variability demonstrated here should also provide a molecular tool for a variety of ecological and ethological studies of the cichlid fishes. □

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Genes required for GABA function in *Caenorhabditis elegans*

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γ-AMINOBUTYRIC acid (GABA) neurotransmission is widespread in vertebrate and invertebrate nervous systems[1]. Here we use a genetic approach to identify molecules specific to GABA function. On the basis of the known *in vivo* roles of GABAergic neurons in controlling behaviour of the nematode *Caenorhabditis elegans*[2], we identified mutants defective in GABA-mediated behaviours. Five genes are necessary either for GABAergic neuronal differentiation or for pre- or postsynaptic GABAergic function. The gene *unc-30* is required for the differentiation of a specific type of GABAergic neuron, the type-D inhibitory motor neuron. The gene *unc-25* is necessary for GABA expression and probably encodes the GABA biosynthetic enzyme glutamic acid decarboxylase. The genes *unc-46* and *unc-47* seem to be required for normal GABA release. Finally, the gene *unc-49* is apparently necessary postsynaptically for the inhibitory effect of GABA on the body muscles and might encode a protein needed for the function of a $GABA_A$-like receptor. Some of these genes are likely to encode previously unidentified proteins required for GABA function.

Different classes of GABAergic neurons of the nematode *Caenorhabditis elegans* are required for different behaviours[2]. The single AVL and DVB neurons stimulate the enteric muscles during the defaecation cycle, the four RME motor neurons modulate the head movements necessary for foraging behaviour, and the six DD and 13 VD cells are inhibitory motor neurons required for locomotion. Animals in which the DD and VD neurons have been killed with a laser microbeam simultaneously contract antagonistic dorsal and ventral body muscles[2], causing these animals to shrink. This understanding of the *in vivo* functions of the *C. elegans* GABAergic neurons allowed us to identify and analyse mutant strains with abnormal GABA function.

Mutations in five genes, *unc-25, unc-30, unc-46, unc-47* and *unc-49* (ref. 3), cause animals to shrink[4]. Because the animals shrink, they behave as if they lack DD and VD function. By examining other behaviours of these mutants, we determined whether the GABA functions of AVL, DVB and the RMEs are also defective. We also examined GABA expression and GABAergic nerve process morphology by staining worms with an anti-GABA antibody (Fig. 1). Finally, we assayed GABA receptor function by measuring the sensitivities of the mutants to the GABA agonist muscimol: when wild-type worms are treated with muscimol, muscle contractions cease in all muscles and the worms develop a flaccid paralysis (Fig. 2*a*; and M. Chalfie, personal communication). Muscimol sensitivity seems to be mediated at the muscle membrane[5], so that resistance to muscimol indicates a defect in the postsynaptic response to GABA. These five genes can be divided into three classes based on the aspect of GABA neurotransmission for which they are required: neuronal differentiation, presynaptic function, and postsynaptic function.

Differentiation. The gene *unc-30* is required for the differentiation of the two anatomically related[6] classes of type-D GABAergic neurons, the DD (dorsal D) and VD (ventral D) ventral cord motor neurons. *unc-30* mutants display the shrinking locomotory defect of animals in which the DD and VD neurons have been killed by a laser microbeam[2], but not the foraging defect of animals that lack the RME neurons or the defaecation cycle defects of animals that lack the AVL and DVB neurons (Table 1). Antibodies against GABA failed to stain the DD or VD motor neurons in *unc-30* mutants but did stain all other GABAergic neurons normally (Fig. 1*b*), again indicating that of the 26 GABAergic neurons, only the VD and DD neurons are abnormal. Reconstructions of the VD and DD neurons of *unc-30* animals from electron micrographs of serial sections revealed that the VD and DD neurons form synapses with incorrect targets, despite being grossly normal in pathfinding (J. G. White, E. Southgate, J. N. Thomson and S. Brenner, manuscript in preparation). *unc-30* animals appeared to be normal in postsynaptic GABA function: *unc-30* animals responded to muscimol like wild-type animals (Fig. 2*a*). These observations indicate that *unc-30* is required for the differentiation of the DD and VD classes of GABAergic neurons.

Presynaptic function. The gene *unc-25* is necessary for the expression of GABA in all GABAergic neurons. Our behavioural studies indicated that *unc-25* animals have defects in all of the functions mediated by GABAergic neurons (Table 1). No additional behavioural defects were observed in these mutants, indicating that there are no gross abnormalities in the non-GABAergic nervous system. GABA expression is eliminated in *unc-25* mutants as assayed by immunocytochemistry (Fig. 1*c*). Postsynaptic GABA function apparently remains intact in *unc-25* mutants, since they were sensitive to the GABA agonist muscimol (Fig. 2*a*). These results suggest that it is the reduced level of GABA in *unc-25* animals that causes the behavioural defects observed, but these results do not exclude the possibility that *unc-25* mutations cause defects in other aspects of GABAergic function in addition to GABA expression.

To demonstrate that other presynaptic GABAergic functions are intact in *unc-25* mutants, we showed that restoring GABA levels in the AVL and DVB neurons was sufficient to restore function to these neurons. When *unc-25* animals were bathed in GABA, the AVL and DVB neurons accumulated GABA as detected by immunocytochemistry (Fig. 1*d*), and the enteric muscle contractions mediated by AVL and DVB were restored

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TABLE 1 Mutant phenotypes

Genotype	Behaviour: Locomotion (DDs and VDs)	Behaviour: Defecation (AVL and DVB)	Behaviour: Foraging (RMEs)	GABA expression: DDs and VDs	GABA expression: AVL and DVB	GABA expression: RMEs	GABA expression: RIS	Muscimol sensitivity	Proposed site	Proposed defect
Wild type						+	+	+		
unc-30						+	+	+	Presynaptic	DD, VD differentiation
unc-25	Shrinking	Expulsion defective			None	None	None	+	Presynaptic	GABA synthesis
unc-47	Shrinking	Expulsion defective				+ +	+ +	+	Presynaptic	GABA release
unc-46	Shrinking	Weak expulsion defective	Weak loopy			+	+	+	Presynaptic	GABA release
unc-49	Shrinking	+	+			+	+	Resistant	Postsynaptic	$GABA_A$ receptor

One plus sign means, like wild type; for GABA expression, two plus signs indicate abnormally high intensity of staining. Locomotion was assayed by touching worms on the nose with a platinum wire while observing them with a dissecting microscope. An animal was scored as a shrinker if it failed to move backwards and it decreased in body length. Defaecation was assayed by observing with a dissecting microscope whether an enteric muscle contraction occurred following a posterior body contraction (PBOC) during the defaecation cycle[13]. Animals scored as defective displayed the expulsion-defective phenotype[13]. Foraging was assayed in a double-blind test. About ten animals of each genotype were placed on agar plates with no bacteria, plates were coded and mixed and foraging was assayed. Foraging was scored as loopy if animals displayed exaggerated bends of the head while moving forward[2]. Data for enteric muscle contractions per posterior body contraction and for foraging behaviour were as follows: wild type: 101/110 = 0.92, non-loopy; *unc-30(e191)* 85/88 = 0.97, non-loopy; *unc-30(e165)* 54/55 = 0.98, non-loopy; *unc-25(e156)* 9/76 = 0.12, loopy; *unc-25(e891)* 11/55 = 0.20, loopy; *unc-25(e265)* 8/55 = 0.15, loopy; *unc-47(e307)* 17/110 = 0.15, loopy; *unc-47(e542)* 16/88 = 0.18, loopy; *unc-47(n2252)* 21/165 = 0.13, loopy: *unc-46(e177)* 33/88 = 0.38, weak loopy; *unc-46(e300)* 32/110 = 0.29, weak loopy; *unc-46(e642)* 62/110 = 0.56, weak loopy; *unc-49(e382)* 54/55 = 0.98, non-loopy; *unc-49(e407)* 87/88 = 0.99, non-loopy; *unc-49(e641)* 85/88 = 0.97, non-loopy. GABA expression was assayed by anti-GABA staining. Muscimol sensitivity was assayed quantitatively based upon its effects on pharyngeal pumping (Fig. 2) or qualitatively based upon body muscle paralysis in the same experiments. All *unc-49* alleles tested conferred resistance to muscimol: *unc-49(e382), unc-49(e407), unc-49(e468), unc-49(e641),* and *unc-49(e929)*. Additional screens for shrinker mutants have identified only mutations in the existing shrinker genes (Y. Jin, J. Kaplan, E.J., S.M. and H.R.H., unpublished observations). Of the six genes previously reported to be able to mutate to produce a shrinker phenotype[4], one *(unc-43)* appears to be different: *unc-43* mutants have a distinctive locomotory defect and do not have obvious abnormalities in foraging or defaecation (data not shown). For this reason, we have not included *unc-43* among the genes described here.

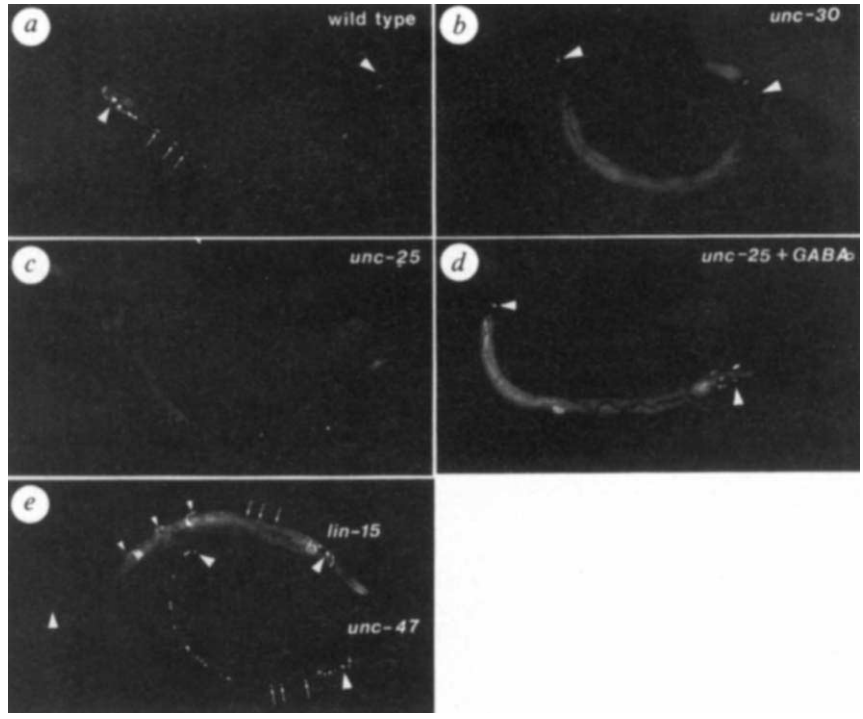

FIG. 1 GABA immunoreactivity in wild-type and mutant *C. elegans*. In all photographs, the ventral cord neurons DD2, VD3 and VD4 (arrows) and the head neuron AVL and the tail neuron DVB (large arrowheads) are indicated when they stained. *a*, Wild type. Twenty-six cells stain: 6 DDs, 13 VDs, 4 RMEs, AVL, DVB and RIS[2]. *b, unc-30(e191).* The DD and VD ventral cord motor neurons did not stain. These cells were still present, as they could be visualized with the nuclear stain 4-6-diamidino-2-phenylindole dihydrochloride (DAPI)[10] (data not shown). The defect in GABA expression in *unc-30* animals is not a consequence of their minor abnormalities in pathfinding, because studies of other mutants have shown that abnormal pathfinding itself does not affect GABA expression in the DD and VD neurons[17]. *c, unc-25(e156).* No cells stained, although they are generated and occupy their normal positions, as assessed by staining cell nuclei with DAPI (data not shown). *d, unc-25(e156)* + GABA. *unc-25(e156)* animals grown overnight in Petri dishes on agar seeded with *Escherichia coli*[3] and containing 100 mM GABA were stained for GABA. In addition to the neurons AVL, DVB and RIS, several normally non-GABA-staining neurons in ganglia of the head and tail accumulated GABA after this treatment. The identities of these cells have not yet been determined. These extra cells also accumulated GABA when wild-type animals were treated with GABA (data not shown). The RMEs stained variably. *e, unc-47(e307)* and *lin-15(n765).* To provide an internal control, *lin-15* animals were fixed and stained in the same test tube as the *unc-47* animals. *lin-15* animals can be distinguished by their multiple pseudovulval protrusions[18] (small arrowheads). GABAergic neurons in *unc-47* animals reproducibly stained more intensely than neurons in wild-type (data not shown) or *lin-15* animals. We stained mutants carrying other alleles of these genes and observed similar staining patterns: *unc-30 (e165, e191, e318, e595, e646, e727, e926,* and *e1169); unc-25 (e156, e265, e591, e891* and *sa4);* and *unc-47 (e307, e542,* and *e707). unc-46(e177, e300* and *e642)* and *unc-49(e382, e407, e468, e641* and *e929)* animals stained the same as wild-type animals (data not shown).

METHODS. Animals were fixed, permeabilized and stained with anti-GABA antisera as previously described[17]. *unc-47* animals consistently stained more intensely than other animals of various genotypes in blind assays of preparations of single strains or mixtures of test animals and morphologically distinguishable control animals stained in the same test tube.

TABLE 2 Effect of exogenous GABA and nipecotic acid on *unc-25* mutants

Genotype and cells killed	None			30 mM GABA			30 mM GABA 10 mM nipecotic acid			10 mM nipecotic acid		
	EMC/def. cycle	No. def. cycles	No. animals	EMC/def. cycle	No. def. cycles	No. animals	EMC/def. cycle	No. def. cycles	No. animals	EMC/def. cycle	No. def. cycles	No. animals
Wild type	**0.97**	33	3	**0.84**	33	3	**1.00**	66	6	**0.97**	33	3
unc-25	**0.12**	77	7	**0.87**	175	14	**0.13**	143	13	**0.17**	99	9
unc-25 AVL⁻DVB	**0.01 + 0.02***	118	7	**0.00**	96	6	—	—	—	—	—	—

Enteric muscle contractions (EMC) were scored for each defaecation cycle (def. cycle). Note that nipecotic acid blocked the restoration of enteric muscle contractions to *unc-25* mutants by GABA but did not act either as an antagonist for GABA (nipecotic acid did not block enteric muscle contraction in the wild type) or as an agonist (nipecotic acid did not restore enteric muscle contraction to *unc-25* mutants on its own). AVL⁻DVB⁻, the neurons AVL and DVB were killed with a laser. EMC/def. cycle, ratio between the number of enteric muscle contractions and the number of defaecation cycles observed; no def. cycles, number of defaecation cycles observed; no animals, number of animals observed. Asterisk, the value following the plus sign represents enteric muscle contractions that occurred between defaecation cycles, that is, more than 10 s following one posterior body contraction and preceding the next. Young adults (about 20 h after the L4 moult) were placed on agar seeded with bacteria, and the defaecation cycle[13] was observed using a dissecting microscope. Animals were placed on agar containing GABA and nipecotic acid for 3 h before observation to allow penetration of the drug. Treated animals were stained after the assay: rescued animals stained and nipecotic acid-blocked animals failed to stain with the anti-GABA antisera. Laser-operated animals were anaesthetized on 5% agarose pads containing 10 mM sodium azide[14] and mounted under a coverslip. Cells were killed using a laser focused through a microscope[7,14]. Wild-type control animals were anaesthetized in parallel with the experimental animals but had no cells killed. The RMEs took up GABA variably, and normal foraging could be restored in *unc-25* animals by exogenous GABA (data not shown). Interestingly, the DD and VD neurons did not take up GABA in *unc-25* animals (Fig. 1*d*), and locomotion was not restored. This observation suggests that these neurons do not express the GABA membrane transporter. Supporting this hypothesis, the equivalent GABAergic neurons of the larger nematode *Ascaris suum* fail to take up radiolabelled GABA in cut animals[15].

(Table 2, row 2). Did GABA rescue AVL and DVB function by a presynaptic or a postsynaptic mechanism? To answer this question, we determined whether rescue required the presence of AVL and DVB and of a functional system of GABA reuptake. Using a laser microbeam[7], we killed AVL and DVB in *unc-25* animals and observed that exogenous GABA no longer restored enteric muscle contractions (Table 2, row 3). We also found that nipecotic acid, which specifically blocks the plasma membrane GABA transporter in invertebrates as well as vertebrates[1], prevented the accumulation of GABA in AVL and DVB (data not shown) as well as the restoration of AVL and DVB function (Table 2). (Nipecotic acid had no effect on the behaviour of wild-type animals.) These observations indicate that if *unc-25* animals are provided with exogenous GABA, AVL and DVB are capable of normal GABA uptake, release and stimulation of the enteric muscles.

Thus, the principal defect in *unc-25* mutants appears to be a simple lack of GABA. GABA is synthesized from glutamate in a single step by the enzyme glutamic acid decarboxylase (GAD) in vertebrates[1] and in *C. elegans*[8]. It seems likely that the *unc-25* gene encodes GAD. Consistent with this hypothesis, GAD activity is greatly reduced in *unc-25* mutant extracts (C. Johnson, personal communication), and a *C. elegans* GAD gene identified on the basis of its sequence similarity to a mammalian GAD gene[9] maps near the *unc-25* gene (A. Coulson, personal communication).

The genes *unc*-46 and *unc-47* seem to be necessary for the normal release of GABA into the synaptic cleft. Like *unc-25* mutants, *unc-46 and unc-47* mutants displayed specific defects in all behaviours mediated by GABAergic neurons (Table 1), although these defects were weaker in *unc-46* animals. *unc-47* animals have an unambiguous presynaptic defect: immunocytochemical studies revealed that all GABAergic neurons in *unc-47* animals contained abnormally high levels of GABA (Fig. 1*e*). GABA expression appeared roughly normal in *unc-46* animals (data not shown), although only severe changes in GABA expression would be detectable by immunocytochemistry. Because both *unc-47* and *unc-46* mutants displayed normal sensitivity to muscimol (Fig. 2*b*), it seems likely that postsynaptic GABA functions are intact in *unc-46* and *unc-47* mutants and that the defects in these mutants are presynaptic. We postulate that mutations in the *unc-47* and *unc-46* genes cause a defect within the GABAergic neurons themselves, such as in the vesicular transporter necessary for uptake of GABA into synaptic vesicles[10] or in a biochemical function required for normal GABA release.

Postsynaptic function. The gene *unc-49* appears to be required for the inhibitory effect of GABA on the body muscles but not for the excitatory effect of GABA on the enteric muscles. First, *unc-49* mutants displayed the locomotory (shrinker) defect seen in animals with the DD and VD motor neurons killed but had normal contractions of the enteric muscles (Table 1). *unc-49* mutants were also normal in foraging. Second, GABA expression was normal in *unc-49* animals (data not shown). Interestingly, *unc-49* animals were resistant to the paralysing effects of muscimol (Fig. 2*b*). This observation indicates that the inhibitory GABA receptor responsible for mediating the effects of muscimol is disrupted. The *unc-49* gene might encode a subunit of an inhibitory receptor such as a $GABA_A$-like receptor[1] or another protein necessary for receptor function.

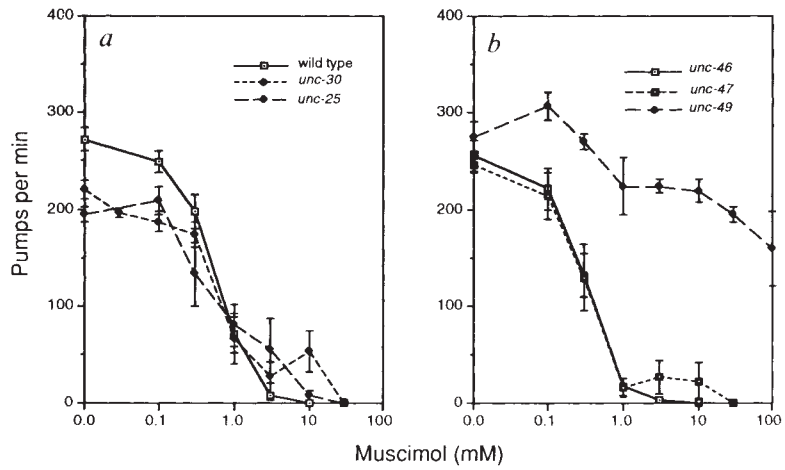

FIG. 2 Effect of muscimol on pharyngeal pumping. *a*, Pharyngeal pumping in wild-type, *unc-30(e191)* and *unc-25(e156)* animals. *b*, Pharyngeal pumping in *unc-46(e177), unc-47(e307),* and *unc-49(e382)* animals. Strains are separated into two graphs for clarity. Error bars indicate the standard error.
METHODS. Muscimol paralysed all muscles in *C. elegans* including the body, enteric, egg-laying, and pharyngeal muscles (data not shown). There are no known GABAergic inputs to the pharyngeal muscles, and we are unsure whether the response of the pharynx to GABA is physiologically significant. We quantified sensitivity to muscimol by counting the pumping rate of the pharynx in increasing concentrations of muscimol. Between 4 and 12 worms of each genotype were placed on NG agar[16] containing muscimol at the indicated concentration and seeded with bacteria. Pumping rates were scored after 30 min by directly counting pumps using a dissecting microscope.

Previous genetic analyses of neurotransmitter function have been based on screens for mutants resistant to specific neurotransmitter agonists or antagonists[11]. By contrast, we identified mutants that phenocopy laser-operated animals lacking particular GABAergic neurons. In principle our approach could identify all genes that are not redundant in function and that are required specifically for GABA function in *C. elegans*. So far we have defined five such genes.

Some of the genes we have identified seem likely to encode familiar molecules. For example, *unc-25* probably encodes the GABA biosynthetic enzyme GAD, and *unc-49* might encode a $GABA_A$ receptor subunit. Although these proteins are biochemically well characterized, the *in vivo* effects of mutations that affect such proteins have not previously been examined. For example, it has been proposed[12] that GABA has a neurotrophic role and is necessary for differentiation in early development. The rescue of AVL and DVB function with exogenous GABA in *unc-25* mutants indicates that GABA may not be necessary for the development of these neurons.

In addition, we have defined several other genes that seem likely to encode proteins that act in GABA presynaptic functions. The gene *unc-30* appears to be necessary for a number of aspects of D-type neuron synaptic development, including neurotransmitter expression and the specification of synaptic partners. The genes *unc-47* and *unc-46* are necessary presynaptically at all GABAergic synapses. These two genes appear to affect GABA release and might define components necessary for GABA vesicular loading, transport or fusion with the synaptic membrane or differentiation of the neuromuscular junction. A molecular characterization of these genes might well define novel proteins involved in GABAergic neurotransmission. □

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The GABAergic nervous system of *Caenorhabditis elegans*

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γ-AMINOBUTYRIC acid (GABA) is the most abundant inhibitory neurotransmitter in vertebrates and invertebrates[1]. GABA receptors are the target of anxiolytic, antiepileptic and antispasmodic drugs[2], as well as of commonly used insecticides[3]. How does a specific neurotransmitter such as GABA control animal behaviour? To answer this question, we identified all neurons that react with antisera raised against the neurotransmitter GABA in the nervous system of the nematode *Caenorhabditis elegans*. We determined the *in vivo* functions of 25 of the 26 GABAergic neurons by killing these cells with a laser microbeam in living animals and by characterizing a mutant defective in GABA expression. On the basis of the ultrastructurally defined connectivity of the *C. elegans* nervous system, we deduced how these GABAergic neurons act to control the body and enteric muscles necessary for different behaviours. Our findings provide evidence that GABA functions as an excitatory as well as an inhibitory neurotransmitter.

Of the 302 neurons in an adult *C. elegans* hermaphrodite[4], 26 are stained by antibodies raised against the neurotransmitter GABA (Fig. 1). These 26 GABAergic cells comprise the six DD, 13 VD, four RME, one AVL, one DVB and one RIS neurons. As described later, we use laser microsurgery to identify roles in behaviour for all of the GABAergic neurons except RIS. We did not observe any behavioural changes in animals in which RIS had been killed.

The DD and VD neurons are motor neurons that innervate the body muscles required for locomotion[4] (Fig. 2*a*). *C. elegans* locomotion involves the propagation of a sinusoidal body wave from one end of the animal to the other. For example, if an animal is touched on the head it backs by producing a body wave of alternating contractions and relaxations of the opposing ventral and dorsal body muscles (Fig. 2*b*). By contrast, if an animal in which the DD and VD neurons have been killed by laser microsurgery is touched on the head, the animal simultaneously contracts its ventral and dorsal body muscles, resulting in a shrinkage in body length rather than in backward movement (Table 1*a*; Fig. 2*c*). Thus, the DD and VD neurons coordinate the wave of body muscle contractions involved in locomotion by preventing the simultaneous contraction of opposing muscles.

Is the role of the GABAergic DD and VD neurons in locomotion mediated by GABA? Mutations in the gene *unc-25* eliminate GABA expression in all 26 GABAergic neurons of *C. elegans*, probably by eliminating the GABA biosynthetic enzyme glutamic acid decarboxylase (ref. 5; and C. Johnson, personal communication). When touched on the head, *unc-25* animals shrink just like laser-operated animals that lack the DD and VD function (Fig. 2*c*; Table 1*a*), indicating that GABA is required for DD and VD function.

Can the known synaptic connectivity of the DD and VD neurons account for their function in locomotion? Based on the connectivities of these neurons, White *et al.*[4] suggested that the DDs and VDs act as contralateral inhibitory neurons[4]. Electrophysiological studies by Stretton and co-workers[6,9] of the larger parasitic nematode *Ascaris lumbricoides* have provided functional evidence for such a role for the *Ascaris* GABAergic motor neurons that correspond to the *C. elegans* VD neurons. Our studies of the behaviours of operated and mutant animals provide functional evidence for such a role for the *C. elegans* DD and VD neurons and strongly support the model that GABA is essential for contralateral inhibition. Specifically, it seems that the DD and VD motor neurons provide positive feedback to

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