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Reversal of a Muscle Response to GABA during *C. elegans* Male Development

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In the *C. elegans* hermaphrodite the expulsion step of defecation depends on the coordinated contraction of three enteric muscle groups: the anal depressor muscle, the intestinal muscles, and the sphincter muscle. These muscles are activated by excitatory GABA neurotransmission. Mutations in 13 genes that affect activation of these enteric muscles have previously been identified. We show that the larval male defecates by contracting the same set of enteric muscles, and that these contractions require 12 of these 13 genes. However, near the end of the last larval stage, the male anal region undergoes a developmental change, including dramatic hypertrophy of the anal sphincter muscle and the opening of a cloacal canal. We find that this modified sphincter must now relax to permit defecation. In contrast to the larval male, we find that in the adult male only 2 of the 13 genes required for enteric muscle contraction, *unc-25* and *unc-47,* are important for sphincter muscle relaxation. *unc-25* and *unc-47* are required for the synthesis and utilization of GABA. We also find that two other genes, *unc-46* and *unc-49,* previously implicated in the inhibitory action of GABA on body-wall muscle, are also required for normal adult male sphincter relaxation. In these mutants, failure to relax the sphincter muscle results in a constipated phenotype, and killing the sphincter muscle rescues this phenotype. We also find that a GABA agonist or GABA itself can suppress the adult male sphincter relaxation defect of *unc-25* mutants. Thus, we demonstrate that GABA is required for excitation of the hermaphrodite and larval male sphincter muscle, while GABA is required for inhibition of this sphincter muscle in the adult male. We conclude that the morphological changes that take place during the developmental transition from the larval to adult male are accompanied by changes in response of the anal sphincter muscle to GABA, presumably at the level of the receptor.

[Key words: behavioral genetics, defecation, GABA, GAD, motor control, motor program, muscle contraction, nematode, sexual dimorphism]

Defecation in *C. elegans* is a readily analyzed sexually dimorphic behavior. The muscles and some neurons required for hermaphrodite defecation have been identified (Thomas, 1990; McIntire et al., 1993a,b). The anatomy and muscular organization of the hermaphrodite and male have been described (Sulston and Horvitz, 1977; Sulston et al., 1980), and the structures of the entire hermaphrodite nervous system (White et al., 1986) and part of the male nervous system (Sulston et al., 1980) have been reconstructed using electron microscopy. This knowledge should allow the identification and analysis of differences in defecation between the male and hermaphrodite.

Hermaphrodite defecation is carried out by a simple motor program, which is composed of three muscle group contractions and occurs every 45 sec (Thomas, 1990; Liu and Thomas, 1994). The three muscle-group contractions are pBoc (posterior body-wall muscle contraction), aBoc (anterior body-wall muscle contraction) and Exp (expulsion or enteric muscle contraction) (Croll, 1975; Thomas, 1990). Mutations have been identified that affect several aspects of the motor program and cycle periodicity (Thomas, 1990; Liu and Thomas, 1994). In particular, genes that affect gamma-amino butyric acid (GABA) neurotransmission have been shown to be required for enteric muscle contraction in hermaphrodites (Thomas, 1990; McIntire et al., 1993a,b).

GABA is used widely as an inhibitory neurotransmitter both in vertebrates (e.g., Tobin, 1991; DeLorey and Olsen, 1992) and in invertebrates (e.g., Lunt, 1991). In *C. elegans*, as in the related nematode *Ascaris* (Walrond et al., 1985; Walrond and Stretton, 1985a,b), GABA is thought to act on body-wall muscles in its familiar inhibitory mode to promote the reciprocal inhibition important for normal locomotion (McIntire et al., 1993b). GABA-defective mutants have a "shrinker Unc" (uncoordinated) locomotion phenotype that is due to defective reciprocal inhibition of the body-wall muscles, permitting simultaneous contraction of opposing muscle groups (McIntire et al., 1993a,b). The shrinker Unc genes *unc-25* and *unc-47* have been implicated in GABA synthesis and utilization, respectively (McIntire et al., 1993a). *unc-25* has been shown to encode glutamic acid decarboxylase (GAD; Y. Jin and H. R. Horvitz, personal communication), the biosynthetic enzyme for GABA. *unc-47* mutants display markedly increased GABA immunoreactivity, suggesting a vesicle-loading defect (McIntire et al., 1993a). As in vertebrates, muscimol acts as a potent agonist of inhibitory GABA receptors (McIntire et al., 1993a). Mutants for the shrinker Unc gene *unc-49* are resistant to muscimol. Since a $GABA_A$ subunit has been shown to bind muscimol in vertebrates (Deng et al., 1986; Casalotti et al., 1986), this resistance suggests that *unc-49* encodes a component of a $GABA_A$ receptor (McIntire et al., 1993a). The phenotypically similar *unc-46* may also function postsynaptically.

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For the hermaphroditic enteric muscles GABA appears to act as an excitatory transmitter (McIntire et al., 1993a). The anal canal in the hermaphrodite is sealed by structural features of the anal region. Specialized enteric muscles (the anal depressor muscle, intestinal muscles, and anal sphincter muscle) are contracted to break this seal and expel gut contents during defecation (Thomas, 1990). The GABA-containing motor neurons AVL and DVB (McIntire et al., 1993a,b), and the *aex* genes (aBoc and expulsion defective; Thomas, 1990) are required for this enteric muscle contraction (EMC). Since *unc-25* (GAD) and *unc-47* mutants are defective in this contraction, GABA is implicated in enteric muscle excitation (Thomas, 1990; McIntire et al., 1993a). Since no other neurons that inhabit the region of the enteric muscles are required for their contraction (McIntire et al., 1993b), GABA probably acts directly to excite the muscles. The *unc-25* and *unc-47* defects in both inhibitory and excitatory GABA function are consistent with their proposed presynaptic function.

We have analyzed defecation in the *C. elegans* male, and the role of GABA in this behavior. A structural change in the male anal region at the end of the last larval stage (L4) is accompanied by a change in the mode of defecation behavior. In contrast to the contraction of the enteric muscles (including the sphincter muscle) that implement expulsion in the hermaphrodite and larval male, the adult male must relax the sphincter muscle for defecation to occur. In addition, the anal depressor muscle reorients and functions in male mating behavior, but not in defecation. Phenotypic analysis of mutants implicated in GABA function (Thomas, 1990; McIntire et al., 1993a,b) indicates that GABA is required for both larval male enteric muscle excitation and adult male sphincter relaxation. These results suggest that a remodeling of the excitability of the sphincter muscle accompanies the structural change of the male anal region at the end of the last larval stage.

Materials and Methods

General methods. General methods of maintenance of *C. elegans* and genetic nomenclature are as previously described (Brenner, 1974; Horvitz et al., 1979). We obtained from the MRC, England, the canonical isolates of all the MRC alleles of *unc-25*, *unc-46*, *unc-47*, and *unc-49*. Observations and photographs using Nomarski optics were performed as described previously (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1986). Unless stated, genetic manipulations and experiments were performed at 20°C. All experiments requiring males were performed in a *him-5* or *him-8* mutant strain (high incidence of males) to facilitate production of males. *him* mutations cause high rates of X-chromosome nondisjunction, which produces the XO males (Hodgkin et al., 1979).

Phenotype observations. At least 10 young to midadult males were observed using a dissecting microscope to arrive at a consensus description of the constipated (Con) phenotype as weak, moderate, or severe Con (Table 1). The Con phenotype was determined by relative distention of the gut lumen in wild-type and mutant animals (Thomas, 1990). Observation of the pre-anal area showed that the intestinal lumen occupies approximately 10% of the width of the body in adult wild-type males (non-Con), 20% in adult *unc-49* males (weak Con), 50% in adult *unc-46* males (moderate Con), and 70% of the width of the body in adult *unc-25* and *unc-47* males (severe Con) (e.g., see Fig. 2). The Con phenotypes of these different adult male genotypes were distinguished in a blind test. In addition, the defecation behavior was scored by direct observation for several defecation cycles in all cases. However, due to the frequent activity of the male-specific diagonal muscles used in mating, consistent quantitative analysis of release was not possible.

Hermaphrodite Con phenotypes were scored as previously described (Thomas, 1990). For every genotype examined, the average degree of hermaphrodite constipation corresponded with the percent enteric muscle contraction (%EMC) (Table 1). Note that *aex* mutant animals (see introductory paragraphs) were generally more constipated than expulsion-defective (Exp) animals with a similar %EMC, presumably due to the additional effect of the aBoc defect on gut clearance. For both male and hermaphrodite Con mutants, animals appeared temporarily less Con just after release of some of the gut contents (Thomas, 1990). This represents temporal variation in phenotype, not incomplete expression of the phenotype (expressivity), since direct observation in all cases confirmed that such animals quickly became Con again with further feeding. The locomotion defects of *unc-49* animals were compared to those of *unc-25*, *unc-47*, *unc-46*, and wild-type animals.

Of the genes required for hermaphrodite defecation, only *exp-2* is not required for larval male EMCs or adult male intestinal muscle contractions. We observed that while *exp-2* mutant hermaphrodites were severely EMC defective, both larval and adult *exp-2* males were approximately wild type for EMCs. We speculate that this may represent sexual dimorphism of the expression of the *exp-2(sa26)* gene product. Since we have only one allele of *exp-2*, this phenomenon was not examined further.

Laser kills. Cells were killed using a laser microbeam (model VSL-337, Laser Science, Inc., Newton, MA) focused through a microscope, as previously described (Avery and Horvitz, 1989). AVL and DVB were killed in mid- to late L1 males. The K blast cell, the precursor of both DVB and a hypodermal cell, was killed in two of eight animals, and DVB was killed directly in the others. The anal sphincter was killed in late L1 animals, and the anal depressor and intestinal muscles were killed in early L1 animals. Cell nuclei were identified based upon previously described locations (Sulston and Horvitz, 1977; Sulston et al., 1983) The efficacy of all kills was confirmed 2 to 4 hr after operation by identifying refractile dying cells with no apparent extraneous damage. For all experiments anesthetized but unoperated animals were used as controls for the effect of anesthetic on behavior.

Using cell kills, we attempted to identify neurons required for sphincter relaxation in adult males. Based upon GABA immunoreactivity in *C. elegans* males (E. Jorgensen and H. R. Horvitz, personal communication) and positions of male tail cells (Sulston, et al., 1980), we chose DVE and DVF as likely candidates for these neurons. Cell nuclei were identified based upon locations described by Sulston et al. (1980). Although *aex* adult males (either from AVL, DVB kills or from *aex* mutations; see Results) were not constipated, we were concerned that AVL and DVB might be redundant in function with the unknown neurons that act to relax the sphincter. Therefore, to address this possibility, laser kills of DVE and DVF were performed in *aex-1(sa27)* or *aex-1(sa3)* mutant backgrounds, where AVL and DVB functions appear to be eliminated (see Results). Precursor blast cells of DVE and DVF, including the immediate precursor of both, were killed. The following cell or cells were killed in an *aex-1* background: B.pp, B.ppp, B.ppa, B.pppp, B.ppa, and B.ppp, B.ppa and B.pppp. Laser-operated animals were grown and defecation phenotypes observed. These experiments eliminated DVE and DVF singly or together, and none of these kills caused an adult male Con phenotype.

High-magnification observation of enteric muscle contractions. We used freeze-frame video observations (60 frames per second) in conjunction with Nomarski microscopy to observe enteric muscle contractions at high magnification. Muscle contractions were observed in unoperated wild-type hermaphrodites and males or hermaphrodites and males with the anal depressor muscle, the sphincter muscle, or the intestinal muscles killed using a laser microbeam. Contraction of the intestinal and anal depressor muscles were clear under most circumstances. We believe that the sphincter muscle contracted in these animals, but the contractions of the sphincter were difficult to distinguish confidently from those of the intestinal muscles. However, in several hermaphrodites and fourth larval stage males in which the anal depressor and intestinal muscles had been killed, sphincter muscle contraction was clear, and its timing was approximately the same as that expected for the intestinal muscles.

GABA and muscimol tests. To make drug plates, NG agar (Wood, 1988) was mixed with either 50 mM muscimol (Research Biochemicals, Inc., Natick MA) or 150 mM GABA (Sigma Chemical Co., St. Louis, MO) stock solutions in M9 buffer (Wood, 1988). Plates were poured with a final concentration of 1.6% agar and either 10 mM muscimol or 30 mM GABA (previously published effective concentrations; McIntire et al., 1993a,b). These drug concentrations are high in comparison to concentrations required in most systems. A number of drugs have been described whose action in *C. elegans* requires high concentrations (e.g.,

Table 1. Comparison of Con phenotypes in hermaphrodite, larval male, and adult male mutants

Gene	Alleles	Hermaphrodite Con phenotype	Hermaphrodite % EMC	Larval male Con phenotype	Larval male % EMC	Adult male Con phenotype
wild type		non-Con	84 (525)	non-Con	87 (61)	non-Con
aex-1	*sa9, sa27*	severe Con[a]	0 (109)[a]	severe Con	10 (62)	non-Con
aex-2	*sa3, sa21*	severe Con[a]	0 (104)[a]	severe Con	6 (64)	non-Con
aex-3	*ad418, sa5*	weak Con[a]	17 (128)[a]	weak Con	32 (54)	non-Con
aex-4	*sa22*	severe Con[a]	11 (184)[a]	severe Con	13 (69)	non-Con
aex-5	*sa23, sa41*	severe Con[a]	8 (101)[a]	severe Con	19 (59)	non-Con
aex-6	*sa24*	severe Con[a]	10 (110)[a]	severe Con	14 (61)	non-Con
egl-2	*n693sd*	severe Con	0 (170)	severe Con	0 (57)	non-Con
egl-23	*n601sd*	mod Con[b]	3 (184)	mod Con[b]	2 (45)	non-Con
egl-36	*n728sd*	weak Con	30 (150)	weak Con	34 (59)	non-Con
exp-1	*sa6*	mod Con[a]	13 (128)[a]	mod Con	0 (60)	non-Con
exp-2	*sa26sd*[c]	severe Con[a]	0 (110)[a]	non-Con[d]	80 (46)[d]	non-Con
unc-25	*e156, e265, e610, sa4, s94ts*[e]	mod Con[a]	12 (133)[a]	mod Con	7 (55)	severe Con
unc-47	*e307, e542, sa93*	mod Con[a]	17 (127)[a]	mod Con	7 (59)	severe Con
unc-46	*e177, e300, e642*	non-Con	35 (123)	non-Con	26 (58)	mod Con
unc-49	*e382, e407, e468, e641, e929*	non-Con	85 (103)	non-Con	98 (40)	weak Con
unc-30	*e191*	non-Con	100 (106)	non-Con	96 (46)	non-Con

egl, egg-laying defective; exp, expulsion defective. All males examined were from a *him* mutant strain. Phenotypes were scored as described in Materials and Methods (mod = moderate). The canonical mutation of each locus (the first allele listed) was scored for percentage enteric muscle contraction (% EMC) per defecation cycle in hermaphrodites and larval males. The numbers in parentheses are the number of defecation cycles scored for that genotype.

[a] As reported originally in Thomas (1990).

[b] *egl-23(n601)* hermaphrodites were observed to leak frequently during the motor program, presumably due to weak activation of the enteric muscles, and were, therefore, not severely Con.

[c] *exp-2(sa26)/+* animals were analyzed, since *sa26* confers recessive lethality (Thomas, 1990).

[d] Hermaphrodite and larval male *exp-2* EMC frequencies are substantially different. We also observed EMCs in *exp-2* adult males (though not quantitatively) and found that contractions occur frequently, as in larval males (see Materials and Methods).

[e] *sa94* animals grown at 25°C.

Lewis et al., 1980; Horvitz et al., 1982; Trent et al., 1983). This is probably due to impermeability of the cuticle to the drug solutions (Lewis et al., 1980). Plates were seeded with *E. coli* OP50 and allowed to incubate 3 or more days. Adult males and hermaphrodites were transferred from normal NG agar plates to muscimol plates and observed every 15 min for 3 hr. Animals were scored for constipation, locomotion, and pumping behaviors. Adult males were scored for leaking during defecation cycles (slight release of gut contents, but substantially less than during a normal expulsion). For GABA assays, adult males and hermaphrodites were transferred from normal NG agar plates to GABA plates and observed hourly for 6 hr. Males were scored for constipation, and hermaphrodites were scored for enteric muscle contractions (EMCs). Males were scored for leaking during defecation cycles.

Strains used. The following single and double mutant strains were used in this analysis: CB1490 *him-5(e1490)*, JT5302 *egl-2(n693)*, JT9 *aex-1(sa9)*, JT3 *aex-2(sa3)*, DA510 *aex-3(ad418)*, JT5244 *aex-4(sa22)*, JT23 *aex-5(sa23)*, JT24 *aex-6(sa24)*, JT6 *exp-1(sa6)*, JT5132 *+/eT1; exp-2(sa26)/eT1 let(n886)*, JT6716 *egl-36(n728)*, JT5302 *egl-23(n601)*, CB156 *unc-25(e156)*, CB177 *unc-46(e177)*, CB382 *unc-49(e382)*, CB845 *unc-30(e191)*, JT5580 *aex-1(sa9); him-5(e1490)*, JT6349 *aex-1(sa27); him-5(e1490)*, JT5578 *him-5(e1490); aex-2(sa3)*, JT6350 *him-5(e1490); aex-2(sa21)*, JT5583 *him-5(e1490); aex-3(ad418)*, JT6351 *him-5(e1490); aex-3(sa5)*, JT5584 *him-5(e1490); aex-4(sa22)*, JT5577 *aex-5(sa23); him-5(e1490)*, JT6352 *aex-5(sa41); him-5(e1490)*, JT5581 *aex-6(sa24); him-5(e1490)*, JT5874 *egl-2(n693sd) him-5(e1490)*, JT5593 *him-8(e1489); egl-2(n693sd)*, JT5654 *egl-23(n601sd); him-5(e1490)*, JT5950 *him-5(e1490); egl-36(n728sd)*, JT5579 *exp-1(sa6); him-5(e1490)*, JT5630 *+/eT1; him-8(e1489); exp-2(sa26sd)/eT1 let(n886)*, JT5397 *unc-25(e156); him-5(e1490)*, JT6354 *unc-25(e265); him-5(e1490)*, JT7689 *unc-25(e610); him-5(e1490)*, JT6355 *unc-25(sa4); him-5(e1490)*, JT6356 *unc-25(sa94ts); him-5(e1490)*, JT5354 *unc-46(e177) him-5(e1490)*, JT5431 *unc-46(e300) him-5(e1490)*, JT5664 *him-8(e1489); unc-46(e642)*, JT5400 *unc-47(e307); him-5(e1490)*, JT6357 *unc-47(e542); him-5(e1490)*, JT6358 *unc-47(sa93); him-5(e1490)*, JT5396 *unc-49(e382); him-5(e1490)*, JT6359 *unc-49(e407); him-5(e1490)*, JT6360 *unc-49(e468); him-5(e1490)*, JT6361 *unc-49(e641); him-5(e1490)*, JT6362 *unc-49(e929); him-5(e1490)*, JT6365 *unc-30(e191); him-5(e1490)*, JT7630 *unc-49(e382) unc-25(e156); him-5(e1490)*, JT7631 *unc-47(e307) unc-49(e382); him-5(e1490)*, and JT7243 *unc-49(e382); unc-46(e177) him-5(e1490)*.

Allele assignments. The *unc-25(sa94)* and *unc-47(sa93)* alleles were isolated by Dennis WC Liu in our lab. Each was assigned to its respective gene on the basis of map position and failure to complement *unc-25(e156)* or *unc-47(e307)*, respectively. The *unc-25(sa94)* mutation was found to be temperature sensitive, with moderate defects at 15° and severe defects at 25° (our unpublished observations). The allele *e610* was originally assigned to *unc-49* by S. Brenner (J. Hodgkin, personal communication). However, when we obtained from the MRC strain collection the canonical strain CB610 that carries *e610*, we found that it displayed a phenotype similar to that of *unc-25* and *unc-47* mutants. On the basis of its hermaphrodite defecation defect, smooth body posture (see Results), muscimol-induced flaccid paralysis (see Results), and complementation testing with *unc-49(e382)*, *unc-25(e156)*, and *unc-47(e307)*, we reassigned *e610* to the *unc-25* gene.

Construction of double mutants. Males mutant for the various genes

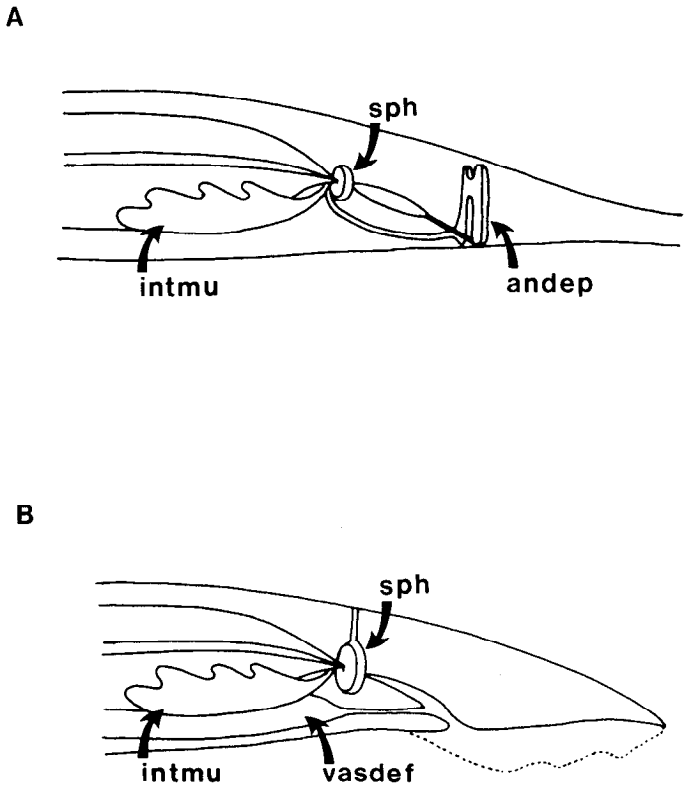

Figure 1. A schematic diagram of the hermaphrodite and adult male tail regions (*andep* = anal depressor muscle, *intmu* = intestinal muscle, *sph* = sphincter muscle, *vasdef* = vas deferens). The myofilaments in the anal depressor run dorsoventrally, those in the intestinal muscles run anteroposteriorly, and those in the sphincter are circumferential. The *dotted line* represents the male tail fan, which is not in the plane shown here. *A,* Hermaphrodite and larval male. *B,* Adult male.

of interest were obtained from *him-5* double mutant strains. In the few cases in which the gene of interest was linked to *him-5*, double mutant strains were, instead, constructed with *him-8*. Double mutant strains were constructed using standard methods (Brenner, 1974). For example: *him-5(e1490)* males were mated to *unc-25(e156)* hermaphrodites and non-Unc F1 hermaphrodite L4 progeny were picked singly to plates. From these F1s, several Unc F2 hermaphrodite progeny were picked singly to plates and their broods were observed for the presence of a high incidence of males (Him), indicating that the *him-5* mutation was homozygous in the parent. In each case, one such strain was selected for freezing and analysis.

Results

Sexually dimorphic organization of the anal region

While most of the male defecation motor program is similar to that of the hermaphrodite, the different physical constraints of the tail region require different mechanisms for clearing the gut contents. The morphology of hermaphrodite anus (Fig. 1*A*) causes it to be sealed in the absence of muscle function (a structural seal), and contraction of the enteric muscles is required to open the anus (Thomas, 1990; our unpublished results). In contrast, the adult male anus is an open cloaca (Fig. 1*B*; Sulston et al., 1980). Despite this, no leakage of gut contents is observed except during activation of the defecation motor program, suggesting that there is a muscle-regulated seal of the gut lumen in the adult male. In order to investigate this sexual dimorphism, we have studied the cellular and genetic properties of male defecation.

During larval growth, the male anus and defecation muscles are similar to those of the hermaphrodite (Sulston et al., 1980), but late in the last larval stage (L4) the male tail undergoes dramatic alterations that transform it into a specialized mating structure (Sulston et al., 1980). Most of the muscular changes involve the addition and organization of male-specific muscles used in mating, but two changes affect muscles used in defecation. First, the anal depressor changes one of its attachment sites, from the wall of the anus to the spicules used in mating (not shown in Fig. 1), and thus becomes a spicule protractor with no plausible function in defecation (Sulston et al., 1980). Second, the anal sphincter, which in the larval male is similar to the hermaphrodite sphincter, dramatically enlarges and forms a dorsal body-wall attachment (Fig. 1; Sulston et al., 1980).

Based upon these changes in muscular morphology in the tail, it has been proposed (Sulston et al., 1980) that the hypertrophied adult male sphincter muscle seals the anus, presumably by tonic contraction, and that this sphincter muscle must be relaxed for defecation. In order to test this model, we directly observed the contraction of the enteric muscles, using Nomarski optics and freeze-frame video (see Materials and Methods). We were able to see the contraction of the anal depressor and intestinal muscles in adult hermaphrodites and larval males, and the contraction of the intestinal muscles in adult males. However, the activity of the smaller sphincter muscle was masked by the more dramatic intestinal and anal depressor muscle contractions. Therefore, we analyzed animals in which the anal depressor and intestinal muscles had been killed using a laser microbeam (see Materials and Methods). Under these conditions, we clearly observed contraction of the sphincter muscle during hermaphrodite and larval male defecation, but not during adult male defecation. (The fact that the sphincter contracts during this last step in hermaphrodite and larval male defecation seems counterintuitive: our observations indicate that the sphincter muscle is elastic and dilated just prior to enteric muscle contraction. We think that the subsequent sphincter contraction either contributes to the lumen pressure during expulsion or that it limits outflow.) These observations support the model that relaxation of the sphincter mediates defecation in the adult male, though we were unable to observe this relaxation directly. In the following sections, we present several observations that demonstrate that GABA action on the sphincter changes from excitatory to inhibitory at the transition from the larval to the adult male.

Mutations causing male constipation

Genes that control the defecation motor program in the *C. elegans* hermaphrodite have been described (Thomas, 1990). Mutations in 13 of these genes perturb activation of the enteric muscles, and thereby cause hermaphrodites to be constipated (Con). In order to assess the importance of this set of genes in male defecation, double mutants were constructed with a *him* mutation (high incidence of males; Hodgkin et al., 1979). Throughout this article all males analyzed, whether mutant in other genes or not, are males from a *him* mutant strain.

The approximate degree of constipation in each mutant hermaphrodite or male was scored as described in Materials and Methods. The degree of constipation (Con) in mutant hermaphrodites was shown to be negatively correlated with the percent enteric muscle contraction (%EMC) of total defecation cycles observed (i.e., the higher the frequency of release of the gut contents, the less Con animals were; compare columns 3 and 4 in Table 1). Similarly, we found a negative correlation between the Con phenotype and %EMC in larval males (compare col-

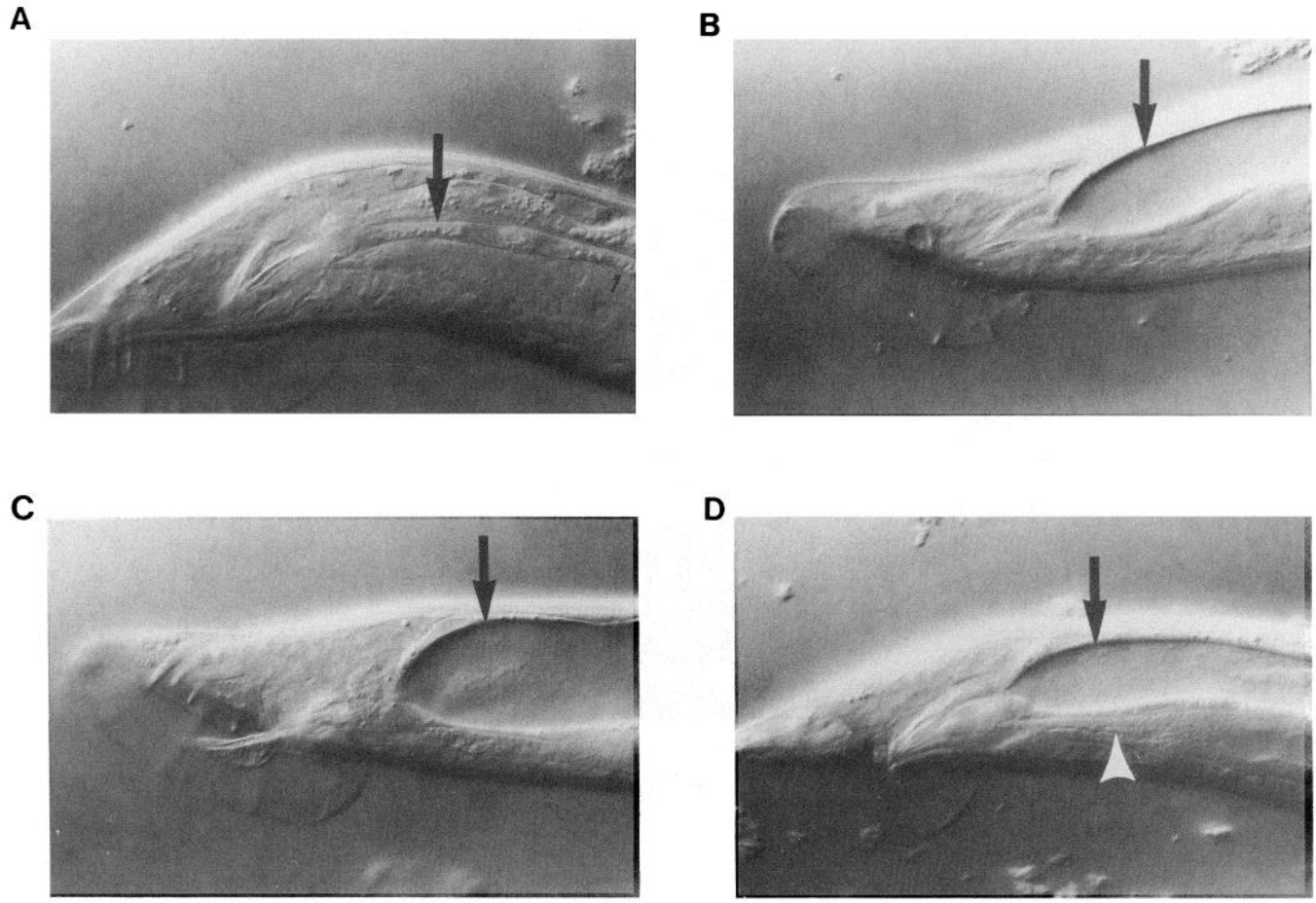

Figure 2. Nomarski photographs of the tail region in wild-type and mutant males. *A, him-5. B, unc-25; him-5. C, unc-47; him-5. D, unc-46 him-5.* The *large black arrows* in each frame indicate the dorsal edge of the intestinal lumen. The *white arrow in D* indicates the lumen of the vas deferens.

umns 5 and 6 in Table 1). In contrast, most mutants that cause constipation in hermaphrodites and larval males do not cause detectable constipation in adult males (column 7 in Table 1). Adult male EMCs could not be quantitatively measured due to the frequent activity of the mating muscles in the tail region (see Materials and Methods). We therefore qualitatively assessed intestinal muscle contractions in adult males (as described above, the anal depressor muscle no longer functions in defecation in the adult male). In general, we found that adult male intestinal muscle contractions were absent from the motor program approximately as often as seen in larval males and hermaphrodites of the same genotype (data not shown; *exp-2* was the single exception, see Materials and Methods). For example, *aex-1* adult males were defective for most intestinal muscle contractions, yet this defect conferred no Con phenotype. We conclude that contraction of the intestinal muscles in the adult male is under the same genetic control as in the hermaphrodite and larval male, but that this contraction is not required to prevent constipation in adult males. These observations indicate that adult males use a strategy for defecation different from that of hermaphrodites and larval males, in accord with the developmental changes seen in the larval to adult male molt.

Strikingly, mutations in only 2 of the 13 genes necessary for hermaphrodite EMC, *unc-25* and *unc-47*, caused a Con phenotype in adult males (Table 1, see also Fig. 2). Two observations indicate that this result is not explained by these *unc-25* and *unc-47* mutations causing a generally more severe Con phenotype than other mutations. First, all of the mutations that cause the most severe defects in the hermaphrodite and larval male cause no discernible Con phenotype in the adult male (Table 1; see above). Second, additional alleles of each gene were analyzed, and no differences among these alleles were observed (Table 1).

unc-25 and *unc-47* are required for function of GABA (McIntire et al., 1993a). GABA is a putative excitatory transmitter for hermaphrodite EMCs (Thomas, 1990; McIntire et al., 1993a) and an inhibitory transmitter for locomotion and head-foraging movements (McIntire et al., 1993b). Mutations in three additional genes, *unc-30, unc-46*, and *unc-49*, have shrinker Unc locomotion phenotypes very similar to those of *unc-25* and *unc-47*, indicating a defect in the GABA-dependent reciprocal inhibition process (McIntire et al., 1993b). Neither *unc-46* nor *unc-49* hermaphrodites were significantly Con, though *unc-46* hermaphrodites had a moderate EMC defect (Table 1; McIntire et al., 1993a). In contrast, we observed that *unc-46* and *unc-49* mutant adult males were Con, although less so than *unc-25* and *unc-47* adult males (Table 1, see also Fig. 2). These observations suggest that GABA is required for adult male defecation. Throughout this study, we used mutations in *unc-30* as a control, since both hermaphrodites and males carrying mutations in *unc-30* were shrinker Unc but not Con, and both appeared to have normal EMCs. It has been shown that *unc-30* is required for the

Table 2. Phenotype of anal depressor kills and sphincter kills in larval and adult males

Genotype	No kill	Control kill (Anal depressor)		Sphincter kill	
	Adult males	Larval males	Adult males	Larval males	Adult males
+	non-Con	sev Con (2/2)	non-Con (2/2)	non-Con (5/5)	non-Con (5/5)
unc-25(e156)	sev Con	sev Con (3/3)	sev Con (3/3)	mod Con (6/6)	non-Con (6/6)
unc-47(e307)	sev Con	sev Con (2/2)	sev Con (2/2)	mod Con (5/5)	non-Con (5/5)
unc-46(e177)	mod Con	sev Con (3/3)	mod Con (3/3)	non-Con (5/5)	non-Con (5/5)

Phenotypes were scored as described in Materials and Methods (mod = moderate, sev = severe). All strains shown have a *him* mutation (see Materials and Methods). Numbers in parentheses indicate the number of animals tested. Each phenotype was based on at least three observations of each animal, separated by at least 0.5 hr. In addition to qualitative scoring of the Con phenotype, defecation cycles were observed in at least two animals for each test.

normal differentiation of the type-D GABAergic motor neurons used in locomotion, but not for the AVL and DVB GABAergic motor neurons that control enteric muscle contraction (McIntire et al., 1993a). *unc-30* encodes a homeodomain protein, presumably used to specify the fates of the type-D motor neurons (Jin, Hoskins, and Horvitz, 1994).

Based upon these phenotypic observations, we hypothesized that the genes required for defecation fall into three classes. First, 11 genes are required to prevent hermaphrodite, but not male, constipation. We hypothesized that these genes are required either specifically for excitatory GABA function or for the function of AVL and DVB, the hermaphrodite motor neurons for EMC. Second, *unc-25* and *unc-47* are required for both male and hermaphrodite defecation because they are necessary for all GABA neurotransmission (Thomas, 1990; McIntire et al., 1993a). Third, *unc-46* and *unc-49* function specifically in inhibitory GABA neurotransmission and thus are required only to relax the adult male sphincter muscle. These hypotheses made several specific predictions that are tested in the next sections.

Evidence that GABA acts to relax the male sphincter

Our hypotheses predicted that the anal sphincter muscle would be required in the wild-type adult male to seal the intestine between defecations. To test this, the sphincter muscle was killed with a laser microbeam in wild-type larval males. Most of such animals were viable and not Con as larvae or adults (Table 2), though occasional animals fatally herniated at the anus, usually during the L4 molt. Presumably, the intestinal seal formed by the anal sphincter is not essential for viability. As predicted, males lacking a sphincter muscle leaked intestinal contents at variable points throughout the defecation cycle (not necessarily accompanied by defecation muscle contractions), whereas males with intact sphincters expelled gut contents only during the appropriate time of motor program activation. The fact that a sphincter-dependent seal of the intestine is present during the intercycle period suggests that the sphincter muscle is tonically contracted in order to seal the anus.

Our hypotheses also predicted that the anal sphincter muscle would be required for the Con phenotype of *unc-25*, *unc-46*, and *unc-47* adult males. As predicted, when the sphincter muscle was killed in *unc-25*, *unc-46*, or *unc-47* animals the adult male Con phenotype was suppressed (Table 2) and animals leaked intermittently. These animals still fed normally, had an otherwise normal defecation motor program, and survived for at least several days as adults. As controls, we killed the anal depressor muscle (which is attached to a mating spicule in the adult male) in wild-type and mutant males. As expected, this kill did not relieve the defecation defect in mutant males, nor did it cause a defecation defect in adult wild-type males. The parallel between larval male and hermaphrodite defecation was shown by the fact that wild-type larval males without an anal depressor muscle were severely Con (Table 2), as was seen in hermaphrodites without an anal depressor (data not shown). These observations demonstrate that the sphincter muscle is necessary for expression of the Con phenotype in *unc-25*, *unc-46*, and *unc-47* adult males, and that the depressor muscle is not required for adult male defecation. We did not perform these experiments with *unc-49* males because we judged that their weaker Con phenotype would make results difficult to interpret.

Pharmacological rescue of the Con mutant phenotype

Our hypotheses also predicted that the GABA agonist muscimol would act to relax the anal sphincter muscle. We tested whether muscimol would rescue the Con phenotype of *unc-25*, *unc-47*, or *unc-46* adult males. We found that mutant adult males placed on plates containing 10 mM muscimol became substantially less Con (Table 3*A*). In addition, these animals were observed to leak gut contents from the anus, as did wild-type animals exposed to muscimol (similar to adult males without the anal sphincter muscle). Muscimol causes paralysis of animals within 30 min of addition (data not shown), and rescues the Con phenotype of mutant animals with a similar time course. The Con phenotype was not entirely rescued by muscimol, perhaps because addition of muscimol decreased the frequency and strength of motor program activation, and thus the gut was not completely cleared. One caveat to this experiment is that pharyngeal pumping is suppressed by the addition of muscimol, possibly affecting the degree of constipation. However, the severe constipation of *egl-2(n693)* and *aex-1(sa3)* hermaphrodites was not suppressed by muscimol, indicating that paralysis of the pharynx need not decrease the severity of the Con phenotype. After 2 hr on muscimol, the Con phenotype in mutant males had returned, presumably reflecting the desensitization of the animals to the drug (Table 3*A*). The muscimol-induced paralysis of body-wall and pharyngeal muscles desensitizes with a similar time course (data not shown).

Neurons necessary for male defecation

One observation that was potentially incompatible with our hypothesis that the adult male sphincter is relaxed by GABA was the fact that *aex* mutant adult males were not Con. *aex* mutants (*a*Boc and *ex*pulsion defective) may eliminate function of AVL

Table 3. Pharmacological rescue of mutant phenotypes

A

Genotype	Muscimol treatment		
	0 hr	0.5 hr → 1.5 hr	2+ hr
+	non-Con	non-Con	non-Con
unc-25(e156)	severe Con	weak Con	severe Con
unc-47(e307)	severe Con	weak Con	severe Con
unc-46(e177)	moderate Con	weak Con	moderate Con

B

Genotype	Adult males		Hermaphrodites	
	non-GABA plates	GABA plates	non-GABA plates	GABA plates
+	non-Con	non-Con	non-Con	non-Con
unc-25(e156)	severe Con	weak Con	moderate Con	non-Con
unc-47(e307)	severe Con	severe Con	moderate Con	moderate Con
unc-46(e177)	moderate Con	moderate Con	non-Con	non-Con

All strains shown have *him* mutations (see Materials and Methods). *A*, Muscimol rescue of mutant male constipation. Twenty adult males for each genotype were placed on plates with 10 mM muscimol and scored as described in Materials and Methods. *B*, GABA rescue of *unc-25* male constipation. Ten adult males of each genotype were placed on plates with 30 mM GABA and scored as described in Materials and Methods. The EMC rescue of *unc-25* hermaphrodites was confirmed directly by the observation of defecation cycles (data not shown).

and DVB, the motor neurons for aBoc and expulsion (Thomas, 1990; McIntire et al., 1993a,b), and GABA from these neurons is required for hermaphrodite EMCs (McIntire et al. 1993a,b). Our observations suggest that GABA from these neurons is also required for larval male EMCs (Table 1). To test directly the importance of AVL and DVB for larval and adult male defecation, we killed AVL and DVB in eight wild-type males. These animals were phenotypically similar to mutants defective in *aex* genes, in that both larval and adult males lacked aBoc and intestinal muscle contractions, but only larvae were Con (Table 4). These observations further corroborate the close parallels between the physical lesion of AVL and DVB and the genetic lesion of the *aex* mutants, suggesting that the *aex* mutations functionally eliminate AVL and DVB. The fact that AVL and DVB are not required for adult male sphincter relaxation may explain why *aex* mutants are not defective in this behavior. We also conclude that GABA from a source other than AVL and DVB is required to relax the adult male sphincter muscle, since *unc-25* (GAD defective) but not *aex* or AVL/DVB-killed adult males are Con (Table 4). We hypothesize that another GABAergic neuron or neurons in the adult male mediate relaxation of the sphincter. These additional GABAergic neuron(s) may be redundant with AVL and DVB for sphincter relaxation, but they are not sufficient to induce intestinal or aBoc muscle contraction without AVL and DVB. Consistent with this conclusion, a number of additional, as yet unidentified GABAergic neurons have been observed in the adult male tail (E. Jorgensen and H. R. Horvitz, personal communication; also see Laser kills in Materials and Methods).

Evidence for unidentified components of inhibitory GABA neurotransmission

While *unc-25* and *unc-47* adult males (defective for presynaptic GABA function) were severely Con, *unc-46* and *unc-49* adult males were only moderately or mildly defective, respectively (Table 1). In addition, we observed that the body posture of *unc-49* animals was more loopy than that of the other shrinker Uncs, with deeper flexures (more like the wild type), which can be interpreted as an incomplete defect in muscle response to reciprocal inhibition. The typical body posture of *unc-25*, *unc-46*, *unc-47*, and *unc-30* mutant animals was smooth (without deep flexures), which is attributed to the complete elimination of reciprocal inhibition of the body-wall muscles (McIntire et al., 1993b). We observed these phenotypic differences for every available allele of these genes in blind tests. We hypothesize that the weaker Con phenotypes of *unc-46* and *unc-49* adult males, and the weaker locomotion phenotype of *unc-49* animals, reflect a residual inhibitory GABA response in these mutants.

We tested this hypothesis by building double mutants between mutations in the presynaptic, GABA-defective genes *unc-25* and

Table 4. Laser kills of AVL and DVB motor neurons in males and hermaphrodites

Animals	Larval male phenotype	Adult male phenotype	Adult hermaphrodite phenotype
aex mutants (many)	severe Con	non-Con	severe Con
AVL^-, DVB^- (8)	severe Con	non-Con	severe Con
unc-25 or *unc-47* (many)	moderate Con	severe Con	moderate Con

The Con phenotype of larval and adult Aex animals and AVL^- DVB^- animals was examined. The number in parentheses indicates the number of animals examined. AVL and DVB were killed in eight males and eight hermaphrodites.

unc-47, and mutations in *unc-46* and *unc-49*. We found that such double mutant adult males were as severely Con as the *unc-25* and *unc-47* single mutants. We also found that both male and hermaphrodite *unc-25* and *unc-47* double mutants with *unc-49* had the same smooth body posture of *unc-25* and *unc-47* single mutants. Therefore, the weaker Con phenotypes of *unc-46* and *unc-49* mutant males, and the weaker locomotion phenotype of *unc-49* animals are GABA dependent. We conclude that the *unc-49* and *unc-46* adult male sphincter muscles retain some GABA responsiveness.

An interesting possibility is that *unc-46* and *unc-49* are partially redundant for GABA response in the adult male sphincter muscle, and that eliminating both of these gene products would confer a more severe adult male Con phenotype. Therefore, we constructed the *unc-49*; *unc-46 him-5* triple mutant, and found that adult males were only as Con as *unc-46* adult males, as confirmed by a blind experiment. This result demonstrates that *unc-46* and *unc-49* are not redundant for adult male sphincter muscle relaxation, and suggests that other, as yet unidentified genes are responsible for the residual response of the sphincter to GABA.

The role of GABA in development of the adult male sphincter neuromuscular junction

GABA has been proposed to have a neurotrophic role in the vertebrate nervous system (Meier et al., 1991; Messersmith and Radburn, 1993). The *unc-25* gene product is required for the production of GABA (McIntire et al., 1993a), and has been shown to encode glutamic acid decarboxylase (GAD), the biosynthetic enzyme for GABA (Y. Jin and H. R. Horvitz, personal communication). Previous experiments showed that exogenous GABA rescued the hermaphrodite EMC defect of *unc-25* animals, but not the EMC defect of *unc-47* hermaphrodites. It was concluded that GABA is not required for development of the excitatory neuromuscular junction between AVL/DVB and the enteric muscles (McIntire et al., 1993b). However, GABA did not rescue the locomotion defect of *unc-25* animals. One interpretation of this result is that GABA plays a developmental role in the formation of the neuromuscular junction between type-D motor neurons and body-wall muscles. Alternatively, the type-D motor neurons in *C. elegans* may be incapable of GABA uptake, like the corresponding neurons in *Ascaris* (Guastella and Stretton, 1991). We tested whether GABA has a role in the development of the inhibitory GABA neuromuscular junction at the adult male sphincter. When exogenous GABA was added to mutant adult males, we observed partial rescue of the *unc-25* (GAD) adult male Con phenotype, but no rescue of the *unc-46* and *unc-47* adult male Con phenotypes (Table 3*B*). Since sphincter muscle relaxation can be partially restored after development of the nervous system, we conclude that GABA is not essential for the development of this neuromuscular junction. These results also suggest that the unidentified neuron(s) required for adult male sphincter relaxation are capable of GABA uptake.

Discussion

We have described sexual dimorphism for the defecation behavior in the nematode *C. elegans*. Hermaphrodite animals expel gut contents using excitation of the enteric muscles by GABA (Thomas, 1990; McIntire et al., 1993a,b). Larval males use this same mechanism, but undergo a dramatic morphological change at the end of the last larval stage (Sulston et al., 1980). By examining mutants defective in GABA neurotransmission, we show that this morphological change is accompanied by a change in the response of the anal sphincter muscle to GABA (from excitatory to inhibitory). It is probable that the sphincter muscle at this time undergoes a significant change in the expression of genes regulating the excitability of the cell. We speculate that this is at least partly at the level of receptor expression. In addition, the sphincter muscle may begin to express the genes required for its tonic contraction, though it is not clear whether the tonic contraction of the sphincter muscle is adult male specific. Since the phenotypes involved are easily scored and the behaviors are not essential for viability, this system presents an opportunity for detailed analysis of a developmental switch in the excitable state of a cell.

We have also gained additional insight into the role of GABA in the function of the GABAergic synapse. First, our results corroborate an inhibitory role for GABA at the *C. elegans* neuromuscular junction. Second, our results support the model of a presynaptic role of *unc-25* and *unc-47* and a postsynaptic inhibitory role of *unc-49* (McIntire et al., 1993a). In contrast, our results tend not to support a proposed role for *unc-46* in presynaptic GABA function (McIntire et al., 1993a). If this were so, one might expect an EMC defect in *unc-46* hermaphrodites to be as severe as that of *unc-25* and *unc-47*, since the locomotion defects of mutants in these genes seem indistinguishable. However, this was not the case. Also, *unc-46* mutant animals showed no perturbation anti-GABA immunofluorescence as was seen in *unc-25* and *unc-47* animals (McIntire et al., 1993a). We consider the alternative model that *unc-46* is required postsynaptically, perhaps for both excitation and inhibition, with a more stringent requirement in inhibition than in excitation.

We also present evidence of residual inhibitory GABA function in *unc-46* and *unc-49* adult male sphincter muscle relaxation, and in *unc-49* reciprocal inhibition of body-wall muscles. The fact that *unc-46* and *unc-49* are not redundant for sphincter muscle relaxation, in conjunction with the fact that these weaker phenotypes are GABA dependent, suggests that components of postsynaptic inhibitory GABA action other than *unc-46* and *unc-49* are sufficient to provide some response to GABA. An order of phenotypic severity similar to that observed for adult male constipation exists for the GABA-mediated head-foraging movements. *unc-25* and *unc-47* mutants are strongly defective, *unc-46* mutants are weakly defective, and *unc-49* mutants are not detectably defective (McIntire et al., 1993a). This leads us to speculate that unknown components of postsynaptic inhibitory GABA function may also exist for the head-foraging behavior.

The rescue of *unc-25* EMC defects (McIntire et al., 1993a) and adult male sphincter relaxation defects (this study) by exogenously added GABA suggests that functional excitatory and inhibitory GABA neuromuscular junctions can form in the absence of detectable GABA. However, in contrast to the complete rescue of the EMC defect, the rescue of the *unc-25* adult male Con defect by exogenous GABA was partial, leaving open the possibility that GABA plays some role in the development of an inhibitory synapse.

In conclusion, we have extended the analysis of the function of several genes in the GABAergic synapse and the role of GABA in the development of the synapse. We have established that the defecation behavior in *C. elegans* is sexually dimorphic, and that an intriguing change in the excitability of the anal sphincter muscle occurs during male development.

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