The Caenorhabditis elegans Gene unc-25 Encodes Glutamic Acid Decarboxylase and Is Required for Synaptic Transmission But Not Synaptic Development

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The neurotransmitter GABA has been proposed to play a role during nervous system development. We show that the Caenorhabditis elegans gene unc-25 encodes glutamic acid decarboxylase (GAD), the GABA biosynthetic enzyme. unc-25 is expressed specifically in GABAergic neurons. Null mutations in unc-25 eliminate the UNC-25 protein or alter amino acids conserved in all known GADs, result in a complete lack of GABA, and cause defects in all GABA-mediated behaviors. In unc-25 mutants the GABAergic neurons have normal axonal trajectories and synaptic connectivity, and the size and shape of synaptic vesicles are normal. The number of synaptic vesicles at GABAergic neuromuscular junctions is slightly increased. Cholinergic ventral nerve cord neurons, which innervate the same muscles as GABAergic ventral cord neurons, have normal morphology, connectivity, and synaptic vesicles. We conclude that GAD activity and GABA are not necessary for the development or maintenance of neuromuscular junctions in C. elegans.

Key words: GABA; γ-amino butyric acid; GAD; glutamate decarboxylase; neuromuscular junctions; C. elegans


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cle contraction depends on the balance of antagonistic inputs from cholinergic and GABAergic synapses.

Killing the DD and VD GABAergic motor neurons causes a locomotory behavior known as “shrink,” in which the animal simultaneously hypercontracts both ventral and dorsal body muscles (Hodgkin, 1983; McIntire et al., 1993b). Shrinker mutants define several genes required for the development and function of these neurons (McIntire et al., 1993a). It was proposed that one of these genes, unc-25, encodes the biosynthetic enzyme for GABA for three reasons. First, unc-25 mutations abolish all GABA functions as defined by laser killing of GABA-expressing neurons (McIntire et al., 1993a). Second, the 26 GABAergic neurons lack GABA immunoreactivity in unc-25 mutant animals (McIntire et al., 1993a). Third, addition of exogenous GABA restores GABA immunoreactivity to AVL and DVB and rescues the defecation defect, suggesting that a lack of GABA is the only defect in these neurons in unc-25 mutant animals (McIntire et al., 1993a).

We show in this paper that unc-25 encodes GAD and is likely to be the only GAD gene in C. elegans and that the lack of GABA in unc-25 mutant animals does not affect axonal morphology or the ratio of excitatory to inhibitory neuromuscular junctions.

MATERIALS AND METHODS

Genetic methods. Worms were maintained at 20°C as described by Brenner (1974), unless noted otherwise. All unc-25 mutations reported here were induced by ethyl methanesulfonate: e156, e265, and e591 were isolated by Brenner (1974) in screens for locomotory-deficient mutants; n2323, n2324, n2328, n2569, and n2638 were isolated by J. Kaplan and E. Jorgensen; sa94 was isolated by J. Thomas in screens for defection-defective mutants; and n2379, n2380, n2381, n2384, n2383, and n2385 were isolated by Y. Jin in screens for shrinker mutants.

Isolation and subcloning of genomic DNAs. cm9e10 was generated by C. Martin and M. Chalfie and was obtained from the C. elegans Genome Sequencing Center at the Medical Research Council, Cambridge, UK. We first examined DNAs from cosmids covered by Y37D8 for hybridization with the cm9e10 insert and failed to identify any positive clones. We then hybridized DNAs from cosmids covered by Y37D8 for hybridization with the cm9e10 insert and failed to identify any positive clones. We then used the cDNA insert in cm9e10 to probe a C. elegans genomic λ phage library, kindly provided by Browning and Strome (1996). Two positive clones, YJD2 and YJC6, were isolated from 75,000 phage plaques (20 × genomic equivalents). Subsequent purifications of phage DNAs and subcloning into plasmids were performed following standard procedures (Sambrook et al., 1989).

Characterizations of GAD cDNAs. We determined the complete sequence of both strands of the cDNA insert in cm9e10 by generating nested ExoIII deletion DNA fragments and using the ABI PRISM cycle sequencing system. The sequence was analyzed on a model 373A sequencer at the National Institutes of Health (NIH) sequencing facility in Washington, DC, following the manufacturer’s instructions. This cDNA clone contains an insert of 1400 bp with a poly(A) tail at the 3’ end. On a Northern blot, this cDNA detected a 1.8 kb mRNA transcript (data not shown). To isolate full-length cDNAs for GAD, we used the cm9e10 DNA insert to probe two C. elegans cDNA libraries made from mixed-stage poly(A)+ RNA: a λ ZAP II cDNA library constructed by Barstead and Waterston (1989) and a λgt11 cDNA library constructed by Okkema and Fire (1994). The longest GAD cDNA we isolated, pSC180, contained a 1.8 kb insert in which an in-frame ATG is present seven nucleotides from the beginning of the cDNA.

Germline transformation. Germline transformation was performed using standard procedures (Mello et al., 1991). PR4F, which contains the dominant mutation rol-6(su1006) (Kramer et al., 1990), was used as a coinjection marker when either N2 or unc-25 animals were used as the host for transformation. pln-15EK, which contains the entire gene for lin-15 (Clark et al., 1994), was used as a coinjection marker when lin-15(n765) was used as the host.

Sequence analysis of unc-25 alleles. The genomic structure of the wild-type unc-25 gene was determined by using primers corresponding to exonic sequences to amplify genomic DNA and cDNA by PCR. The sequences of the PCR products were then determined and compared, revealing that unc-25 is composed of eight exons. We then amplified genomic DNAs including all exonic and exon/intron boundaries sequences from unc-25 mutant animals and determined their sequences using the fmol DNA cycle sequencing system (Promega, Madison, WI). Specific primer sequences are available on request.

Reporter gene constructs. In general, all reporter constructs were prepared by simple ligation between desired unc-25 DNA fragments and lacZ or green fluorescent protein (GFP) reporter vectors (Fire et al., 1998; Chalfie et al., 1994). To tag GAD with GFP at the amino terminus, we amplified the GFP using a primer that changes the stop codon of GFP to Xhol site allowing the insert to be cloned into the XhoI site in the first exon of unc-25 and generating the plasmid pSC317. GFP was thereby inserted in-frame at the amino terminus of GAD after residue 12.

Electron microscopy. Adult worm micrographs were cut with a scalpel in 8% glutaraldehyde and 0.7% osmium tetroxide in 0.1 M cacodylate, pH 7.4, on ice. After 2 hr worms were moved to 2% osmium tetroxide in 0.1 M cacodylate and left at 4° overnight. Processing and sectioning were performed as described by McIntire et al. (1992). Worms were sectioned until the region between the pharynx and the reflex of the gonad had been reached. Thereafter, roughly 1000 serial sections of 60 nm thickness were cut, mounted on slot grids, and photographed. The connectivity of the C. elegans nervous system is largely invariant, and cells can be identified by comparing reconstructions to the published wild-type reconstructions. Moreover, synapses are en passant; thus, complex dendritic arbors and axonal termini are absent. In this study, motor neurons were first identified by the order of the cell bodies along the ventral cord, the orientation of axons in regard to the cell body, the positions of their axons in the ventral nerve cord, their connectivities, and the morphologies of their synapses. In Figure 6, only the VA, VB, VD, and DD processes are shown. The axons of these motor neurons cluster around the neuromuscular junctions but can be readily distinguished even in single sections. Specifically, motor neuron axons are ordered in typical ventral nerve cord somatic positions at the ectoderm of the ventral nerve cord, with the DD neuron dorsal-most, VD below DD, VA next, and VB ventral-most. Second, the connectivities of these neurons differ: the DD neurons receive inputs from the VA and VB neurons, the VDs only form neuromuscular junctions in the ventral nerve cord, and the VAs and VBs form dyadic synapses to the DD neurons and the muscles. Third, the morphologies of the synapses differ: VD neurons have large varicosities and small active zones centered and oriented directly on the muscle; and VA and VB neurons have small varicosities with large active zones that are oriented dorsally. Position along the ventral cord was confirmed by noting the positions and identities of the other ventral cord motor neurons. Data for synaptic morphology were collected by examining two N2 and three unc-25(e156) animals. Serial reconstruction was made from one N2 and one unc-25(e156) animal.

RESULTS

unc-25 is a C. elegans GAD gene

unc-25 was previously mapped genetically on the right arm of chromosome III (Brenner, 1974). A partial cDNA clone, cm9e10, encodes a protein with sequence similarity to GAD and hybridizes to the YAC clone Y37D8, which is in the region of unc-25 on the physical map (Fig. 1A) (Waterston et al., 1992). We used cm9e10 as a probe to screen a C. elegans genomic library constructed in λ phage and isolated two positive clones (see Materials and Methods). Injection of DNA from either phage clone into the germ line of unc-25(e156) mutant worms produced stable
transgenic lines in which the unc-25 mutant phenotype was restored to wild type, indicating that the genomic DNA in the phage clones contained the unc-25 gene. We localized the rescuing activity to a 12 kb genomic DNA fragment (Fig. 1B). This 12 kb DNA contains a predicted gene corresponding to the cm9e10 cDNA. In addition, all known unc-25 alleles contained mutations in this gene (see below and Table 1). We conclude that unc-25 encodes a C. elegans GAD-like protein.

**C. elegans GAD is equally similar to GAD$_{65}$ and GAD$_{67}$**

We constructed a full-length cDNA for unc-25 and determined its sequence (see Materials and Methods). The unc-25 cDNA predicts a protein of 508 amino acids. The predicted UNC-25 protein shares 44% amino acid identity with human GAD$_{65}$ and 46% with human GAD$_{67}$ (Fig. 2). The C-terminal 440 amino acids are highly conserved, and in this region the identity between the C. elegans and human GAD proteins is close to 65%. The landmark structural feature of GAD, a tetrapeptide Asn-Pro-His-Lys (NPHK) involved in binding pyridoxal phosphate, is conserved in C. elegans GAD. The overall structure of C. elegans GAD is closer to that of the Drosophila GAD than to that of the vertebrate GADs in that the C. elegans GAD lacks a long N-terminal extension.

The similarity of unc-25 to human GADs is revealed further at the level of genomic structure. Most exon boundaries are conserved between the human GAD$_{65}$ and GAD$_{67}$ genes (Bu and Tobin, 1994). The unc-25 gene is composed of eight exons (Fig. 1B). Remarkably, four exon–intron boundaries occur at the same positions as those found in human GAD$_{65}$ and GAD$_{67}$ (Bu and Tobin, 1994) (Fig. 1B), suggesting that GAD gene structure is conserved in evolutionarily distant phyla.

### unc-25 mutations affect conserved residues in GAD

Fifteen unc-25 mutations were isolated from various genetic screens (see Materials and Methods). We determined the molecular lesions in these alleles (Table 1). Twelve of the unc-25 mutant alleles cause severe defects in locomotion and defecation. The phenotype caused by these strong alleles is indistinguishable from the phenotype caused by a strong allele in trans to a deficiency (McIntire et al., 1993a). Three of these strong unc-25 alleles are nonsense mutations, whereas the other nine are missense mutations changing amino acid residues conserved among known GADs (Fig. 2). The nonsense mutation n2324 changes Trp291 to an amber stop codon and is likely to result in a premature protein that lacks the C-terminal half of the protein, including the NPHK tetrapeptide, the cofactor binding site. This mutation is thus likely to cause complete loss of unc-25 function.

Three alleles, sa94, n2379, and n2569, caused temperature-sensitive locomotory defects: these mutants displayed nearly wild-type locomotory movement and worms were weakly constipated; +, partial rescue, nearly wild-type locomotory movement and worms were weakly constipated. Independently established transgenic lines (10–20) were scored with each construct.
mutations may interfere with the regulation of GAD activity but not abolish GAD function.

unc-25 is expressed exclusively in GABAergic neurons

To determine the expression pattern of unc-25, we made a series of reporter gene constructs using the GFP (Chalfie et al., 1994) (Fig. 3). We found that all of the GABAergic neurons and only these cells express unc-25 (Fig. 4A), and the unc-25 expression was visible as soon as these neurons were generated. This expression pattern indicates that the GABA immunoreactive cells accumulate GABA via de novo synthesis rather than via uptake of GABA released by neighboring cells. Furthermore, our reporter gene analysis suggested that UNC-25 expression in different classes of GABAergic neurons is regulated at both the transcriptional and post-transcriptional levels. Specifically, reporter constructs containing either the entire unc-25 genomic sequences (pSC317) or genomic sequences up to exon 6 (pSC100) were expressed in all 26 GABAergic neurons. Reporter constructs containing shorter genomic sequences that included the putative 5' regulatory region and various lengths of genomic sequences up to exon 5 (pSC380, pSC379, pSC98, and pSC315) were not expressed in the RIS, AVL, and DVB neurons. However, the expression of unc-25 reporter gene constructs in these three neurons did not depend on specific intronic or exonic sequences. We used an unc-25 genomic fragment that contained only the 5' regulatory region and the first 13 amino acid residues in exon 1 to drive a GFP reporter gene in which multiple synthetic introns were inserted into the GFP coding sequence (pSC381) (A. Fire, personal communication). This construct expressed GFP in all 26 GABAergic neurons (data not shown). Although these experiments are subject to the general caveat that overexpression of a reporter gene may not accurately represent endogenous gene expression, this analysis suggests that the 5' region of unc-25 contains the information required for expression in all GABAergic neurons and that the expression of unc-25 in AVL, DVB, and RIS may additionally require RNA processing. Such post-transcriptional regulation might be achieved through regulated nuclear RNA export and/or RNA stability (Johnson, 1994; Re-thmeier et al., 1997).

The two isoforms of vertebrate GAD differ in their subcellular locations (Erlander et al., 1991). To determine where UNC-25 is localized within a cell, we inserted GFP in-frame into the amino terminus after amino acid Val12 (pSC317). The transgene containing this construct rescues the Unc-25 phenotype, indicating that the GFP insertion did not disrupt the function of UNC-25 and therefore that this transgene was expressed at sites at which UNC-25 function is needed. GFP was observed throughout cell bodies and axonal branches and was enriched in synaptic regions (Fig. 4B,C). To evaluate whether the synaptic localization of UNC-25::GFP is caused by an association with synaptic vesicles, we examined the expression of this transgene in unc-104 mutant animals, which accumulate synaptic vesicles in cell bodies because of defects in a kinesin-like molecule (Hall and Hedgecock, 1991; Otsuka et al., 1991). We found that in unc-104 animals, the synaptic punctate expression of GFP diminished and GFP became uniformly distributed in the axonal branches and highly concentrated in the cell bodies (Fig. 4D), suggesting that some fraction of UNC-25::GFP was associated with vesicles. Because all synaptic vesicles are retained in cell bodies in unc-104 mutants (Hall and Hedgecock, 1991), this analysis suggests that UNC-25 is present in both nonvesicular- and vesicular-bound forms, although it is possible that the nonvesicular localization is caused by overexpression from the transgenic array.

Axonal outgrowth and synapse formation are normal in unc-25 mutants

To investigate whether GABA plays a role in axon guidance, we examined the morphology of the GABAergic neurons in unc-25 mutant animals using the GFP reporter transgene with multiple synthetic introns (pSC381). GFP expressed from this transgene was found throughout GABAergic neuron cell bodies and axons (data not shown), indicating that lack of GABA has no effect on the divisions of the precursor cells that generate the GABAergic DD and VD neurons. Moreover, in unc-25 mutant animals, the number and positions of the motor neurons in the ventral nerve cord, as examined using Nomarski optics, were the same as in wild-type animals (data not shown), indicating that lack of GABA has no effect on the divisions of the precursor cells that generate the GABAergic DD and VD neurons.

To determine whether GABA plays a role in neuronal connectivity, we first compared in unc-25(e156) and wild-type animals the expression of a transgene, juIs1, in which GFP was fused to the C. elegans SNB-1 protein, a homolog of the synaptic vesicle protein synaptobrevin (Nonet et al., 1998), and driven by the unc-25 promoter (Jorgensen et al., 1995). Punctate fluorescent clusters of GFP were seen along the dorsal and ventral nerve cords, corresponding in position to the synaptic varicosities of the.
DD and VD neurons, respectively (Jorgensen et al., 1995). We detected no abnormality in \textit{unc-25(e156)} animals in either the shape or the density of the fluorescent clusters (Fig. 5 A, B), suggesting that the synaptic termini of these neurons were largely normal, although the intensity of the fluorescent clusters was slightly stronger in \textit{unc-25} mutants than that in wild-type animals. These experiments indicated that the distribution of GABAergic synapses was roughly normal in a \textit{unc-25} mutant. However, these experiments did not demonstrate that synaptic connectivity is normal in a \textit{unc-25} mutant. Specifically, they did not examine whether these synapses were directed to their normal muscle targets, nor did they determine whether the density of cholinergic synapses to the muscle was normal.

To examine the synaptic connectivity of a \textit{unc-25} mutant, we fixed a \textit{unc-25(e156)} animal and a wild-type animal and prepared electron micrographs from serial sections of each. The morphologies of the GABAergic and cholinergic neuromuscular junctions were normal in the \textit{unc-25} animal. Specifically, the cholinergic VA and VB and the GABAergic VD neuromuscular junctions were neither enlarged nor diminished in the \textit{unc-25} mutant compared with the wild type (Fig. 5 C, D), and the diameters of synaptic vesicles were also unchanged (Table 2).

In the absence of inhibitory input, the muscles in \textit{unc-25} mutants should receive an excess of excitatory cholinergic input. Are cholinergic inputs pruned to restore the muscles to a normal level of excitation? To examine this question, we reconstructed a segment of the ventral nerve cord between the DD2 commissure and the DD2 cell body (Fig. 6) and counted the neuromuscular junctions from the cholinergic motor neurons VA4 and VB3 and the GABAergic motor neuron VD4. We found that the total number of synapses was approximately the same for the two strains in the reconstructed segment: VD4 formed 24 neuromuscular junctions in this interval in both the wild-type and the \textit{unc-25(e156)} animal. There were similar numbers of synapses from the cholinergic neurons in the wild-type (52) and \textit{unc-25(e156)} (48) individuals. Based on these numbers, the ratio of the VA and VB neuromuscular junctions to VD neuromuscular junctions was 2.2 in the wild-type and 2.0 in the \textit{unc-25(e156)} animal. We conclude that there was no compensation in synaptic density of the GABAergic neurons or the cholinergic neurons in response to the lack of GABA in the \textit{unc-25} mutant.

Another possibility is that rather than remodeling neuromuscular junctions, the nervous system may compensate for reduced neurotransmission by changing the strength of existing synapses. Although the rate of release of synaptic vesicles cannot be measured in \textit{C. elegans} at this time, the number of synaptic vesicles...
can be examined directly. We found that the mean number of synaptic vesicles at the midpoints of the GABAergic neuromuscular junctions was slightly increased in the unc-25 animals (37) compared with that in the wild-type animals (27) (Table 2) ($p = 0.045$). Although the increase is small, these data may indicate that a lack of transmission at these synapses causes a compensatory increase in the number of vesicles available for release.

**DISCUSSION**

The C. elegans gene unc-25 encodes a neuronal-specific GAD, the biosynthetic enzyme for the neurotransmitter GABA. Null mutations in unc-25 abolish GABA expression and cause animals to display behaviors indistinguishable from those in which the GABAergic neurons are killed by a laser. unc-25 missense mutations affect amino acid residues that are conserved among members of the GAD family. Using both light and electron microscopy, we found that in unc-25 mutant animals GABAergic neurons exhibit normal axonal morphology and synaptic connectivity, and the size and shape of both synaptic vesicles and neuromuscular junctions are normal, indicating that GABA is not necessary for the development of these neurons and the maintenance of neuromuscular junctions in vivo.

**Regulation of GAD**

Although GABA synthesis in the brain has been studied extensively (for review, see Martin and Rimvall, 1993), to date there has been no structure/function analysis of GAD or other decarboxylases besides the interaction between the NPHK tetrapeptide and the cofactor pyridoxal phosphate. For example, little is known about the residues needed for GAD catalytic activity. We have identified 12 unc-25 missense mutations that indicate the functional importance of specific amino acids for GAD activity. Nine of these alleles are strong mutations and behave genetically as null mutations (McIntire et al., 1993a). All cause amino acid

**Table 2. Synaptic vesicle densities and diameters in unc-25 and wild-type animals**

<table>
<thead>
<tr>
<th></th>
<th>SVs per midsynaptic profile$^{a,b}$</th>
<th>Number of synapses</th>
<th>SV diameter (nm)$^{b}$</th>
<th>Number of SVs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type ACh synapses</td>
<td>27.4 ± 2.3</td>
<td>32</td>
<td>30.1 ± 0.4</td>
<td>109</td>
</tr>
<tr>
<td>Wild-type GABA synapses</td>
<td>27.4 ± 3.4</td>
<td>14</td>
<td>29.6 ± 0.3</td>
<td>196</td>
</tr>
<tr>
<td>unc-25(e156) ACh synapses</td>
<td>26.9 ± 2.6</td>
<td>14</td>
<td>31.6 ± 0.5</td>
<td>170</td>
</tr>
<tr>
<td>unc-25(e156) GABA synapses</td>
<td>37.0 ± 3.3</td>
<td>14</td>
<td>31.6 ± 0.6</td>
<td>134</td>
</tr>
</tbody>
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SVs, Synaptic vesicles.

$^a$Number of synaptic vesicles in a cross section at the center of the active zone.

$^b$Mean ± SEM.
substitutions at positions conserved among known GADs. These residues might define sites important either for catalysis or for protein structure or stability. Three weak unc-25 alleles caused a constitutive loss of enteric muscle contractions but only a temperature-sensitive locomotory defect. These three alleles alter amino acids adjacent to the pyridoxal 5'-phosphate binding site, indicating that the amino acids around the cofactor binding site may contribute either to the binding of the cofactor or to the correct conformation of the catalytic site around the bound glutamate. These three unc-25 temperature-sensitive alleles may generate either temperature-sensitive proteins or proteins with lowered activity. In the latter case, more neurotransmitter would be required at higher temperatures, and these reduction of function mutations would simply be revealing this temperature-sensitive process.

unc-25 is transcribed and translated exclusively in the 26 GABAergic neurons. However, the regulation of unc-25 expression among GABAergic cells may differ. Using reporter gene constructs, we found that unc-25 expression in the RIS, AVL, and DVB GABAergic neurons required the presence of introns in addition to the 5' regulatory regions. This intron requirement does not seem to be sequence- or gene-specific, because adding synthetic introns into the GFP reporter gene driven by the unc-25 promoter caused GFP to be expressed in these neurons. Introns are known to facilitate RNA processing by regulating RNA export, splicing, and polyadenylation (Gallie and Young, 1994; Jarrous and Kaempfer, 1994; Damert et al., 1996; Rethmeier et al., 1997), and they also play roles in translation (Chapman and Walter, 1997). GABA is used in many different types of neurons in the nervous systems of many animals. The expression of vertebrate GADs in different types of neurons appears to be differentially regulated at both the mRNA and protein levels (Esclapez et al., 1994; Hendrickson et al., 1994; Houser and Esclapez, 1994). Although we do not know how post-transcriptional regulation is achieved in the AVL, DVB, and RIS neurons, the multi-level regulation of unc-25 we observed in C. elegans may reflect a general mechanism used by different types of neurons in complex nervous systems.

**Evolution of GAD**

In contrast to vertebrates, C. elegans may have only a single GAD gene. This conclusion is based on three observations. First, mutations in unc-25 eliminate all GABA immunoreactivity and the known functions of all GABAergic neurons (McIntire et al., 1994).
1993a,b). Second, unc-25 is expressed in all 26 GABAergic neurons. Third, the sequence of 82% of the C. elegans genome has been determined, and the sequences of many mRNAs have been partially determined as expressed sequence tags, yet no other gene is as similar to the vertebrate GADs as is unc-25. Most GAD activity in Drosophila can be attributed to a single gene, GAD1 (Jackson et al., 1990; Kulkarni et al., 1994), although other minor GADs may contribute to GABA synthesis in some tissues (Phillips et al., 1993) (M. Phillips, personal communication). Neither the C. elegans GAD gene nor the Drosophila GAD1 gene more closely resembles either the mammalian GAD65 or GAD67. Thus, the duplication and divergence of these mammalian genes probably occurred after the divergence of vertebrates from arthropods and nematodes.

The C. elegans GAD protein is strongly conserved with the vertebrate and Drosophila GAD proteins in its C portion. Even the locations of some exon/intron boundaries are maintained between species as distant as C. elegans and human. GAD65 and GAD67 each have a region of ~100 amino acids at the amino terminus that is not conserved between the two forms. This amino-terminal region is absent in both the C. elegans and Drosophila GADs. In GAD65 this region contains two cysteines that can be palmitoylated and a region that is critical for membrane binding. Although the amino terminus of the C. elegans and Drosophila GADs and of human GAD67, also contain multiple serines that could potentially be phosphorylated, GAD67 and UNC-25 are found in both synaptic regions and cytoplasm, suggesting that these serines may not be involved in an interaction with synaptic vesicles. Together, both the protein sequence comparisons and the subcellular expression patterns suggest that UNC-25 may resemble an ancestral member of the GAD family.

**Function of GAD in nervous system development**

Because there appears to be a single GAD gene in C. elegans, null mutations in unc-25 are likely to define all GABA-dependent functions. unc-25 animals exhibit hypercontraction of the body muscles, hyperflexions of the head during foraging, and a severe reduction in contractions of the enteric muscles. Our data indicate that these defects are a consequence of a lack of neurotransmitter function in the mature nervous system rather than of connectivity defects caused by absence of GABA during development. We reach this conclusion for several reasons. First, we detected no abnormalities in the axonal trajectories of the GABAergic neurons in unc-25 mutants. Second, the density of synaptic varicosities is normal in GABAergic neurons as analyzed by light microscopy. Third, we observed no abnormalities in neuromuscular connectivity or in the differentiation of neuromuscular junctions using electron microscopy. Fourth, we observed previously that the AVL and DVB neurons are capable of importing GABA and that the acute restoration of GABA to these cells by bath application can rescue the function of these neurons, suggesting that fully functional synapses are formed in unc-25 mutants and that neurotransmission fails only because GABA is absent (McIntire et al., 1993a). Fifth, in unc-49 mutant animals, which are defective in a GABA receptor (B. Bamber and E. J., unpublished observations) and hence likely to be defective in GABA function, the axonal morphology of GABAergic neurons (McIntire et al., 1993a) and the presynaptic termini of the DD and VD GABA neuromuscular junctions as revealed by a synapse-specific GFP marker are normal (Y. J., unpublished results). These data indicate that the behavioral defects of unc-25 animals are caused by a lack of GABA function in an otherwise normal nervous system.

Although the connectivity of the nervous system is unchanged in unc-25 mutants, we noted that there is a slight increase in the number of synaptic vesicles at the GABAergic neuromuscular junctions in unc-25(e156) animals. This increase in synaptic vesicle number may be the result of a feedback mechanism. Specifically, the muscle cells may detect that GABA transmission is inadequate and hence may signal the motor neuron to make more synaptic vesicles and perhaps to increase the probability of release of these vesicles. However, because these vesicles lack GABA, increased synaptic release will not lead to increased transmission at these mutant synapses.

Our observations appear to contrast with several reports that GABA can affect the development and differentiation of the mammalian CNS. Exposing explants of rat embryonic cortex to GABA causes a decrease of the number of cortical cells synthesizing DNA and presumably undergoing cell divisions (LoTurco et al., 1995). However, we did not observe an increase or decrease in the number of ventral cord neurons in unc-25 mutants. Manipulations of GABA transmission in vivo by the addition of agonists or antagonists can alter axonal pathfinding during retinal development in the rabbit (Messersmith and Redburn, 1993). By contrast, we see no changes in the axonal trajectories of motor neurons in unc-25 mutants. Why might our results differ? One possibility is that our experiments were conducted in vivo in a mutant lacking GABA, whereas the other experiments were conducted either by adding exogenous GABA to cell cultures in vitro or by interfering with GABA transmission pharmacologically.

Our observations also appear to contrast with studies of the regulation of the size of mature vertebrate neuromuscular junctions. Pharmacological perturbations of vertebrate neuromuscular junctions cause the nervous system to be remodeled to compensate for these changes in neurotransmission. Specifically, reducing the effective level of neurotransmitter to the chick hindlimb muscles by blocking acetylcholine receptors with α-bungarotoxin causes the motor neuron to sprout and form additional synapses (Dahm and Landmesser, 1991). Increasing neurotransmitter activity by adding the acetylcholine agonist carbachol causes a compensatory reduction in the density of synapses made by the chick lumbosacral motor neurons (Lance-Jones and Landmesser, 1981). These experiments indicate that the density of synapses may change to maintain a constant level of input into the muscle. Given these data, we might have expected that in a unc-25 mutant a compensation for the lack of GABA neurotransmission would be hypertrophic arborization of the GABA neurons or an increase in the number of GABAergic neuromuscular junctions along the ventral cord. However, we found neither.

Differences between our studies and those of vertebrate neuromuscular junctions include the organism, the neurotransmitter examined, and whether pharmacological intervention was used. For example, unlike in vertebrates, in C. elegans muscles send processes to neurons, and neuromuscular junctions are formed en passant; chemotropic interactions between nerves and muscles could be different as a consequence. Second, although acetylcholine has been reported to have a role in the development of the vertebrate neuromuscular junction, no such function has been assigned to GABA for synaptic development in the CNS; perhaps GABA does not have such a role in either C. elegans or vertebrates. Finally, the vertebrate studies that indicated a role for
neurotransmitters in the development of neuromuscular junctions were based on pharmacological manipulations, and it is conceivable that targets other than those intended were perturbed. A genetic study of a vertebrate, like our study of *C. elegans*, suggested that neurotransmitter function is not necessary for the formation of normal neuromuscular junctions: in a zebrafish mutant lacking a muscle acetylcholine receptor, motor neurons have morphologically normal patterns of innervation and normal neuromuscular junctions (Westerfield et al., 1990).

Based on our findings, we conclude that neither synaptic development nor synaptic maintenance depends on GABA neurotransmission at neuromuscular junctions in *C. elegans*.

**REFERENCES**


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