the DB motor neurons are required for forward locomotion and innervate the dorsal muscles (*d*orsal *B*-type neurons). The cholinergic DB motor neurons also innervate the VD motor neurons which release the inhibitory neurotransmitter GABA on the ventral muscles.[9] Thus, muscles on one side will be contracted due to release of acetylcholine and the muscles on the other side will be relaxed due to release of GABA, and a bend will form in the worm. How differential activation of the circuits causing contraction of the dorsal versus ventral muscles (shown as black and gray respectively) is accomplished is unknown but it has been hypothesized that long undifferentiated extensions of the cholinergic neurons may provide proprioceptive feedback to these motor neurons.

motor control.[10] By contrast, the aminergic neurotransmitters are largely expressed in the neurons in the head and appear to alter the rate of movement and possibly act at the level of the command interneurons (B. Sawin, personal communication). Other muscles, such as the vulval muscles, or specialized body muscles of the male are innervated by serotonergic motor neurons and respond directly to serotonin.[11,12]

Neuromuscular development

Neuromuscular development begins about halfway through embryogenesis after the completion of all cell divisions (Figure 4). The muscles attach to the epidermis when the embryo is still an ovoid of cells. As the embryo elongates, the contractile apparatus assembles and muscles begin to spontaneously contract. These contractions are probably important for the morphogenesis of the worm because muscle function is required for elongation beyond the 'two-fold' stage.[13] During this initial elongation ('comma' to 'two-fold'), the nerve ring also becomes the first neuronal tissue to organize. Subsequently, a single interneuron pioneers the ventral cord[14] and shortly thereafter three classes of ventral cord motor neurons extend processes in the ventral and dorsal cords. Around the 'three-fold' stage, neuromuscular junctions form and the embryo begins rapid coordinated movements within the eggshell.[14]

Developmental plasticity

Changes that occur later in development indicate that the motor system expresses a degree of developmental plasticity. At the end of the L1 larval stage additional body wall muscles and five additional classes of motor neurons are born.[1] These muscles and motor neurons are incorporated into the embryonic motor system and new neuromuscular junctions form, implying a continued responsiveness between the muscles and neurons. Moreover, some rudimentary plasticity is probably required throughout the life of the animal

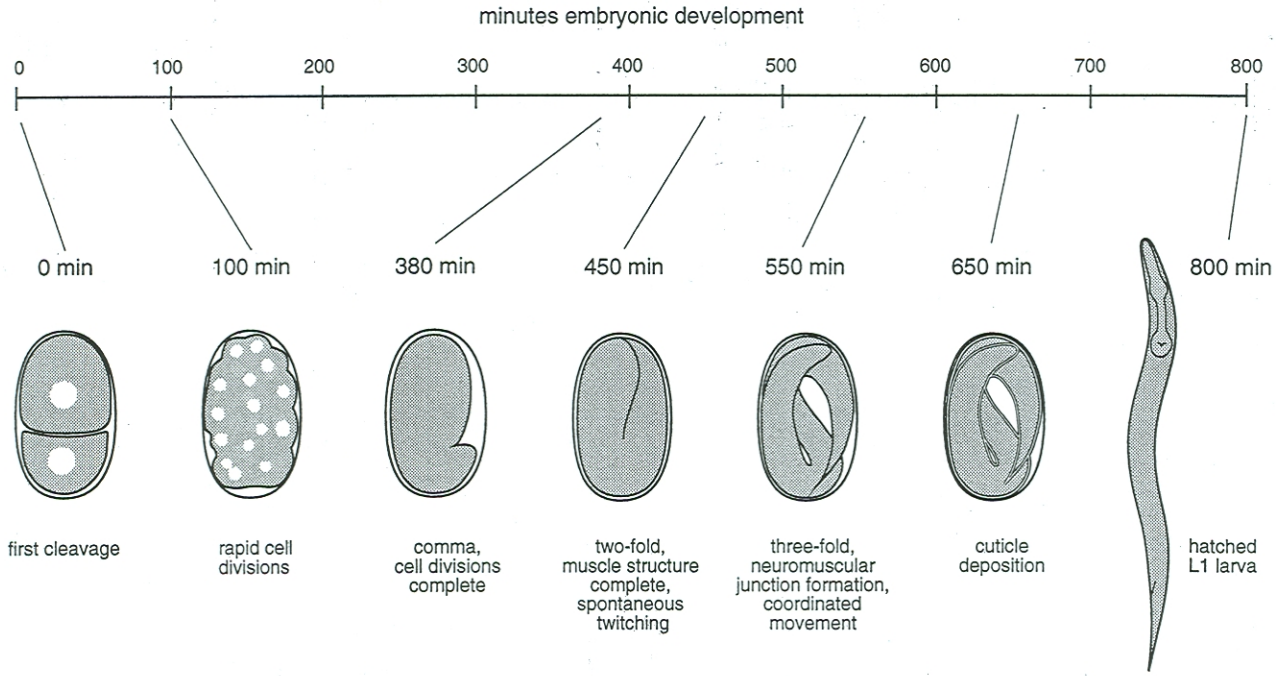

Figure 4. *C. elegans* embryonic development. Times are indicated in minutes after the first cleavage. Muscles have migrated to their final position and have attached to the underlying epidermis at 350 minutes.[56] The muscles flatten and become polarized by 430 minutes, the time at which the first spontaneous contractions are observed. This early stage of elongation is called '2-fold' because the developing worm is folded back on itself in a 'U' shape. At 550 minutes neuromuscular junctions form which coincides with the first signs of rapid coordinated movement.[14] Adapted from Williams and Waterston 1994.[13]

to maintain synaptic density during the greater than five-fold increase in length between hatching and adulthood. Finally, the distribution of synapses along the ventral cord indicates an interaction between adjacent motor neurons. The fields of innervation of neighboring motor neurons of a single class usually do not overlap.[2] Such a distribution may be formed by competition between members of a class for targets.

A more concrete example of developmental plasticity accounts for the rewiring of the DD GABAergic motor neurons (Figure 5). The embryonic class of GABAergic neurons, the DD motor neurons, originally innervate the ventral muscles and receive input on the dorsal side. At the end of the L1 larval stage the DD motor neurons rewire their synaptic contacts so that they innervate the dorsal muscles and receive input from newly-differentiated neurons on the ventral side.[15] A second class of GABAergic neurons, the VD motor neurons, are born during this period and innervate the ventral muscles. It is possible that this new input into the ventral muscles causes the DDs to rewire. However, analysis of mutants indicates that the rewiring of the DD neurons does not depend on the VD innervation of the ventral muscles.[16] Nor is new input to the DD neurons from the ventral cholinergic motor neurons, VA and VB, required for DD rewiring.[15] It is more likely that this rewiring is an intrinsic program of the DD neurons initiated by the developmental stage of the animal.

Genetic control of neuromuscular connectivity

The selection of synaptic partners during neuromuscular junction formation requires active participation by both the pre- and postsynaptic cells. Genetic analyses of uncoordinated mutants has identified some of the regulatory molecules required in motor neurons to select appropriate inputs and outputs for these cells. Once a *C. elegans* motor neuron has extended a process and possesses a neuronal morphology, homeodomain proteins are required to specify the proper synaptic partners. *unc-4* is required for the selection of synaptic inputs into the A-type motor neurons used in backward locomotion.[17,18] UNC-30 is a homeodomain protein related to UNC-4 and is required for GABA expression and neuromuscular junction formation in the GABAergic motor neurons, the DDs and VDs.[19-21] The *unc-55* gene, although not yet cloned, is required in the VD neurons to select ventral instead of dorsal muscle targets.[16] In the future, analysing the targets of these putative transcription factors may identify the molecules that directly regulate neuromuscular junction formation.

Neuromuscular junction formation in *C. elegans* and vertebrates differ in one striking regard: in nematodes the muscle extends a process, the muscle arm, to the nerve cord to meet the appropriate motor neuron and form a neuromuscular junction at the edge of the nerve bundle. Thus, the migrating muscle arms must obey spatial and identity cues to form proper synapses. Two *C. elegans* mutants suggest that chemotropism governs the muscle arm interaction with the motor neuron. First, in the *unc-6* mutant the axons that normally run in the dorsal nerve cord are displaced ventrally (Figure 6).[22] In this mutant the

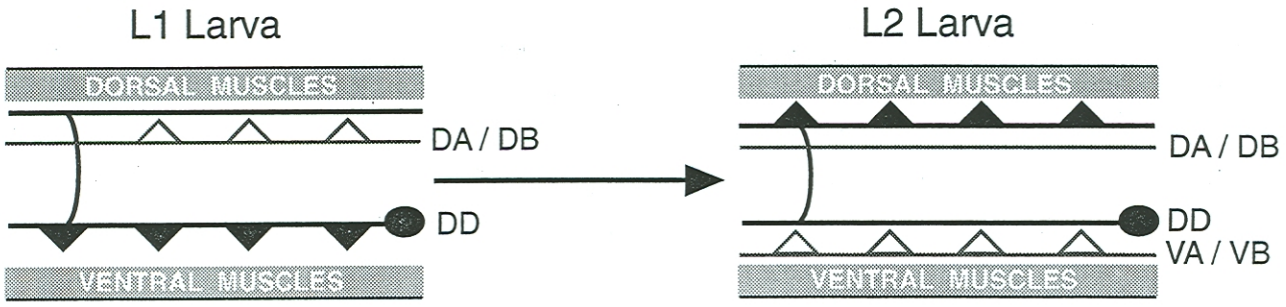

Figure 5. Synaptic rewiring during larval development. In the first stage larva (L1) there are only three classes of motor neurons in the body; the DA, DB and DD neurons. The DD neurons receive input from the DA and DB neurons and innervate the ventral muscles. In the second stage larva five new types of motor neurons are added to the ventral cord; the VA, VB, VC, VD and AS neurons. The DD motor neurons change their synaptic connectivity such that they now receive input from cholinergic motor neurons in the ventral cord and innervate the dorsal muscles. The VD neurons are wired reciprocally, that is, they receive input on the dorsal side and innervate the ventral muscles.

dorsal muscles send their arms across the lateral epidermis to locate the misplaced axons. Second, worms that lack a neuronal specific kinesin encoded by the gene *unc-104* have relatively normal axon morphology but fail to form normal synapses and accumulate vesicles in the neuronal cell bodies instead of transporting them along the axon. Muscle arms in *unc-104* mutants project to the vesicle-rich cell bodies instead of the axons. Both phenotypes suggest that a chemotropic molecule, perhaps stored in vesicles, is released by axons, and guides the muscle arms to the motor neurons. However, the secreted signal that mediates this interaction is not likely to be the neurotransmitter since lack of acetylcholine (J. Rand, personal communication) or GABA[23] does not lead to a change in the frequency of neuromuscular junctions. Alternatively, what we see in the adult mutant may be the result of promiscuous muscle arm projection to many targets during development, but that only the correct projection persists, stabilized by adhesive interactions during neuromuscular formation. In either case, these mutants have revealed an interactive process between the muscle arms and the motor neurons.

Genetic analysis of neurotransmission

A combination of molecular and genetic studies have led to the identification and characterization of molecules that play roles in transmitter release in *C. elegans*. Sydney Brenner originally identified a large number of mutants with uncoordinated locomotion, called *uncs*,[24] many of which are now known to have defects in the functioning of neuromuscular junctions. Many of these mutants with severely disrupted nervous systems are viable and can be propagated and easily studied. The paradoxical non-essential nature of a tissue to which *C. elegans* devotes 30% of its somatic cells is a consequence of the fact that the organism is treated so luxuriously in the laboratory environment. The animals lay in their food — a bacterial lawn — so they do not need to actively search for their next meal. Feeding is mediated by a primarily myogenic program and requires almost no neuronal function,[25] and the animals are self-fertilizing which eliminates a requirement for mating to maintain a strain.

Neurotransmitters

Our understanding of neurotransmission in *C. elegans* has been guided by analysis of mutants with defects in particular neurotransmitter systems. *cha-1* encodes the acetylcholine synthetic enzyme choline acetyltransferase and *unc-17* encodes an acetylcholine vesicular transporter.[26,27] These two mutants are phenotypically

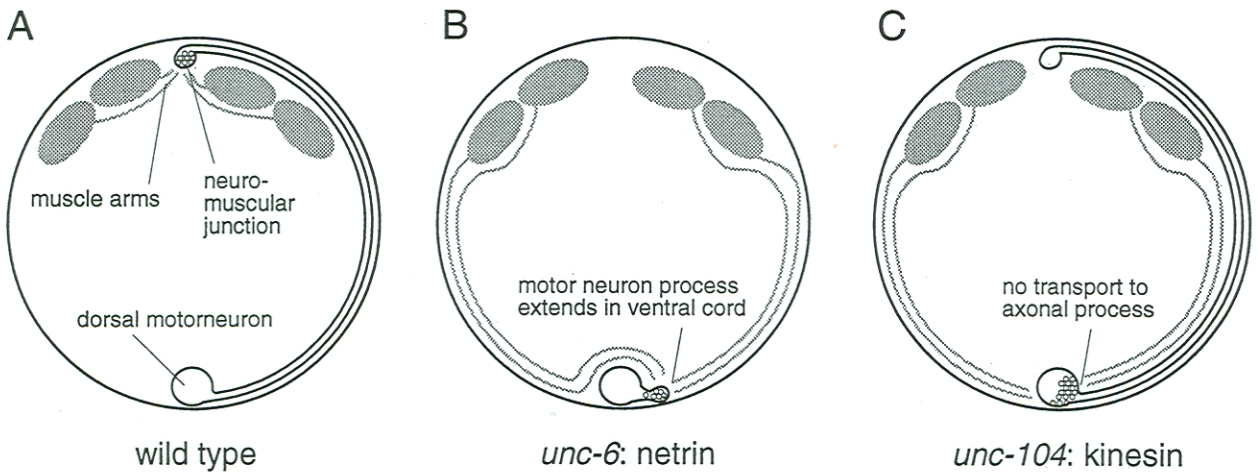

Figure 6. Muscle arm connectivity. (A) In nematodes, muscle cells send processes called arms to the nerve cords where they form neuromuscular junctions with the motor neurons. (B) In *unc-6* mutants the motor neurons do not send processes to the dorsal cord and the dorsal muscles send their arms to the misplaced processes near the ventral cord. Recently, vertebrate homologs of UNC-6, called netrins, have been discovered which are also chemotropic factors for commissural axons.[57] (C) In *unc-104* mutants the dorsal motor neurons send their processes to the dorsal cord but in the absence of kinesin heavy chain do not transport vesicles to the axons. The muscles send arms to the cell bodies in the ventral cord.

very similar and represent the most severe phenotype associated with synaptic defects in *C. elegans*. Both mutants proceed through embryogenesis and hatch. However, the L1 larvae are practically incapable of movement and arrest and die as young larvae. By contrast, mutants lacking GABA neurotransmission survive to adulthood and exhibit distinctive locomotory abnormalities.[21] *unc-25* mutants lack the GABA synthetic enzyme glutamic acid decarboxylase (ref 21; Y. Jin and H.R. Horvitz, personal communication). Abnormal GABA accumulation in the neurons of *unc-47* mutants make this an excellent candidate to encode the GABA vesicular transporter.[21] Finally, mutants with severely depleted levels of biogenic amines have been characterized (ref 28; G. Garriga, personal communication). Phenotypically, these mutants have only subtle behavioral defects such as altered foraging or motivational state (J. Kaplan, B. Sawin, personal communications). These mutants illustrate the spectrum of phenotypic defects that can be associated with defects in neurotransmission and were the basis upon which exploration of genes required for the functioning of all synapses was initiated.

Summary of biochemical analysis of exocytosis

The mechanisms of exocytosis and endocytosis in the presynaptic cell have been the focus of a large number of physiological, morphological and biochemical studies. In summary, a reserve pool of synaptic vesicles are stored near synaptic junctions and can be mobilized to replenish vesicles as they are released. Vesicles that are capable of being released are docked at the active zone, and the influx of calcium is the signal that allows these vesicles to fuse with the plasma membrane. Vesicular components are then recycled by endocytosis to regenerate mature synaptic vesicles. Recently, converging work in several disparate fields has led to a model for the molecular mechanism controlling the release of vesicles. The most intriguing finding of these studies is that secretion at the nerve terminal uses many of the same proteins or related proteins to those found in Golgi transport and in yeast secretion (reviewed in ref 29). Briefly, the model proposes that vesicle docking is regulated by the interaction of synaptic vesicle proteins with plasma membrane proteins (see Figure 7 for details).[6,30] Fusion of the synaptic vesicle to the plasma membrane is thought to be mediated by the displacement of the calcium sensor from the complex by soluble proteins. However, it must be emphasized that this is an ad-hoc model; there are no data to support the precise order of molecular events, and data about the roles of some of these molecules remain contradictory. We eventually need to test the functions of these molecules *in vivo* to characterize their roles in the exocytotic process.

Genetic analysis of transmitter release in C. elegans

Genetic analysis in the nematode is beginning to contribute to our understanding of synaptic function. Mutants with defects in presynaptic function were filtered out of the large collection of uncoordinated mutants using three criteria:[31,32] first, increased levels of acetylcholine in a mutant implied that release of acetylcholine was impaired. Second, resistance to inhibitors of cholinesterase, such as aldicarb or trichlorfon, suggested that these mutants had decreased levels of acetylcholine in the synaptic cleft. By inhibiting the breakdown of acetylcholine these pesticides cause the accumulation of toxic levels of acetylcholine in wild-type animals. Third, sensitivity to cholinergic agonists such as levamisole suggested that these mutants were normal for postsynaptic functions such as the muscle-specific acetylcholine receptors. Molecular characterization showed that these genes encode such synaptic proteins as the vesicular acetylcholine transporter[26] and synaptotagmin[33] and thereby have validated this genetic strategy to the identification of synaptic components. Analysis of these *C. elegans* mutants has provided insight into the in-vivo role of synaptic components identified by biochemical techniques, and has revealed new proteins likely to be involved in synaptic function.

Docking

A small group of *C. elegans* mutants which share a similar lethargic phenotype represent good candidates to encode molecules participating in the docking process. The mammalian homolog of one of these gene products, UNC-18 (also called n-sec1 or Munc-18), has already been implicated biochemically in the regulation of vesicle docking. Specifically, n-sec1/Munc18 binds syntaxin with a 1000-fold greater affinity than does the synaptic vesicle protein VAMP, syntaxin's partner in the biochemically defined docking complex (Figure 7).[34,35] *In vitro*, VAMP is easily competed off of syntaxin by sub-stoichiometric concentrations of n-sec1. These experiments suggest that binding of the UNC-18 to the plasma membrane protein syntaxin prevents docking of the synaptic

vesicle to the active zone. One might expect that in the absence of the UNC-18 protein that vesicle docking will occur more frequently resulting in an increase in neurotransmitter release and a depletion of vesicles at the synapse. However, analysis of *unc-18* mutants has not confirmed these expectations. Transmitter release appears to be vastly reduced in *unc-18* mutants since they exhibit almost no coordinated movement, accumulate acetylcholine, and are resistant to acetylcholinesterase inhibitors.[31,32] This can be observed at an ultrastructural level as well: *unc-18* mutants accumulate synaptic vesicles at terminals (E. Jorgensen, E. Hartwieg, H.R. Horvitz, unpublished results). In contrast to the binding studies, these data suggest that UNC-18 *facilitates* synaptic vesicle docking events. Three other *C. elegans* mutants, *unc-13*, *unc-64* and *ric-4* share many phenotypic properties with *unc-18* mutants in that they are strongly paralysed, accumulate acetylcholine, and are strongly resistant to inhibitors of cholinesterase.[31,32,36] Thus these gene

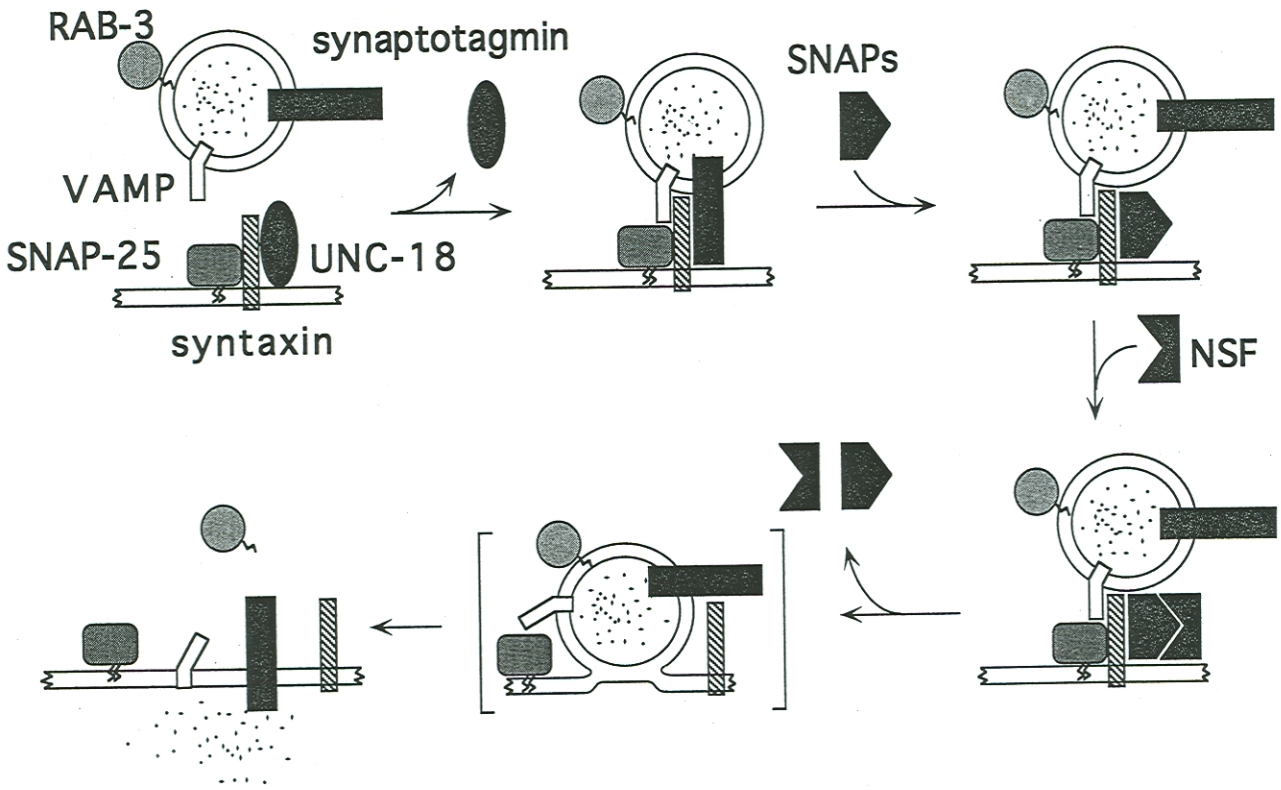

Figure 7. A model describing synaptic vesicle docking and fusion. The hypothesis is based on biochemical experiments describing interactions among proteins in detergent solubilized extracts.[6,34,58] The model proposes that interactions between the synaptic vesicle-associated proteins VAMP and synaptotagmin and plasma membrane proteins syntaxin and SNAP-25 are proposed to form a complex upon vesicle docking. UNC-18, a neuronal isoform of a yeast secretory protein, SEC1, is postulated to dissociate from syntaxin during formation of this complex.[34,35] SNAPs then bind to the complex displacing synaptotagmin in the process. The release of the calcium-binding protein synaptotagmin from this complex has been equated with the calcium-dependent initiation of fusion, although displacement of synaptotagmin by SNAP is not calcium dependent *in vitro*.[6] NSF subsequently binds to SNAP to form a larger complex which probably consists of multiple NSF, SNAP, VAMP, syntaxin and SNAP-25 molecules in unknown stoichiometry. ATP hydrolysis by NSF disrupts the biochemically defined complex and is proposed to mediate the fusion of the plasma and vesicle membranes to release neurotransmitter *in vivo*. The role of RAB-3 in exocytosis is not yet established, but it is known to become dissociated from the synaptic vesicle membrane during the fusion process.[39] Abbreviations: NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; VAMP, vesicle-associated membrane protein (also called synaptobrevin); SNAP-25, synaptosome-associated protein 25 kD. Adapted from Pevsner *et al*, 1994.[34]

products are good candidates to encode molecules which are also involved in regulating docking. While *unc-64* and *ric-4* have not been molecularly characterized, *unc-13* encodes a large molecule with intriguing properties.[37] Recombinant UNC-13 protein fragments bind phorbol esters in the presence of calcium. One possibility is that UNC-13 acts as a calcium-modulated regulator of docking. Phorbol esters have been shown to potentiate release;[38] although it has been assumed that protein kinase C is the phorbol ester target in these experiments, it is possible that UNC-13 is the relevant target in the synapse. The characterization of interacting proteins and the electrophysiological and ultrastructural characterization of null mutants should clarify UNC-13's role in the release process.

Genetic studies of mutants in both the mouse and *C. elegans* suggest that the role of Rab3A in neurotransmitter release may be different than the role of rab proteins in yeast secretion. Small GTP-binding proteins of the rab family have been proposed to be required for secretion based on the essential nature of these proteins in yeast secretion and because a unique member of this family is associated with each discrete step of secretory pathways in eukaryotic cells (reviewed in ref 39). The *C. elegans* protein RAB-3 is most closely related to Rab3A, the rab family member associated with synaptic vesicles. In stark contrast to the absolute requirement for rab proteins in yeast secretion, mouse *rab3A* mutants are phenotypically normal.[40] Similarly, *C. elegans rab-3* mutants display only mild behavioral defects (M. Nonet, M. Kilgard and B. Meyer, unpublished results). However, synaptic terminals of *rab-3* mutants are nearly three-fold depleted of vesicles arguing that RAB-3 does not simply play a stimulatory role in vesicle docking (E. Jorgensen, E. Hartwieg, H.R. Horvitz, unpublished observations). Instead, the ultrastructural data suggests that RAB-3 might also play a role in regulation of the size of the reserve pool of synaptic vesicles, perhaps by regulating formation of vesicles from synaptic endosomes. Electrophysiological defects in *rab-3A* mice are limited to minor depressions in synaptic transmission after repetitive stimulation,[40] and are also consistent with a role in regulating synaptic vesicle reserves. The dispensability of RAB-3 could also be explained by the presence of a protein with redundant functions at the nerve terminal. Only a single *rab-3*-like gene has been identified in *C. elegans*. Therefore, if redundancy accounts for the mild nature of the defect in *rab-3* mutants, the other protein is likely to be distinct from the rab3 family.

Fusion

A variety of biochemical studies indicate that the synaptic vesicle-associated protein synaptotagmin is the calcium sensor that initiates fusion of the vesicle membrane to the plasma membrane. It has been proposed that synaptotagmin inhibits constitutive fusion of the membranes and that the binding of calcium by synaptotagmin removes this blocking activity and thereby promotes release.[41] Removal of an inhibitor of synaptic vesicle fusion should lead to constitutive release of vesicles and an *increase* in transmitter release. However, the defects in *C. elegans* mutants lacking synaptotagmin are inconsistent with the simplest version of this hypothesis. First, synaptotagmin (*snt-1*) mutants accumulate acetylcholine and are resistant to inhibitors of acetylcholinesterase suggesting release is greatly *reduced* in these animals.[33] Second, an ultrastructural characterization of *snt-1* animals revealed that vesicle populations at synaptic terminals are depleted (E. Jorgensen, E. Hartwieg, H.R. Horvitz, unpublished observations). The 'missing' vesicle membrane is probably dispersed in the plasma membrane in the vicinity of the terminals since immunocytochemical examinations of *snt-1* animals detect relatively normal levels of other synaptic vesicle-associated proteins in synaptic regions (M. Nonet, unpublished results). Together these findings suggest that in synaptotagmin mutants synaptic vesicle retrieval is impaired. The recent identification of synaptotagmin as a high affinity receptor for the clathrin AP-2 complex supports this notion.[42] However, since a block in endocytosis would limit availability of vesicles at the terminal, the phenotype of synaptotagmin mutants does not preclude the possibility that synaptotagmin plays both a role in endocytosis and in exocytosis. In fact, genetic and physiological analyses of mouse and Drosophila mutants indicate that synaptotagmin plays a role in exocytosis.[43-46] Genetic lesions separately disrupting these two functions would provide a convincing demonstration of synaptotagmin's role in both processes *in vivo*. In addition, further genetic and molecular analysis of mutants with phenotypes similar to *snt-1* (for example, *unc-11*, *unc-26* and *unc-41*) should help refine our understanding of the role of synaptotagmin at presynaptic terminals (see Figure 8, for summary of *C. elegans* mutants).

Postsynaptic defects

Significant progress has also been made in character-

ization of postsynaptic elements of neuromuscular junctions. The most extensive work has focused on characterization of mutants resistant to the anthelmintic levamisole which acts as a cholinergic agonist.

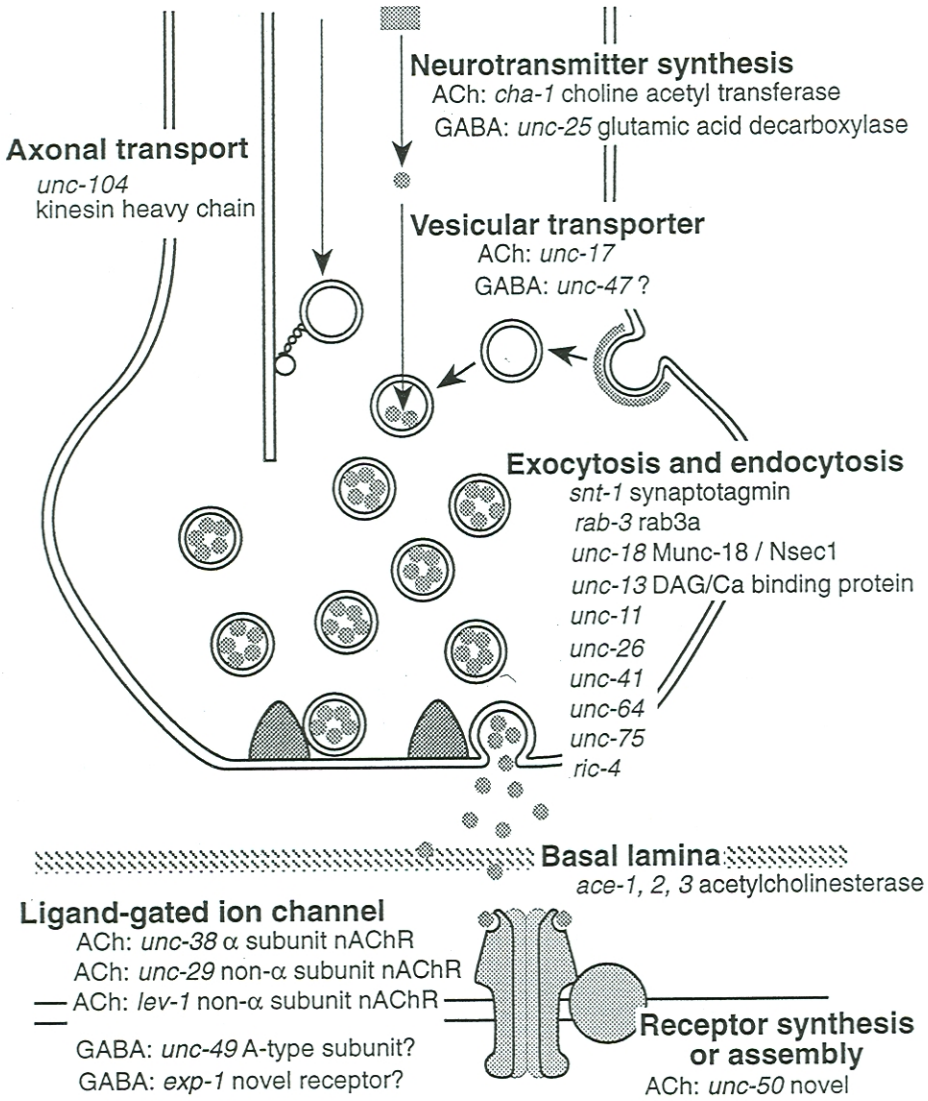

Figure 8. *C. elegans* mutations that disrupt aspects of neuromuscular synaptic transmission. *C. elegans* genes involved in axonal transport, neurotransmitter synthesis, vesicular transport, exocytosis and endocytosis are depicted in a schematic diagram of a presynaptic terminal. Similarly, genes encoding ligand-gated ion channels and a component involved in receptor assembly are depicted at the postsynaptic terminal. Gene products are described in the cases where these have been molecularly characterized. In the cases where genes regulate aspects of a single type of transmitter, the transmitter is noted. Additional genes are thought to be involved in various steps in neurotransmission, but these have not been characterized in enough detail to position them as acting at a specific step in neurotransmission. These include multiple *eat* mutants, *lev-8*, *lev-9*, *lev-10*, *ric-1*, *ric-3*, *ric-6*, *ric-7*, *unc-2*, *unc-31*, *unc-32*, *unc-36*, *unc-46*, *unc-63*, *unc-65* and *unc-75* (refs 31, 32, 59), E. Jorgensen and H.R. Horvitz, unpublished results). Three letter abbreviations for *C. elegans* genes: *ace*, acetylcholinesterase abnormal; *eat*, eating abnormal; *cha*, choline acetyltransferase abnormal; *exp*, explusion defective; *lev*, abnormal levamisole sensitivity; *ric*, resistance to inhibitors of cholinesterase;*rab*, rab GTP-binding protein related; *snt*, synaptotagmin related; *unc*, uncoordinated. Basal lamina is indicated as hatched region.

Mutants resistant to levamisole define genes encoding at least three acetylcholine receptor subunits that are probably expressed in body wall muscle. *unc-38* encodes an α-like acetylcholine receptor subunit, and *unc-29* and *lev-1* encode non-α subunits.[47] These three genes represent only a fraction of the acetylcholine receptor subunits in *C. elegans* as other, possibly neuronal, acetylcholine receptors have been identified by molecular techniques (ref 47; A. Coulson, personal communication). Additionally, other levamisole-resistant mutants probably define non-receptor molecules involved in receptor transcription or assembly. Most notably, *unc-50* mutants lack levamisole binding in in-vitro assays[48] and encodes a novel non-receptor protein (M. Hengartner, N. Tsung, H.R. Horvitz, personal communication). Additional levamisole-resistant mutants (*lev-8, lev-9, lev-10, unc-63* and *unc-74*) remain to be characterized at a molecular level.[49]

Two genes probably encode distinct classes of postsynaptic GABA receptors. First, *unc-49* likely encodes a $GABA_A$ receptor subunit expressed in muscle since *unc-49* mutants are resistant to the GABA agonist muscimol, and the *C. elegans* genome project has identified a $GABA_A$ receptor gene near the map location of *unc-49*.[21] Pharmacological evidence also indicates that the *exp-1* gene[50] may encode a postsynaptic component required for GABA-mediated stimulation of enteric muscle contractions (E. Jorgensen, H.R. Horvitz, unpublished data).

Future perspectives

While genetic studies have dominated the analysis of neuromuscular development and synaptic transmission in *C. elegans* in past years, future analysis will involve a larger repertoire of tools. A combination of molecular approaches are presently being applied to analyse the worm genome. A large number of genes have been identified by a combination of genome and random cDNA sequencing. 2712 unique cDNAs and over 7% of the *C. elegans* genome have been sequenced to date.[51,52] The complete sequence of the genome is anticipated within five years. Analysis of this sequence has identified several players in neuronal development and function including neurotransmitter receptors, and relatively divergent forms of the synaptic vesicle proteins VAMP/synaptobrevin and synaptotagmin (the *C. elegans* mapping and sequencing consortium, personal communication). Directed knockout procedures have been developed for *C. elegans* so that the function of these proteins can be probed *in vivo*.[53] This is particularly important in the analysis of synaptic function for which biochemical studies have advanced far ahead of genetic studies.

Although the small size of *C. elegans* has made the organism particularly advantageous for the geneticist, its diminutive size has posed a serious problem for the study of the neuromuscular junction. Because the neurons are only three micrometres in diameter, it has not yet been possible to conduct electrophysiological studies. Recently, Raizen and Avery have managed to develop extracellular recording techniques that can analyse the physiology of the pharynx.[54] This preparation is sensitive enough to detect not only the electrical activity of the pharyngeal muscle but of the pharyngeal nervous system as well. While this work has been able to dissect some of the inhibitory and excitatory inputs that modulate the intrinsic muscle activity that drive contractions of the pharynx, the method is limited to examination of pharyngeal muscle. Additionally, recordings measuring absolute voltage changes are not possible in this preparation.

Detailed electrophysiological studies will require the development of intracellular methods. One tool that may be very useful in this regard is the expression of the Green Fluorescent Protein (GFP) in identified cells so that recordings can be made from these marked living cells.[55] Initial forays recording from such cells in dissected preparations have been successful (V. Maricq and S. Lockery, personal communication). Eventually, however, the application of voltage-sensitive dyes may be the most successful method in measuring electrical activity from intact worms.

The molecular dissection of the process of neuromuscular development and neurotransmission in a simple invertebrate may at first seem superfluous. However, the nematode remains the only organism in which a complete molecular, cellular and functional description of a nervous system can be reasonably accomplished. Analysis of *C. elegans* mutants provides a practical method of determining the in-vivo role of the molecules that are expressed in the nervous system and should nicely complement the biochemical studies in vertebrates. The molecules required for nervous system development and function are strikingly conserved between invertebrates and vertebrates, from molecules controlling chemotropic guidance of axons to those regulating synaptic vesicle release. Such conservation suggests that analysis of *C. elegans* neuronal mutants will have practical implications for studies of the vertebrate nervous system.

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