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The *Caenorhabditis elegans lim-6* LIM homeobox gene regulates neurite outgrowth and function of particular GABAergic neurons

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SUMMARY

We describe here the functional analysis of the *C. elegans* LIM homeobox gene *lim-6*, the ortholog of the mammalian *Lmx-1a* and *b* genes that regulate limb, CNS, kidney and eye development. *lim-6* is expressed in a small number of sensory-, inter- and motorneurons, in epithelial cells of the uterus and in the excretory system. Loss of *lim-6* function affects late events in the differentiation of two classes of GABAergic motorneurons which control rhythmic enteric muscle contraction. *lim-6* is required to specify the correct axon morphology of these neurons and also regulates expression of glutamic acid decarboxylase, the rate limiting enzyme of GABA synthesis in these neurons. Moreover, *lim-6* gene activity and GABA signaling regulate neuroendocrine outputs of the nervous system. In the chemosensory system *lim-6* regulates the asymmetric expression of a probable chemosensory receptor. *lim-6* is also required in epithelial cells for uterine morphogenesis. We compare the function of *lim-6* to those of other LIM homeobox genes in *C. elegans* and suggest that LIM homeobox genes share the common theme of controlling terminal neural differentiation steps that when disrupted lead to specific neuroanatomical and neural function defects.

Key words: LIM homeobox, *Caenorhabditis elegans*, Axogenesis, GABA, Uterus

INTRODUCTION

Dedicated neural circuits which mediate specific behaviors have been defined in *Caenorhabditis elegans*. The description of the complete neural connectivity of *C. elegans* as well as the behavioral defects induced by laser or genetic ablation of specific neurons has led to the definition of neural circuits that mediate, for example, mechanosensory, locomotory, thermosensory and rhythmic defecation behaviors (Avery and Thomas, 1997; Chalfie et al., 1985; Hart et al., 1995; McIntire et al., 1993a,b; Miller et al., 1992; Mori and Ohshima, 1995; Thomas, 1990; White et al., 1992). Not surprisingly, specific neurotransmitter synthesis, synaptic signaling, and receptor signaling pathways have been implicated in the function of most of the pathways (Bargmann and Kaplan, 1998). But genetic analysis has also revealed that transcriptional control mechanisms regulate both early stages of neural cell fate determination and events late in neural differentiation and in the mature nervous system. For example, the *unc-4* homeobox gene determines the pattern of interneuron connectivity to motor neurons from its site of expression within motorneurons (Miller et al., 1992; White et al., 1992), and the *unc-55* nuclear hormone receptor is required for the correct wiring of VD type motorneurons (Zhou and Walthall, 1998). The analysis of several LIM homeobox genes also revealed that they regulate terminal steps in the differentiation of specific components of the mechanosensory (Way and Chalfie, 1988, 1989) or thermosensory circuits (Hobert et al., 1998, 1997).

LIM homeobox genes represent a large subfamily of homeobox genes; although they act in a variety of different developmental contexts, a common theme is their regulatory role in neural development (Dawid et al., 1998). In *Drosophila*, a requirement for LIM homeobox genes in axonal fasciculation, pathfinding and neurotransmitter choice was demonstrated for the *apterous*, *lim3* and *islet* genes, respectively (Benveniste et al., 1998; Lundgren et al., 1995; Thor and Thomas, 1997; Thor et al., 1999). In vertebrates, LIM homeobox genes appear to play a similar role in terminal steps of neural differentiation, although it is clear that they have also been recruited to several additional functions outside the nervous system, such as early embryonic inductions or limb patterning (Dawid et al., 1998). In the vertebrate spinal cord, the temporal and spatial patterns of LIM homeobox gene expression implicates them in control of motorneuron differentiation (Tsuchida et al., 1994). Loss of function of the mouse *Isl-1* gene leads to a failure of motorneuron differentiation (Pfaff et al., 1996), *Lhx-3* and *Lhx-4* determine motorneuron subtype identities (Sharma et al., 1998) whereas loss of function of the *Lhx-2* gene causes a variety of brain developmental defects (Porter et al., 1997).

Here we analyse the function of the *C. elegans* LIM homeobox gene *lim-6* and compare it with its mammalian orthologs *Lmx-1a* and *Lmx-1b*. The Lmx-1a LIM homeodomain protein was originally identified as a regulator of insulin gene expression (German et al., 1992). The closely related *Lmx-1b* gene (100% identity in homeodomain) is expressed in the central nervous system, including regions of the hindbrain and in particular domains in the spinal cord (Chen et al., 1998; Matise and Joyner, 1997; Riddle et al., 1995). *Lmx-1b* mutant mice display CNS patterning defects (R. Johnson, personal communication). However, the analysis of *Lmx-1b* has so far been focused on its function outside the nervous system, mainly in limb and kidney development, where it is required for specific patterning and differentiation events (Chen et al., 1998; Dreyer et al., 1998; Riddle et al., 1995; Vogel et al., 1995). Moreover, Nail Patella Syndrome, an autosomal dominant disorder characterized by renal defects and various forms of skin and limb hypoplasias is caused by a heterozygous null mutation in human *Lmx-1b* (Dreyer et al., 1998). Thus the normal two copy gene dosage of *Lmx-1b* is essential for these patterning events. We show that the single *C. elegans Lmx-1a/b* ortholog *lim-6* is expressed in particular neurons, many of which are GABAergic, in the excretory system and in epithelial cells of the uterus. Using a *lim-6* deletion mutant we show that *lim-6* is required for the terminal differentiation of sensory- and motorneurons, and for morphological aspects of uterine development. The behavioral and neuroendocrine defects of the *lim-6* mutant animals are mostly due to defects in maturation of GABAergic neurons that depend on *lim-6* function. The neuronal defects of *lim-6* mutant animals resemble those of other LIM homeobox genes in *C. elegans*, thus pointing to a common theme in the function of this gene family in *C. elegans*. Moreover, the sites of *Lmx-1* and *lim-6* expression also suggest that specific functions of these genes may be conserved during evolution.

MATERIALS AND METHODS

Strains and transgenic lines

Wild type were N2 Bristol, *lim-6(nr2073), unc-25(e156), unc-30(e191), daf-7(e1372), unc-1(e719), unc-33(e204), unc-36(e251), unc-73(e936), unc-76(e911), egl-38(n578), lin-11(n389), lin-17(n671), che-3(e1379), pha-1(e2123ts), aex-2(sa3), sem-4(n2654).*

Transgenic lines were either created in a wild-type background using pRF4 (*rol-6(su1000)*) at 100 ng/μl or *mec-7::GFP* at 50 ng/μl as an injection marker or in a *pha-1(e2123ts)* mutant background using pBX (*pha-1* wild-type expression construct) at 100 ng/μl as the rescuing construct for the *pha-1* lethality (Granato et al., 1994). The lines are:

pha-1(e2123ts); mgEx[lim-6r::GFP; pBX] (4 independent lines)
pha-1(e2123ts); mgEx[lim-6prom::GFP; pBX] (3 independent lines)
pha-1(e2123ts); mgEx[lim-6int3::GFP; pBX] (8 independent lines)
pha-1(e2123ts); mgEx[lim-6up::GFP; pBX] (1 line)
N2; mgEx[GADcompl::GFP; pRF4] (8 independent lines)
mgEx446, mgEx447 = N2; mgEx[fl-lim-6-2; mec-7::GFP] (2 independent lines)
mgEx408, mgEx409 = N2; mgEx[fl-lim-6VP16-2; mec-7::GFP] (2 independent lines)
juIs8: integrated pSC381 (Jin et al., 1999)
oxIs12: McIntire et al. (1997)
nuIs1: Hart et al. (1995)
nuIs26: S. Nurrish and J. Kaplan (unpublished)
lin-15(n765) X; adEx1262 [lin-15(+) gcy-5::GFP] (Yu et al., 1997)
lin-15(n765) X; adEx1297 [lin-15(+) gcy-6::GFP] (Yu et al., 1997)
lin-15(n765) X; adEx1288 [lin-15(+) gcy-7::GFP] (Yu et al., 1997)

Plasmid construction

Unless noted otherwise below, the expression plasmids were constructed by amplifying the respective sequences either from wild-type genomic DNA or the K03E6 cosmid and subcloning of the PCR products into the pPD95.75 expression vector. The respective constructs contain the following sequences (the numbering originates from an arbitrary numbering of the K03E6 cosmid; the ATG startcodon is at position 14254, the stop codon ends at position 10541).

lim-6r::GFP: bp 18290-10544
lim-6prom::GFP: bp 18290-12846
lim-6int3::GFP: bp 12785-11584
lim-6up::GFP: bp 20219-16193
fl-lim-6-2: bp 18290-10544 (+ *unc-54* 3′UTR, without GFP)
lim-6VP16-2: As *fl-lim-6-2*, except that the acidic activation domain of VP16 (aa 411-aa 490) was cloned into a *Bam*HI site that had been engineered at the C terminus of *lim-6*.

GADcompl::GFP: The *GAD/unc-25* gene is from YAC y37d8contig.03366. Its structure was predicted by Genefinder. The GFP fusion was generated by PCR amplification of the complete gene including 1846 bp of upstream sequence (up to the preceding predicted gene) and the full coding sequence. The amplification product was fused to GFP using a PCR fusion approach with overlapping PCR primers (O. H. and G. R., unpublished). The fusion amplification product was directly injected into adult animals. The sites of expression of this *GADcompl::GFP* construct was similar to the expression construct pSC381/juIs8 described by Jin et al. (1999).

Isolation and rescue of *lim-6(nr2073)*

lim-6(nr2073) was kindly provided by NemaPharm, Inc. It was isolated from an EMS-induced *C. elegans* deletion library which included approximately 400,000 mutagenized chromosomes using a PCR based sib-selection procedure (Jansen et al., 1997). The mutant strain was backcrossed five times. Its genotype was confirmed using a triplex PCR with 3 primers, two of which flank the deletion (yielding a PCR product of 0.9 kb on the deleted chromosome), the third is located within the deletion (yielding a PCR product of 1.1 kb on the wild-type chromosome). The exact deletion point was determined by DNA sequencing. All the phenotypes reported here were linked with the *lim-6* deletion *nr2073* through the five backcrosses. Moreover, the *nr2073* mutant phenotypes were complemented by a wild type *lim-6(+)* transgene and phenocopied by expression of a dominant negative *lim-6* gene product (described below). For the rescuing approach, we introduced two independent extrachromosomal arrays, *mgEx446* and *mgEx447*, which contain a wild-type copy of *lim-6*, termed *fl-lim-6-2* into wild-type animals. *fl-lim-6-2* contains the same genomic region as the GFP construct *lim-6r::GFP* shown in Table 1. To cross these arrays into *lim-6(nr2073)* the *mgEx446* and *mgEx447* transgenes were mated into *unc-1(e719)*. Then these animals were crossed with the *lim-6(nr2073)* strain and the closely linked *unc-1* chromosome was segregated away to yield a homozygous *lim-6* mutant carrying the transgene. The genotype was verified by PCR. The resultant strain *lim-6(nr2073) mgEx446/447* at least partially rescues the uterine defects and defecation defects. We noted that the same *mgEx446/447* arrays cause moderate defecation defects in some wild-type animals, suggesting that either overexpression of *lim-6* or promoter titration effects cause similar defects to its loss of function. This observation is a potential explanation for why not all *lim-6(nr2073) mgEx446* animals are completely rescued. We also constructed a *lim-6::VP16* expression construct (*lim-6VP16-2*), which by increasing transactivation function of LIM-6 was expected to enhance rescue (as

exemplified by *ttx-3::VP16*, O. H., I. Mori and G. R., unpublished data). Extrachromosomal arrays expressing the VP16 fusion constructs (*mgEx408* and *mgEx409*) indeed completely rescued the *lim-6* mutant phenotype; 20/20 animals showed intact uterine lumens; 19/20 animals restored *gcy-5* expression in ASER; 3/3 showed intact EMC cycles.

The second approach to show linkage of the defects with *lim-6* consisted of expressing a dominant negative derivative of *lim-6* in which we deleted from the *fl-lim-6-2* expression construct the C-terminal 72 amino acids (including parts of the homeodomain) and replaced it by GFP. Expression was similar to that seen for *lim-6r::GFP* (Table 1). We observed strong and highly penetrate constipation defects and brood size reductions, which thus phenocopies the *lim-6(nr2073)* defects.

Antibody staining

FMRFamide antibody staining was performed as previously described by Schinkmann and Li (1992).

Behavioral assays

The defecation assay was described by J. Thomas (1990). Well fed animals were scored as young adults. Muscle contractions of individual animals were scored using a stereomicroscope at 50x magnification. Each animal was observed for 5 to 15 defecation cycles. The time elapsing between the pBoc and the EMC and the intercycle time was recorded. An EMC was only scored as complete if the release of gut contents was observed.

Egg laying behavior was scored by picking single L4 staged animals to a fresh plate and transferring them every day to a fresh plate until no further progeny was produced. The total amount of progeny was counted.

Dauer arrest was scored with non-starved, non-crowded animals. Adult animals of the respective genotype were allowed to lay eggs for 4-12 hours; those eggs were kept at the respective temperature and the larvae hatching from these eggs were scored for dauer characteristics 3-7 days after the egg lay.

RESULTS

lim-6, the *C. elegans* homolog of Lmx-1a/b, is expressed in the developing nervous system, uterus, and excretory system

lim-6 is one of seven LIM homeobox genes revealed by the *C. elegans* genome sequence. Three of the LIM homeobox genes emerged from genetic analysis of development and neural function, *mec-3*, *lin-11*, and *ttx-3* (Freyd et al., 1990; Hobert et al., 1997; Way and Chalfie, 1988). Most of the *C. elegans* LIM homeobox genes detect clear vertebrate orthologs in the database (Fig. 1). Like all genes from the LIM homeobox class of transcription factors *lim-6* contains two Zinc-finger-like LIM domains and a DNA binding homeodomain (Dawid et al., 1998) (Fig. 1; GenBank accession no. U55375). The isolation of *lim-6* cDNAs and northern blot analysis confirmed that *lim-6* is an actively transcribed locus (data not shown). *lim-6* is highly related to the vertebrate *Lmx-1a/b* LIM homeobox genes (Chen et al., 1998; German et al., 1992; Iannotti et al., 1997). The degree of similarity between *lim-6* and the *Lmx-1* genes in the homeodomain as well as the LIM domains suggests that *lim-6* and *Lmx-1a/b* were derived from a common ancestor and are orthologous genes (Fig. 1B,C). *lim-6* is located on cosmid K03E6 which maps to a region of the X chromosome bearing no obvious genetic candidates for mutations in *lim-6*, based on the function of its vertebrate orthologs or its expression pattern in *C. elegans* (see below).

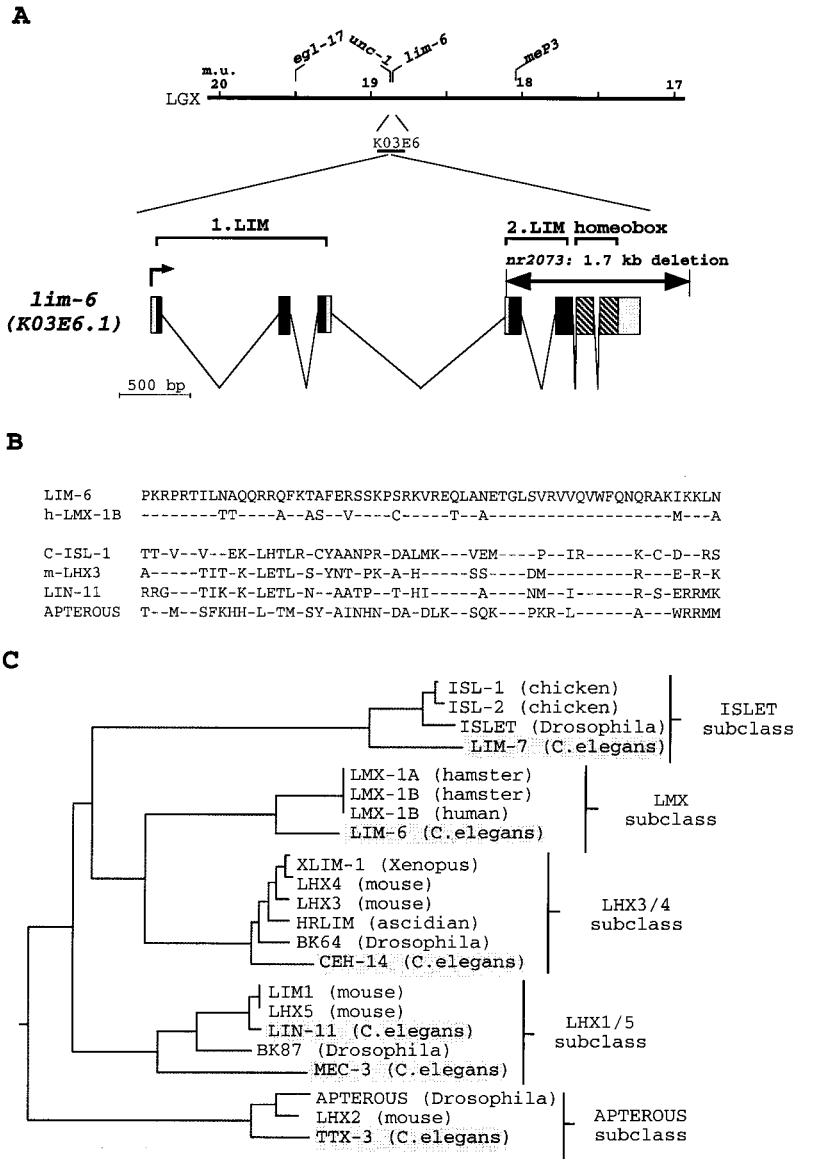
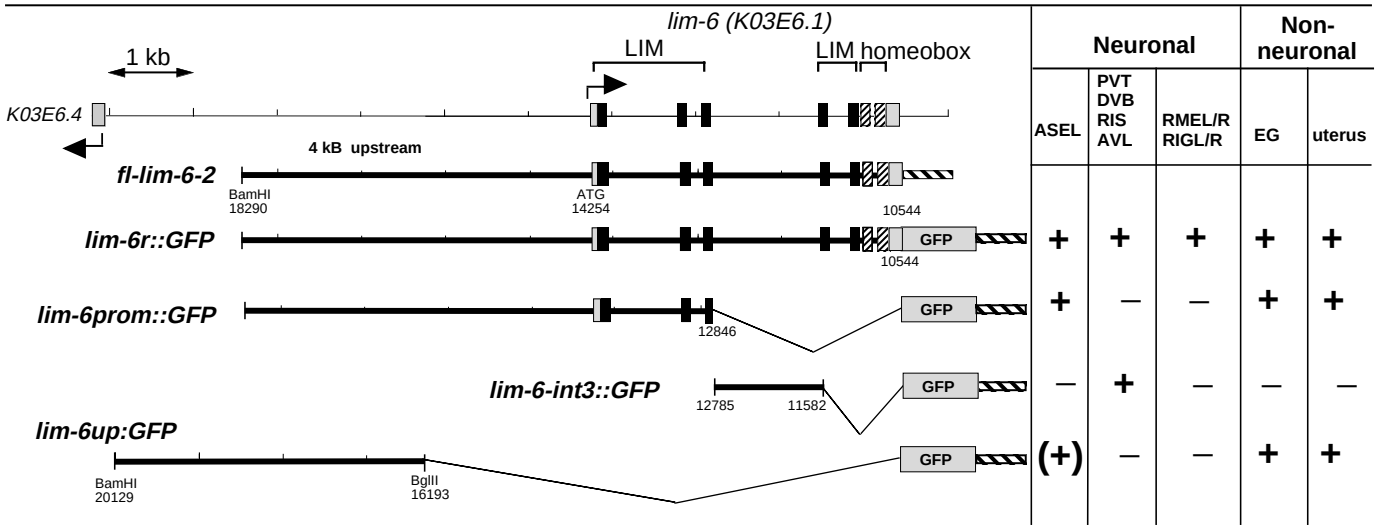


Fig. 1. Genomic structure of *lim-6* and sequence comparisons. (A) Predicted exon/intron structure of *lim-6*. The location of the *nr2073* deletion mutation is schematically shown; the deletion starts at amino acid position 65 and deletes the C-terminal 213 amino acids of the predicted LIM-6 protein. (B) Alignment of the homeodomains of representative proteins from different LIM homeodomain classes. The homeodomain sequences of all vertebrate Lmx-1 proteins identified so far are 100% identical. (C) Sequence relationship of representative members of several LIM homeobox gene classes. The dendrogram was constructed with the homeodomains of the respective proteins from a distance matrix created with the Jukes-Cantor method using the neighbor-joining method (GCG software package); Prd-type homeodomains were used as an outgroup to root the tree (not shown). *C. elegans* proteins are shaded. The accession number for LIM-6 is U55375. All other sequences were retrieved by their sequence names from GenBank.

Table 1. Expression of *lim-6* reporter gene constructs in neuronal and non-neuronal cells*



*Multiple independent transgenic lines were analyzed and found to have identical patterns of expression (see Materials and Methods).
(+) indicates very faint expression.
EG, excretory gland cells.
▨ = *unc-54* 3′UTR.

To reveal the expression pattern of *lim-6*, we fused *lim-6* genomic regions to green fluorescent protein (GFP) and generated transgenic lines bearing these fusion genes (Table 1). One of these reporter genes, *lim-6r::GFP*, contains precisely the same *lim-6* non-coding and coding regions as a genomic region that complements the null phenotype of *lim-6* (*fl-lim-6-2*; Table 1); moreover, most cells expressing *lim-6* reporter genes show defects in *lim-6* null mutant animals (described below). Thus, with the usual caveats of reporter gene expression studies, these observations indicate that the *lim-6r::GFP* constructs reveals authentic sites of endogenous *lim-6* expression.

The *lim-6r::GFP* fusion gene reveals expression in a restricted set of neurons, epithelial cells of the uterus and the excretory system (Table 1; Fig. 2). Reporter gene expression in the nervous system begins late in embryogenesis at about 300 minutes of development, which is after these neurons have been generated and while they initiate neurite outgrowth (Sulston, 1983). After hatching, *lim-6r::GFP* is expressed in one chemosensory neuron, ASEL, and in eight inter- and motorneurons (Fig. 2A-E). Most of these neurons are GABAergic, namely RMEL/R, AVL, RIS and DVB (McIntire et al., 1993b). RMEL/R, AVL and DVB are motorneurons which innervate specific sets of head and enteric muscles, respectively, whereas RIS is an interneuron (White et al., 1986). The other three neurons, PVT and RIGL/R, express the neuropeptide FMRFamide (Schinkmann and Li, 1992). The expression of *lim-6* in all neurons continues throughout adulthood, indicating that *lim-6* may continue to function in the mature nervous system.

The expression of *lim-6* in the uterus is dynamic. *lim-6* reporter gene constructs (*lim-6prom::GFP, lim-6up::GFP, lim-6r::GFP*, Table 1) start to be expressed in two uterine cells during the late L3/early L4 stage and the expression widens during the L4 stage to include the uv2 and uv3 cells, several uterine toroid (ut) cells, which form the lumen of the uterus and at least one cell type (sujn) of the spermatheca-uterine junction (Fig. 2F,G). Occasionally, weaker and less consistent expression can be observed in some cells of the distal side of the spermatheca, which connect the spermatheca to the rest of the somatic gonad. Expression of *lim-6* in the uterus is absent in adults suggesting that *lim-6* function is specific for the stages of uterine development. Consistent with these uterine cell identities, we could not observe any major changes of *lim-6* expression in animals that are mutant for genes that affect other cells of the developing uterus, such as *egl-38*, which affects the fate of the uv1 cells (Chamberlin et al., 1997) or *lin-11*, which affects the utse cells (Newman et al., 1996) (data not shown).

Considering the expression and function of vertebrate *Lmx-1b* in the kidney, it is intriguing that *lim-6* is expressed in the excretory system of *C. elegans*. The *C. elegans* excretory system is composed of four cell types (Nelson et al., 1983), one of which, the A-shaped excretory gland cell, expresses *lim-6* from late embryogenesis throughout adulthood (Fig. 2B).

Analysis of *lim-6* promoter deletion derivatives reveals that the *lim-6* promoter is composed of separable regulatory elements that are specific for individual cell types as shown in Table 1. The regulatory element for uterine and sensory neuron expression is localized upstream of the transcriptional start site, while the regulatory element for *lim-6* expression in several GABAergic neurons is located in the third intron.

Isolation of a *lim-6* deletion mutant

To determine the function of *lim-6*, a *lim-6* deletion mutant was isolated and phenotypically characterized. This deletion allele, *nr2073*, was isolated by NemaPharm, Inc. (Cambridge, MA) using a PCR screen of a mutagen induced deletion library. The *nr2073* allele harbors a 1.7 kb deletion in the *lim-6* gene which deletes three quarters of the *lim-6* coding region, including the second LIM domain and the homeodomain (Fig. 1A). Since

the DNA binding homeodomain represents the crucial functional feature of LIM homeodomain proteins, it is very likely that *nr2073* represents a strong loss-of-function allele, presumably a null allele. After extensive backcrossing of the mutant, we undertook a phenotypic analysis of *lim-6(nr2073)*. *lim-6(nr2073)* animals are viable, move normally and show no gross morphological abnormalities. However, the animals do not expel the gut contents normally and have reproductive defects. In addition, upon a detailed characterization of particular neural markers, *lim-6* mutant animals show defects in the pattern of gene expression and function of GABAergic neurons (see below). The defects of the mutant animals are summarized in Table 2. All of the cellular defects that we observe correspond to the defects expected based on the sites of *lim-6* expression described above. Both the neural and non-neural defects of *lim-6(nr2073)* could be rescued with a genomic *lim-6* transgene that contains all the regulatory elements described above in the *lim-6r::GFP* reporter gene construct (see Material and Methods). Moreover, most of the defects can be phenocopied by expressing, in wild-type animals, a dominant-negative version of the LIM-6 protein under its own promoter (data not shown; see Materials and Methods), further supporting that the *nr2073* deletion allele causes the defects that we observe.

Table 2. Summary of defects caused by the *lim-6* null mutation

Neural defects	Non-neural defects
EMC defects (Exp)	Egg-laying defective/low brood size (Egl)
DVB/AVL axonal defects	Uterine closure
Asymmetric sensory receptor regulation	
Foraging defective (Nup)	
Dauer defects (SynDaf-c)	
unc-25 misregulation	

Phenotypes were analyzed with a 5× backcrossed strain. Linkage of these defects to *lim-6* was demonstrated by rescue and by phenocopying of some of the defects with a dominant negative *lim-6* expression construct. The phenotypes also correlate with the sites of *lim-6* expression.

Behaviors mediated by GABAergic neural circuits are defective in *lim-6* mutants

lim-6 mutant animals display defective defecation behavior, as revealed by the bloated appearance of their gut (Fig. 3A). Periodic contractions of the enteric muscle are required to release the gut content (Avery and Thomas, 1997). The enteric muscle contractions (EMC) are regulated by the motorneurons AVL and DVB which innervate the enteric muscles (Liu and Thomas, 1994; McIntire et al., 1993b). The expression of *lim-6* in AVL and DVB and the bloated visible phenotype of the *lim-6* mutant animals suggested that *lim-6(nr2073)* mutants may have defects in the rhythmically executed EMCs triggered by AVL and DVB. In wild-type animals, the EMCs are intricately linked to two other muscle contraction steps, posterior body wall contractions (pBoc) and anterior body wall contractions (aBoc). The activation of these muscle contractions is precisely

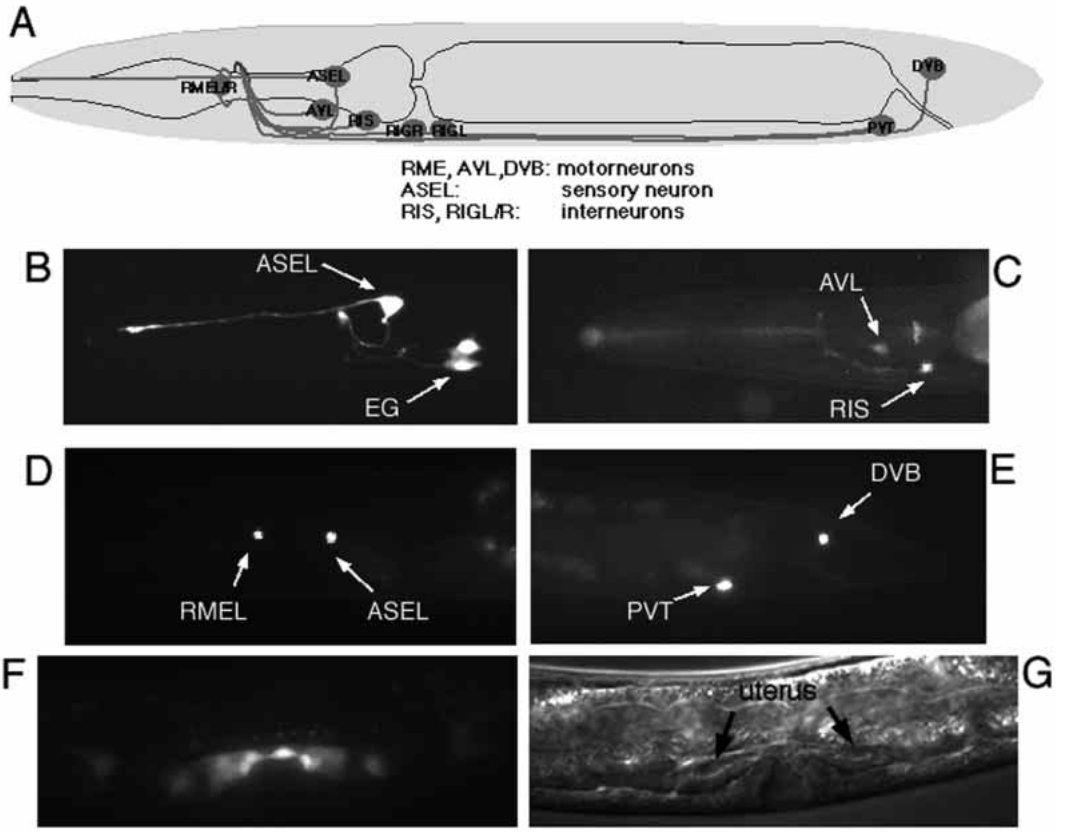


Fig. 2. *lim-6* reporter gene expression in neural and non-neural tissues. (A) Schematic drawing of the position and axonal morphology of all *lim-6*-expressing neurons. (B-F) Expression of the *lim-6* reporter gene constructs in late larval stages. (B) Expression of *lim-6prom::GFP* in ASEL and the excretory gland (EG) cells. Note that there are two EG cell bodies which send out processes which fuse at two different places to yield a binucleate cell. (C) Expression of *lim-6int3::GFP* in AVL and RIS. (D) Expression of *lim-6r::GFP* in the head and (E) in the tail. (F) Expression of *lim-6prom::GFP* in the uterus of an L4 stage animal. (G) DIC photomicrograph of the same animal as shown in F. Note that in B, C and F the GFP reporter gene is localized throughout the cell presumably due to the absence of a nuclear localization sequence in the only partial LIM-6 sequences. In D and E the presence all LIM-6 protein codings sequences reveals its nuclear localization. The sites of expression of the respective reporter gene constructs are summarized in Table 1.

timed and represents an ultradian rhythm that is temperature compensated and can be entrained by external stimuli (Avery and Thomas, 1997; Liu and Thomas, 1994; Thomas, 1990).

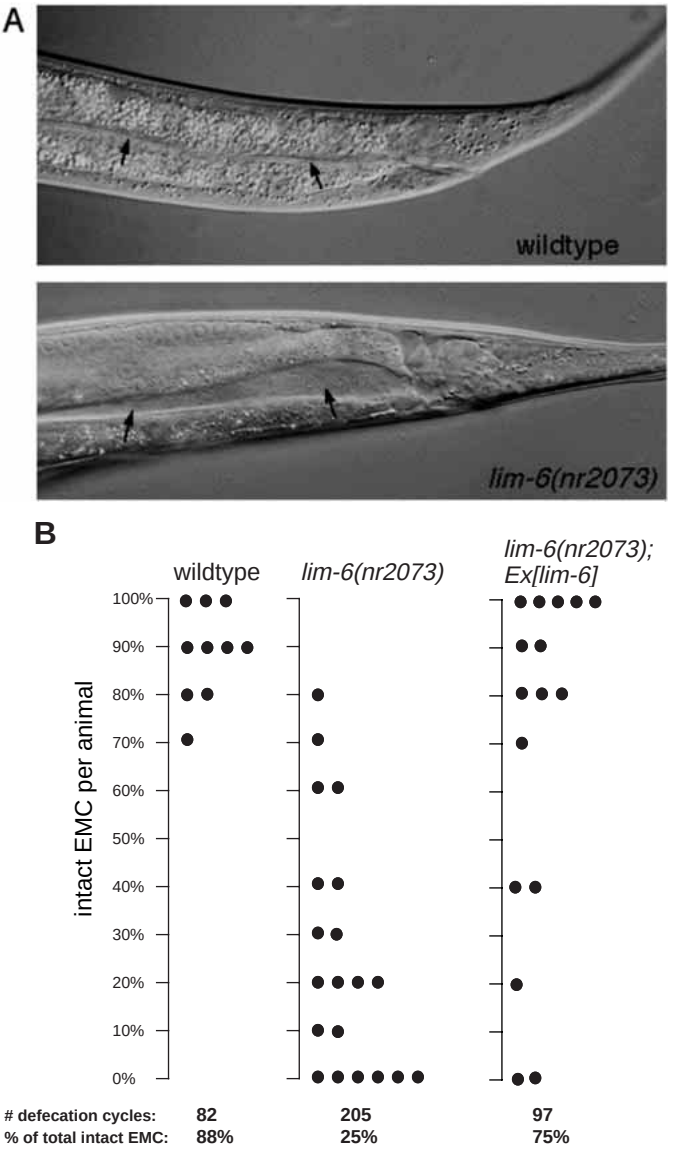


Fig. 3. Enteric muscle contractions are defective in *lim-6* mutant animals. (A) *lim-6* mutant animals are constipated. Micrographs were taken using differential interference contrast (DIC) microscopy under Nomarski settings. The black arrows point to the intestinal lumen, which is bloated in *lim-6(nr2073)* but not wild-type animals. (B) Analysis of the rhythmic defecation motor program. Each dot represents an animal tested. Each animals was assayed for 5-11 defecation cycles. The percentage EMC per animal indicates how often an intact EMC was observed per animal (e.g. if observed for 10 cycles, 9 intact EMCs will make a 90% value). 'Intact EMC' is defined as a EMC that occurred within 3-5 seconds following a pBoc. In *lim-6* mutant animals those 75% EMC that do not count as intact, include two classes: entirely absent (59%) or delayed (16%), meaning that it occurs sometime between 6-15 seconds after the pBoc. Also note that the *lim-6* mutant phenotype is not 100% penetrant. *lim-6* mutant animals were rescued with the extrachromosomal, *lim-6*-expressing array *mgEx446*. Animals that showed partial rescue as manifested by an enteric muscle contraction that was visible but did not lead to the expulsion of significant amounts of gut contents were omitted from the count.

The EMC step of this motor program is activated 3-5 seconds after the pBoc step of the motor program; after a 40-55 second intercycle time, the pBoc (and its closely linked aBoc step) is activated again and is again followed by the EMC and so on. While in wild-type animals the EMC is activated in 88% (n=82) of all cycles with a correct timing of 3-5 sec after the pBoc, in *lim-6* mutant animals the EMC is activated only in 25% (n=205) of the cycles; in 75% of the cycles, the EMC is either entirely absent (59%) or significantly delayed relative to the stereotyped pBoc step (16 %) (Fig. 3). The overall cycle length, i.e. the time between the intact pBoc steps, is not affected.

The GABAergic AVL and DVB neurons are essential for the muscle contraction step of the defecation cycle and proteins involved in GABAergic signaling, such as the GABA-synthesizing enzyme UNC-25 or the GABA transporter UNC-47 are expressed in these neurons and required for the execution of the cycle (Jin et al., 1999; Liu and Thomas, 1994; McIntire et al., 1993b, 1997; Thomas, 1990). The expression of *lim-6* in these neurons suggests that the focus of *lim-6* action in the defecation cycle is these neurons. The impact of *lim-6* loss of function on the neuroanatomy of AVL and DVB is consistent with this model (see below).

The expression of *lim-6* in RMEL/R, two out of a set of four GABAergic head motorneurons (RMEL, RMER, RMED, RMEV) required for head foraging (Hart et al., 1995) indicates a possible *lim-6* function in these neurons. Although we have not tested head foraging behavior in detail, a preliminary analysis suggests that *lim-6* mutant animals do indeed display abnormal nose movements (S. Nurrish, personal communication).

The GABAergic neurons that express LIM-6 are generated but their axons are defective in *lim-6* mutant animals

The *lim-6* mutant defects in GABAergic mediated behaviors suggests that the LIM-6 transcription factor mediates a developmental step in the differentiation of these neurons. We attempted to define the cellular basis of these defects by examining the neural fate and the neuroanatomy of *lim-6*-expressing neurons in the *lim-6* mutant animals. As a first step to monitor the cell fate of the *lim-6*-expressing neurons, we crossed the *lim-6int3::GFP* and *lim-6prom::GFP* reporter gene constructs (Table 1) into *lim-6* mutant animals. The expression of these reporter genes as well as other reporter genes described below is largely unaffected in the *lim-6* mutant, thus revealing that *lim-6*-expressing neurons are generated and continue to survive in the mutant (data not shown).

The cell fate of *lim-6*-expressing neurons was further analyzed using three differentiation markers of the neural types that express *lim-6*. First, we examined the expression of the GABA vesicular transporter *unc-47* (McIntire et al., 1997) in *lim-6(nr2073)* and found it to be correctly expressed in AVL and DVB, as well as the other GABAergic neurons that express *lim-6*, RMEL/R and RIS (Figs 4, 5). Thus, expression of the GABA transporter *unc-47* is not dependent on *lim-6* gene activity. Second, we examined the expression of the glutamate receptor *glr-1*, which is a cell fate marker for the DVB and RME motorneurons (Hart et al., 1995); like *lim-6*, *glr-1* is only expressed in the right and left types of the otherwise fourfold symmetric RME motorneurons. *lim-6* mutant animals

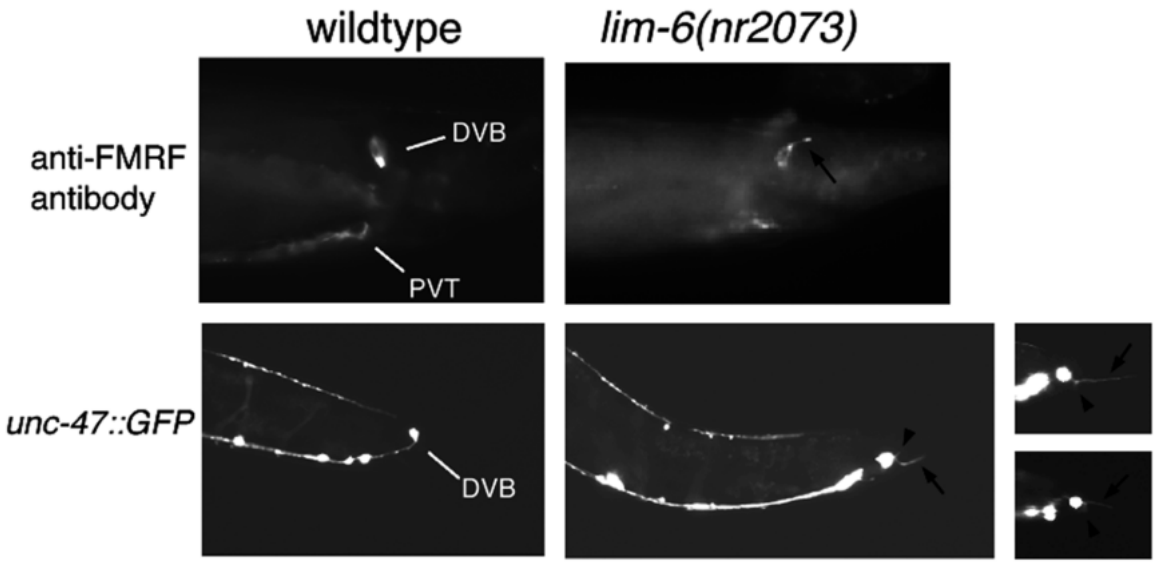


Fig. 4. Neural cell fate and axonal morphology of the DVB motorneuron. The tail region of adult animals are shown. Upper panels: Staining of wild-type and *lim-6(nr2073)* animals with anti-FMRFamide antibody. Lower panels: Expression of the integrated *unc-47::GFP*-expressing array oxIs12 in wild-type animals and after crossing into *lim-6(nr2073)* animals. oxIs12 is also expressed along the VNC in D-type motorneurons (McIntire et al., 1997).Small panels on right: Several cases of DVB axonal defects. Black arrows point to aberrant axonal sprouts. The black arrowheads in the lower panel point to the DVB motorneuron.

containing a chromosomally integrated *glr-1::GFP* reporter gene, *nuIs1*, show unaltered expression of this neurotransmitter receptor in RMEL/R and DVB compared to wild-type animals (data not shown). Third, we observed FMRFamide expression in *lim-6(nr2073)* animals using anti- FMRFamide antibodies. FMRFamides are neuromodulatory peptides expressed in a subset of neurons in *C. elegans*, including the DVB, PVT and RIGL/R neurons (Schinkmann and Li, 1992); FMRFamide expression is correctly specified in DVB, PVT and RIGL/R in *lim-6(nr2073)* mutant animals (Fig. 4). In summary, this analysis demonstrates that *lim-6* is not required for the *lim-6*-expressing neurons to be generated nor to assume certain aspects of their respective identities.

Close examination of the neuroanatomy of the DVB motorneuron, using the *unc-47::GFP* fusion gene reveals severe neuroanatomical defects in the *lim-6* mutant animals (Table 3). 73% of the animals display additional small axons of varying lengths that either emanate directly from the DVB cell body or from the main axonal process in close vicinity of the cell body (Fig. 4). These extra axons often have additional branches. This axonal morphology is very rarely observed in wild-type animals (Table 3). Due to the proximity of AVL to

Table 3. *lim-6* is required for DVB and AVL motorneuron axon morphology

	Normal axon morphology	Additional axons	*n*
DVB motorneuron: sprouting			
Wildtype	93%	7%	121
lim-6(nr2073)	27%	73%	51
unc-25(e156)	100%	0%	21
	Normal axon extension	**Abnormal extension***	***n***
DVB motorneuron: axon extension in ventral nerve cord (VNC)			
unc-30(e191)‡	100%	0%*	23
unc-30(e191); lim-6(nr2073)	48%	52%*	48
AVL motorneuron: axon extension			
unc-30(e191)‡	100%	0%§	40
unc-30(e191); lim-6(nr2073)	28%	72%§	29

DVB and AVL axon morphologies were visualized using the GABAergic neural marker *unc-47::GFP* provided on the integrated array *oxIs12* (McIntire et al., 1997).

*We observed 3 types of abnormal extensions in the VNC: main axon turns at its end or partly runs in an aberrant path outside the VNC (36%), the main axon terminates prematurely at less than half the way from the cell body to its normal termination position at the vulva (52%), the main axon extends beyond its normal termination position (12%).

‡*unc-30(e191)* mutant animals were used to eliminate *unc-47::GFP* expression in the ventral cord D motorneurons, which would obscure the visualization of the AVL and DVB axons in the ventral cord.

§We refer to the following cases as abnormal extension: No AVL axon visible in the ventral nerve cord (note that an AVL axon in the anterior third of the ventral nerve cord would be obscured by the RMEV motorneuron); prematurely terminated AVL axon in the ventral nerve cord; AVL axon in an aberrant path outside the ventral nerve cord. In *lim-6(nr2073)* the *unc-47::GFP* reporter gene is often less strongly expressed in AVL than in wildtype animals. We counted only those cases, in which the *unc-47::GFP* was roughly as strong in AVL as in DVB so that we would not miss the axon in the ventral nerve cord if it existed.

other neurons in the head ganglia, we were unable to observe whether AVL displays similar sprouting defects.

The main axonal trajectories of the DVB and AVL motorneurons are also affected by the *lim-6* null mutant. Both of these neurons are monopolar and extend their single axonal process along the ventral nerve cord (VNC) (White et al., 1986). The visualization of these axons by *unc-47::GFP* is obscured by the processes of the GABAergic D-type motorneurons in the VNC. However, the expression of *unc-47::GFP* in the D-type neurons can be disrupted without affecting expression in the other GABAergic neurons by a mutation in the *unc-30* homeobox gene. In *unc-30(e191)* animals, the only VNC axons that contain *unc-47::GFP* are those of the RMEV, AVL and DVB motorneurons (Basson and Horvitz, 1996; Jin et al., 1994), and the axon projections of these neurons are normal (Fig. 4). We observed, however, that in more than half of the *unc-30(e191); lim-6(nr2073)* mutant animals, the main axonal process of the DVB motorneuron displays abnormalities along the VNC (Fig. 5; Table 3). Either the axon does not terminate at its normal termination position at the vulva, or it takes an abnormal path outside the VNC, or it turns at the vulva and projects aberrantly (Fig. 5). The main axon of the AVL motorneuron, which runs along the entire length of the VNC was more difficult to examine since it is obscured in the anterior quarter of the VNC by the RMEV motorneuron, which also expresses *unc-47::GFP* (but not *lim-6*). Nevertheless, in the majority of *lim-6* mutant animals, the AVL motorneuron does not extend past the RMEV motorneuron (Table 3; Fig. 5). In summary, both the DVB and the AVL motorneurons require *lim-6* either to acquire or maintain intact axon morphology.

The PVT neuron is a presumptive guidepost of the VNC and is possibly involved in directing axons of the ventral cord (Wadsworth et al., 1996). Since PVT expresses *lim-6*, we examined whether a presumptive PVT function in VNC organization requires *lim-6*. VNC organization was monitored

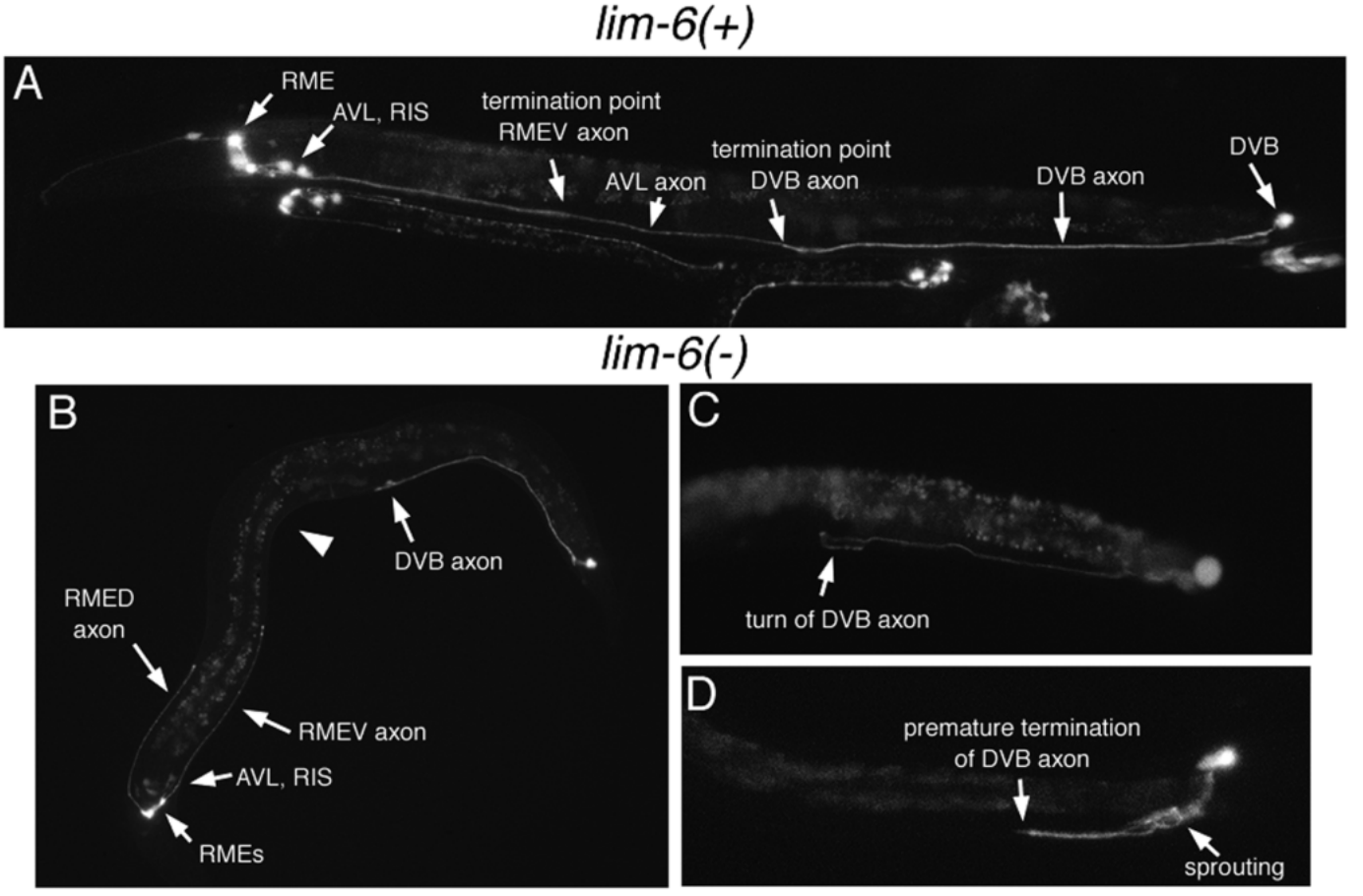


Fig. 5. DVB and AVL motorneuron axons in the ventral nerve cord are defective in *lim-6* mutant animals. GABAergic neurons are visualized with the integrated *unc-47::GFP* reporter gene construct *oxIs12*. In order to visualize the axonal processes of DVB and AVL in the VNC, this analysis was performed in *unc-30(e191)* mutant animals, which lack expression of *unc-47::GFP* in the D-type motorneurons of the VNC. (A) In the presence of wild-type *lim-6*, AVL extends a process along the VNC. Note that the DVB motorneuron terminates around the vulva. This contrasts the report of White et al., which describes that the DVB axons extends throughout the whole VNC into the nerve ring (White et al., 1986). We confirmed that the termination of the DVB neuron around the vulva is not due to the *unc-30* mutation by using a *unc-47::GFP* promoter deletion derivative, *juEx61* (a gift from Y. Jin) that is strongly expressed in DVB but not expressed in the D-type motorneurons and which reveals a similar termination point of the DVB axon around the vulva in wild-type animals (data not shown). (B) In the absence of *lim-6* gene activity, AVL terminates prematurely. The white triangle points to the region of the VNC in which the AVL axon can normally be seen. Note that *unc-47::GFP* expression is often slightly reduced; however, unlike the reduction observed with *unc-25::GFP* (see below) we never observed a complete absence. (C,D) Representative examples of termination defects of the DVB axon in the VNC. Note the additional sprouting seen in D.

using a *cat-1::GFP* reporter gene construct (S. Nurrish and J. Kaplan, personal communication) and a *lin-11::GFP* reporter gene construct (Hobert et al., 1998), each of which is expressed in axons of the right and left VNC. Transgenic *lim-6* mutant animals expressing these reporter genes revealed no obvious defect in VNC organization.

We conclude that *lim-6* is not necessary for neurons to be generated and to execute certain aspects of their differentiation program. However, their patterns of neurite outgrowth depend on *lim-6* gene activity.

lim-6 participates in the cell-type-specific regulation of glutamic acid decarboxylase

Glutamic acid decarboxylase (GAD) is the rate limiting enzyme for synthesis of the neurotransmitter γ-aminobutyric acid (GABA) and is encoded by the *C. elegans unc-25* gene (Jin et al., 1999). A translational fusion of the promoter and the first 13 codons of *unc-25* to GFP is expressed in all GABAergic neurons (Jin et al., 1999). The expression of this fusion gene in *lim-6(nr2073)* mutant animals is significantly decreased in the GABAergic neurons DVB and RIS and to a lesser extent in AVL (Fig. 6; Table 4). *unc-25*::GFP expression is normal in the RMEL/R motorneurons, which normally express *lim-6*, and in the RMED/V motorneuron and the D-type motorneurons of the VNC, neither of which express *lim-6*. A translational GFP fusion to *unc-25*, that encompasses the full coding sequence of *unc-25* (see Material and Methods) showed a similar pattern of expression in GABAergic neurons and was also affected by the absence of *lim-6* gene activity; while 96% (*n*=23) of wild-type animals show strong expression in DVB, only 28% (*n*=25) of *lim-6(nr2073)* mutant animals show equally strong expression, 44% show weak expression and 28% show no expression (data not shown). We conclude that *lim-6* contributes to the cell-type-specific regulation of *unc-25* expression in the DVB, RIS and AVL neurons but because the *lim-6* effects are quantitative, other factors also contribute to *unc-25* expression.

We tested whether the neuroanatomical defects of the DVB motorneuron in the *lim-6* mutant might be related to defects in GABAergic signaling, perhaps due to LIM-6 regulation of *unc-25* expression. For example, active DVB synaptic signaling may be required for normal axon morphology in a manner similar to other activity-dependent structural changes in the nervous system (Dahm and Landmesser, 1988, 1991). To observe DVB morphology in animals lacking GABA signaling, we observed DVB axon morphology using the *unc-47::GFP* reporter gene in GABA-deficient *unc-25* mutant. We found that even in the complete absence of GABA, the DVB motorneuron maintained its correct axon morphology (Table 3). An electron microscopical reconstruction of the synaptic connectivity of *unc-25* mutant animals has led to a similar conclusion (Jin et al., 1999).

It is conceivable that *lim-6* indirectly affects *unc-25* expression through the effects of *lim-6* on axonal pathfinding. However, this possibility is unlikely since several genes known to affect axonal pathfinding of GABAergic neurons, such as *unc-34*, *unc-71* or *unc-76*, do not affect GABA synthesis and thus *unc-25* expression (McIntire et al., 1992). Thus, it is most likely that the effect of *lim-6* on DVB axonal morphology represents a pathway that is parallel to its regulation of *unc-25* expression.

Dauer arrest is regulated by GABA and *lim-6*

Sensory inputs to *C. elegans* not only elicit motor outputs. As in other animals, there are endocrine outputs of the nervous system. For example, sensory detection of a pheromone

Table 4. *unc-25::GFP* (*juIs8*) expression in *lim-6* expressing neurons

	DVB	RIS	AVL	RMER/L
Wild type	100% (*n*=30)	100% (*n*=21)	100%* (*n*=21)	100% (*n*=12)
lim-6(nr2073)	10% (*n*=30)	22%‡ (*n*=23)	61%§ (*n*=23)	100% (*n*=18)

*Expression was not uniform; in 57% of the animals expression was strong, in the remainder the expression was weak but still significant.

‡In these 22% the expression was as strong as in wildtype, in the remaining 78% the expression ranged from very faint to entirely absent.

§These 61% include strong (22%) and weak (39%) expression, in the remainder the expression ranged from very faint to entirely absent.

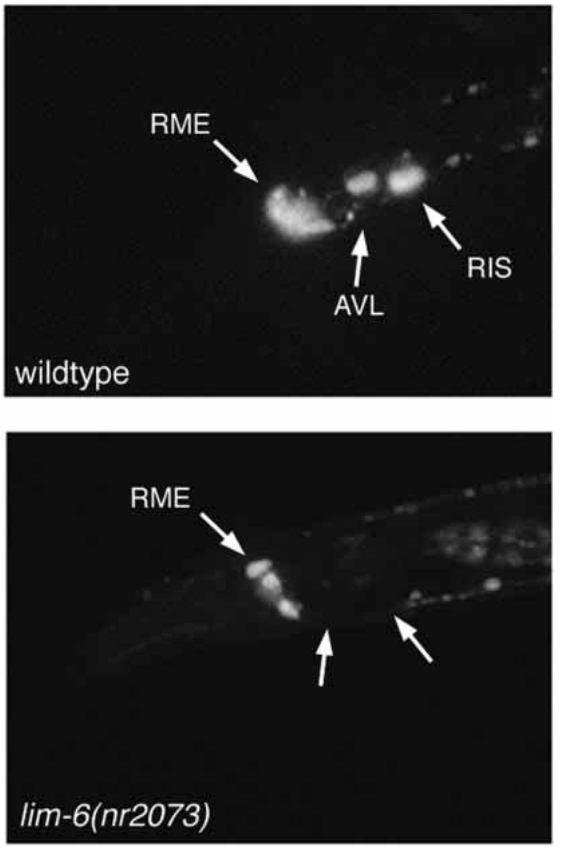


Fig. 6. *lim-6* affects expression of glutamic acid decarboxylase (*unc-25)*. Expression of the *unc-25::GFP* integrated reporter gene array *juIs8* was examined in wild-type (upper panel) and *lim-6(nr2073)* animals (lower panel). Representative example of *juIs8* expression in the AVL, RIS and the four RME neurons are shown. Quantification of the observations are shown in Table 4. Note that the effects of *lim-6* expression on *juIs8* were variable in AVL, as noted in Table 4. The effects of *lim-6* on *juIs8* expression in the DVB motorneuron are not shown but represented in Table 4. The expression of *juIs8* in the D-type motorneurons of the VNC is out of the plane of focus and thus not visible.

controls neuroendocrine outputs mediated by insulin-like and TGFβ-like signaling molecules that in turn regulate the metabolism of the whole animal and regulate whether development is arrested at the dauer diapause stage or continues to the reproductive adult stages (Riddle and Albert, 1997). A variety of sensory inputs are integrated by distinct neural circuits, for example food signals, crowding and temperature, to affect the neuroendocrine control of dauer stage entry (Riddle and Albert, 1997). We noted that unlike wild-type animals *lim-6(nr2073)* animals occasionally form dauers under conditions in which the animals are fairly crowded yet not completely starved; their pheromone responsiveness was unaffected, however (data not shown). As these dauer inducing conditions were difficult to reproduce, we investigated *lim-6* regulation of dauer arrest by sensitizing the genetic background to make the animals more prone to enter the dauer stage. We used a strain mutant for the *daf-7/TGFβ* gene, a neuroendocrine signaling molecule that synergizes with an insulin-like signaling cascade to repress entry into the dauer stage (Kimura et al., 1997; Ogg et al., 1997; Ren et al., 1996). In the absence of *daf-7/TGFβ* signaling, the animals are sensitized to enter the dauer stage; e.g. they now become hypersensitive to specific environmental stimuli such as changes in their ambient temperature (Riddle and Albert, 1997). Consistent with the enhanced dauer arrest under weakly dauer inducing conditions of the *lim-6* mutant alone, *lim-6(nr2073); daf-7(e1372)* double mutant animals are significantly enhanced for dauer arrest compared to *daf-7(e1372)* at 15˚C (Table 5).

In vertebrates, the neurotransmitter GABA is expressed in several neuroendocrine tissues and affects the release of insulin (Gu et al., 1993; Sorenson et al., 1991). Considering the expression of *lim-6* in GABAergic neurons we examined whether defects in GABAergic signaling could phenocopy the effects of *lim-6(nr2073)* on dauer arrest. *unc-25(e156)* mutant animals, which are GABA deficient (McIntire et al., 1993a), are neither defective in dauer arrest, nor do they enter the dauer stage constitutively (Table 5). However, like *lim-6(nr2073)*, the *unc-25(e156)* mutation strongly enhances *daf-7(e1372)* at 15˚C (Table 5). This effect of *unc-25* is not due to a requirement for *unc-25* in the D-type ventral cord motorneurons, since *unc-30*, a gene that abolishes GABAergic cell fate in the D-type motorneurons, but not in the other GABAergic neurons (Jin et al., 1994), does not enhance *daf-7(e1372)* (Table 5). The enhancement of *daf-7(e1372)* by *unc-25(e156)* is unlikely to be a non-specific secondary consequence of constipation, since neither *aex-2(sa3)* mutant animals, which are 100% EMC defective (Liu and Thomas, 1994) nor *sem-4(n2654)* mutant animals, which are 84% EMC defective (Basson and Horvitz, 1996) enhance *daf-7* induced dauer formation to a similar degree as *unc-25* (Table 5). We conclude that GABA signaling normally activates reproductive development or represses dauer arrest. Since the absence of functional D-type motorneurons in *unc-30* does not effect dauer formation, GABA regulation of dauer arrest is most likely mediated through either the RME, AVL, RIS or DVB neurons. As each of these neurons expresses *lim-6* and as the absence of *lim-6* gene activity phenocopies the absence of GABAergic function we consider it most likely that *lim-6* activity in these neurons is required for their functional specification to in turn affect GABA control of dauer arrest. For example, neurons postsynaptic to these GABA neurons may secrete DAF-7 or one of the dozens of insulin-like genes that are likely to regulate dauer arrest (S. Pierce, L. Liu and G. R., unpublished).

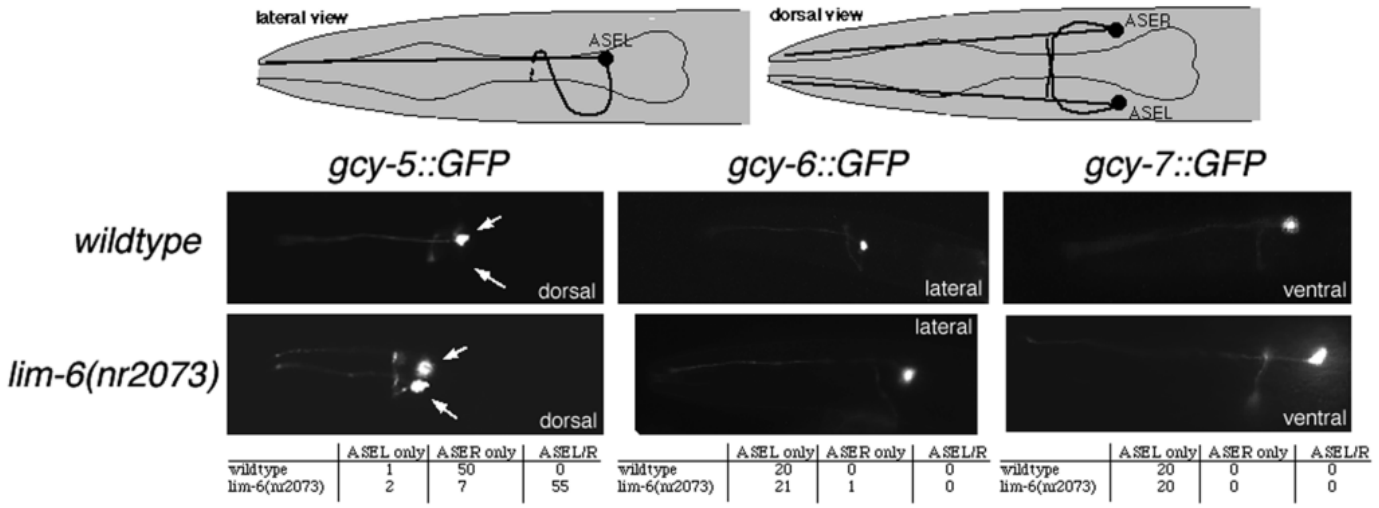
Table 5. GABA and *lim-6* regulate dauer arrest

Genotype	Dauer arrest at 15°C (*n*)	Dauer arrest at 25°C (*n*)
Wild type	0% (278)	0% (228)
lim-6(nr2073)	0% (179)	0% (204)
unc-25(e156)	0% (156)	0% (116)
unc-30(e191)	0% (378)	0% (79)
daf-7(e1372)	0% (379)	97% (64)
daf-7(e1372); lim-6(nr2073)	88% (189)	100% (>100)
daf-7(e1372); unc-25(e156)	73% (127)	93% (244)
daf-7(e1372); unc-30(e191)	5% (552)	100% (61)
daf-7(e1372); aex-2(sa3)	26% (123)	100% (75)
daf-7(e1372); sem-4(n2654)	10% (380)	100% (>100)

lim-6 affects asymmetric sensory receptor expression

Many neurons in the nervous system of *C. elegans* come in bilaterally symmetric pairs (White et al., 1986), yet *lim-6::GFP* reporter gene constructs are only expressed in the left neuron (ASEL) of the ASER and ASEL chemosensory pair (Fig. 2, Table 1; Bargmann and Horvitz, 1991). Using the ASEL-expressed *lim-6prom::GFP* reporter gene construct, we found that ASEL is generated and pathfinds normally in *lim-6* mutant animals (data not shown). We examined the expression of the two ASE differentiation markers *gcy-6* and *gcy-7*, two putative guanylyl cyclase-type sensory receptors that like *lim-6* are exclusively expressed in ASEL, but not in ASER (Yu et al., 1997). We found that *gcy-6* and *gcy-7* GFP reporter gene constructs are correctly expressed in *lim-6* mutant animals (Fig. 7), suggesting that *lim-6* gene activity is not necessary for these aspects of ASEL neural maturation. However, *lim-6* activity is required for the correct expression of another putative guanylyl cyclase-type sensory receptor, *gcy-5*. A *gcy-5* reporter gene construct is normally expressed in the right neuron, ASER, but not in ASEL (Yu et al., 1997). In *lim-6(nr2073)* mutant animals, the *gcy-5* reporter gene is ectopically expressed in ASEL (Fig. 7). Thus, *lim-6* is normally required for repression of *gcy-5* expression in ASEL. The LIM-6 transcription factor could either activate a repressor that directly acts on the *gcy-5* promoter or LIM-6 could directly bind and repress the *gcy-5* promoter. It is not known to what sensory inputs the *gcy-5* receptor responds. Consequently, the physiological significance of disregulated *gcy-5* receptor expression in *lim-6* mutant animals is hard to assess at the moment. We did, however, examine *lim-6(nr2073)* animals for their responsiveness to various water-soluble chemicals that are sensed by ASEL and ASER, but could not detect any defects (data not shown).

The asymmetric expression of *lim-6* in ASEL does not appear to be neural activity dependent. The abrogation of sensory inputs or the disturbance of correct connectivity have no influence on the asymmetric expression, as revealed by crossing *lim-6prom::GFP* in various *che* and *unc* mutants (*che-3*, *daf-19*, *unc-33*, *unc-36*, *unc-73*; data not shown). It may be relevant that besides ASEL most of the neurons that express *lim-6* (RIS, AVL, DVB, PVT) are asymmetrically generated in the sense that they do not come as bilaterally symmetric pairs.



	ASEL only	ASER only	ASEL/R
wildtype	1	50	0
lim-6(nr2073)	2	7	55

	ASEL only	ASER only	ASEL/R
wildtype	20	0	0
lim-6(nr2073)	21	1	0

	ASEL only	ASER only	ASEL/R
wildtype	20	0	0
lim-6(nr2073)	20	0	0

Fig. 7. *lim-6* is required for the asymmetric expression of a putative sensory receptor. (Top) A schematic representation of the ASE sensory neurons (White et al., 1986). The middle panels show the expression of the respective *gcy::GFP* fusion genes in wild-type and *lim-6* mutant animals. The table below shows the penetrance of the defects.

The expression of the vertebrate *lim-6* homolog *Lmx-1* is regulated by a vertebrate wingless homolog (Riddle et al., 1995; Vogel et al., 1995); wingless signaling in *C. elegans* is involved in determining asymmetric features of cell division (Sawa et al., 1996). We tested whether *lin-17*/*wingless* mutants affect the asymmetry of *lim-6* expression, but found no effects (data not shown). Other *wingless* like genes that are present within the *C. elegans* genome (Ruvkun and Hobert, 1998) might be involved in regulating asymmetric features of *lim-6* expression.

lim-6 and excretory gland cell function

The excretory gland cell displays a neuron-like appearance; it contains specific types of secretory vesicles of unknown function and extends thin processes towards the nerve ring, where it receives synaptic input (Nelson et al., 1983). To examine the effects of the *lim-6(nr2073)* mutation on excretory gland cell morphology and function, we attempted to observe the cell with the *lim-6prom::GFP* reporter gene, which labels the entire gland cell in wild-type animals. Interestingly, the *lim-6* reporter gene construct is not expressed in the excretory gland cell of *lim-6* mutant animals, whereas its expression in the other neurons and epithelial cells is unaffected (data not shown). This suggests that LIM-6 may autoregulate its own expression in the excretory gland cell or that there are severe differentiation defects of the excretory gland cell. Because laser ablation of the excretory gland cell has no significant impact on growth, moulting, osmoregulation, fertility, longevity, and dauer larva formation (Nelson and Riddle, 1984), we did not assess the functional consequences of the absence of *lim-6* activity in the excretory gland cell. Vertebrate Lmx-1 is required for the specification of basement membrane structures in the kidney. An ultrastructural analysis of *lim-6* mutant animals will be required to examine whether *lim-6* might have a similar function in *C. elegans*.

We found that the extension of excretory gland cell processes uses guidance mechanisms similar to those used by neurons, since several neural pathfinding genes, such as *unc-33*, *unc-73* and *unc-76* (Hedgecock et al., 1987), affect excretory gland cell morphology as monitored by crossing *lim-6prom::GFP* into these mutants (data not shown). Since *lim-6* is required to specify the correct neuroanatomy of several neurons, as described above, it is conceivable that by regulating a similar set of target genes *lim-6* also affects the process morphology of the excretory gland cell.

lim-6 function in uterine morphogenesis

lim-6 mutant animals have a significantly reduced brood size (Table 6). Moreover, most of the progeny of *lim-6* mutant animals hatch within the parent animal. The uterine expression of *lim-6* prompted us to examine the morphology of the uterus in the *lim-6(nr2073)* mutant. The main tissue types of the uterus are the epithelial uterine toroid (ut) cells, which line the uterine lumen and which express *lim-6* (see above), and specialized utse and uv cells, which constitute the connection between the uterus and the vulva (Newman et al., 1996). Using DIC microscopy we found that the uterine lumen is not formed correctly in 78% of *lim-6* mutant animals and appears to be clogged (Fig. 8; Table 6). The extent of clogging varies from animal to animal and can range from a simple ‘bridge’ as shown in Fig. 8 to a complete closure of the uterine lumen. The tissue that clogs the uterine lumen expresses *lim-6* and could represent ut cells that have not separated correctly (Fig. 8). The *lim-6(nr2073)* egg laying defects could be explained by the animal’s inability to accommodate eggs in the uterus. A hindered passage through the uterus would congest the gonad and inhibit further fertilizations, thus explaining the low brood size of the animals (which is even smaller than those of vulvaless animals, such as *lin-11(n389)*; Freyd, 1991). However, the uterine closure defect is not 100% penetrant, while the low brood size clearly is. This argues for additional defects in the reproductive system. The expression of *lim-6* in the junction cells of the spermatheca described above could indicate that eggs in *lim-6(nr2073)* mutants might have problems in passing through the spermatheca thus reducing fertility. In summary, we have shown that *lim-6* is required for uterine morphogenesis.

Table 6. *lim-6* is required for the development of the reproductive system

	Brood size		
	Wild type	*lim-6(nr2073)*	*lim-6(nr2073); Ex[lim-6]* or *Ex[lim-6::VP16]*
No. of progeny at 22°C	207±42 (*n*=10)	15±10 (*n*=49)	125±26* (*n*=8)
Morphology of the uterine lumen			
both lumenal sides open	96%	22%	100%‡
one lumenal side closed	4%	41%	0%‡
both lumenal sides closed	0%	37%	0%‡
	(*n*=27)	(*n*=27)	(*n*=20)

*The array line *mgEx446* was tested for rescue. 8 out of 12 animals tested showed at least partial rescue of the brood size.

‡Due to the partial rescue of the *lim-6* expression construct we increased the activity of *lim-6* by adding the VP16 transcriptional activation domain. We had previously shown that adding the VP16 domain to the LIM homeobox gene *ttx-3* significantly increases its ability to complement a *ttx-3* null mutant (O. H., I. Mori, G. R., unpublished data). The *lim-6::VP16* construct, expressed from the extrachromosomal arrays *mgEx408* and *mgEx409*, is also very potent in complementation of the other *lim-6* defects (see Materials and Methods).

DISCUSSION

lim-6 is one of seven *C. elegans* LIM homeobox genes revealed by the genome sequence. Most of these LIM homeobox genes detect mammalian orthologs (Fig. 1). The determination of the function of these *C. elegans* LIM homeobox genes with precise cellular identifications and correlations with neuron type and function should broadly indicate the function of these genes across phylogeny. *lim-6* is an interesting case because while the function of the mammalian ortholog *Lmx-1b* was much better understood than *lim-6* before this study, our genetic and cellular analysis suggests new functions to search for in mammals.

Our results show that *lim-6* functions mainly in GABAergic neurons that regulate enteric muscles and endocrine outputs, and uterine development. It is not yet clear which of the *C. elegans lim-6* functions are generalizable to its mammalian orthologs *Lmx-1a* and *Lmx-1b*. It is possible that some of the *lim-6* functions are served by one ortholog and others by the other. Consistent with such a model, the regulation of neuroendocrine signals by both *C. elegans lim-6* (dauer regulation) and mammalian *Lmx-1a* (insulin regulation) suggests common, ancestrally related functions. Similarly, the requirement for *Lmx-1b* in mammalian kidney function and the *lim-6* expression in the *C. elegans* excretory gland cell suggests a common function in excretory system differentiation for this LIM homeobox gene. Our data also suggests that expression of mammalian *Lmx-1a* and *Lmx-1b* in the central and peripheral nervous system should be correlated with GABAergic neural markers. *Lmx-1b* knock-out mice exist (Chen et al., 1998) but have not been analyzed for of GABAergic neuroendocrine or enteric muscle control defects. The *C. elegans* function of this class of LIM homeobox genes suggests that GABAergic function (for example sensitivity to GABA-related drugs such as valium) in the *Lmx-1a* or *Lmx-1b* knockout mice should be assayed.

Our data also suggests that uterine development should be analyzed. The expression of vertebrate *Lmx-1* in the uterus has not been reported. However, *Wnt-7a*, which induces *Lmx-1b* expression in the developing limb (Riddle et al., 1995; Vogel et al., 1995), has recently been shown to act in uterine development (Miller and Sassoon, 1998). As in limb development the expression of *Wnt-7a* in the uterus might similarly be coupled to the induction of Lmx-1b expression in the uterus. However, it is also possible that some of the *lim-6* functions in *C. elegans* may not be primitive and universal.

lim-6 function in the GABAergic nervous system

The most prominent site of expression of *lim-6* in the nervous system are GABAergic neurons; five of the nine *lim-6*-expressing neurons are GABAergic. The complete GABAergic nervous system of *C. elegans* consists of 26 neurons (McIntire et al., 19993b). They can be functionally subdivided into the D-motorneurons of the ventral nerve cord, which are required

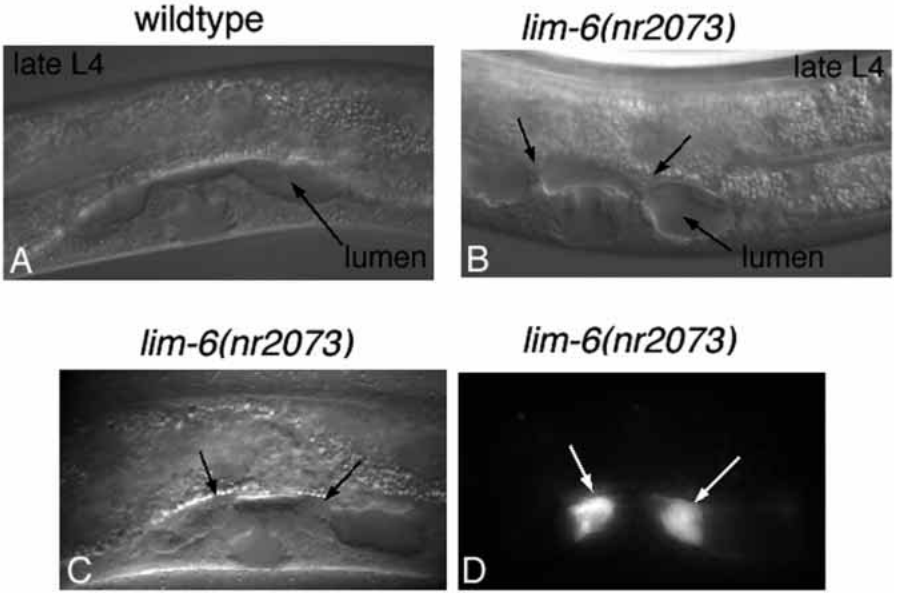


Fig. 8. Uterine defects in *lim-6(nr2073)* mutant animals. (A) Wild-type animal showing the continuous opening of the uterine lumen. (B,C) Two representative photomicrographs (using DIC microscopy) of uterine closure defects in *lim-6(nr2073)*. The upper two arrows point to the uterine closure. Occasionally the whole uterine lumen is closed. (D) *lim-6prom::GFP* in *lim-6(nr2073)*. The animal is identical to that in C.

for locomotion, the AVL/DVB motorneurons required for enteric muscle contraction, the RME motorneurons required for foraging and the RIS interneuron with an unknown function. The *unc-30* homeobox gene is exclusively expressed in and required by one specific subset of the GABAergic neurons, the D-type motorneurons, for the acquisition of the GABAergic neural cell fate (Jin et al., 1994). Interestingly, the expression pattern and function of *lim-6* is almost entirely complementary to the the expression pattern and function of *unc-30* in the GABAergic nervous system; *lim-6* is not expressed in the D-type motorneurons, but is expressed in all other GABAergic neurons (with the exception of two dorsal and ventral types of the fourfold symmetric RME motorneurons). However, *lim-6* does not appear to be the functional counterpart of *unc-30* in these neurons, since unlike *unc-30* the *lim-6* gene is not as tightly required for the expression of all GABAergic-specific cell specializations. For example, the expression of the *unc-47* GABA transporter in the non-D-type GABAergic neurons is largely unaffected and the expression of the *unc-25* /GAD gene is only partially affected by the *lim-6* null mutation. Presumably other transcription factors can also regulate *unc-25* and *unc-47* expression in the non D-type motorneurons, suggesting that the genes that define GABAergic neural identity are regulated by different regulatory mechanisms in different subtypes of GABAergic cells.

The functional and neuroanatomical defects of the AVL and DVB neurons in the *lim-6* null mutant strongly suggest that the function of these neurons is defective. The neuroanatomical defects which manifest as sprouting defects or the inability of the main axonal projection to follow correct paths could either represent an inability of the neuron to correctly engage its axonal outgrowth machinery, or alternatively, could represent a secondary consequence of neural activity defects. Neural activity defects have been shown before to cause axonal misrouting in both *C. elegans* and vertebrates (Coburn and Bargmann, 1996; Coburn et al., 1998; Dahm and Landmesser, 1988, 1991; Shatz and Stryker, 1988). *lim-6* could be required for neural activity of GABAergic neurons by regulating the expression of genes directly involved in generating electric activity or regulating genes required to generate the synaptic connectivity required to receive and transmit electrical signals.

The defects in non-D-type GABAergic function of the *lim-6* mutant can account for the defects in defecation cycle that the animals show. Defecation behavior requires the activation of the defecation motor program. This program represents a rhythmic behavior composed of three stereotyped muscle contractions; one of these rhythmic contractions, the enteric muscle contraction is controlled by two GABAergic motorneurons, AVL and DVB (Liu and Thomas, 1994; McIntire et al., 1993b). Mutations in GABAergic signaling, for example in the *unc-47* GABA transporter or the *unc-25* GABA synthesizing enzyme, cause very similar enteric muscle contraction defects (McIntire et al., 1993a). Similarly, GABAergic neurons are also part of the neural circuit that controls gut function in vertebrates (Jessen et al., 1986) and enteric GABA has been shown to regulate the peristaltic effects in the vertebrate enteric system (Grider and Makhlouf, 1992). Moreover, muscle contractions of the enteric system are also rhythmically active in vertebrates. This rhythmicity is regulated by the electric pacemaker activity of the interstitial cells of Cajal, which function as an intermediary between enteric nerves and smooth muscle cells (Sanders, 1996). It will be interesting to see if human patients with Nail Patella syndrome, a haploinsufficency of *Lmx-1b,* or mouse carrying *Lmx-1a* or *Lmx-1b* knockout mutations, also show defects in enteric motor function, perhaps due to a common GABAergic input to enteric muscles.

Our data favors an output from the GABAergic neurons to the neuroendocrine regulation of dauer formation which depends on *lim-6* activity. We find that *lim-6* loss of function strongly enhances the neuroendocrine signaling defects caused by a *daf-7/TGFβ* mutation. This neuroendocrine function of *lim-6* is likely to be due to its function in GABAergic neurons, since *unc-25* mutant animals with defects in GABA synthesis show the same phenotype. Because animals with defective D-type motor neurons do not have these neuroendocrine defects, these data argue that it is the *lim-6*-regulated GABAergic neurons that regulate *C. elegans* endocrine signaling. Although we can not exclude that *lim-6* affects dauer arrest through other, as yet unidentified sites of expression, the simplest model is that *lim-6* specifies functional aspects of the non-D-type GABAergic neurons that in turn regulate dauer arrest via GABAergic signaling.

The expression of *lim-6* in the GABAergic neuroendocrine system also reveals an interesting similarity to the expression of its vertebrate homlog *Lmx-1a*. Vertebrate *Lmx-1a* participates in the control of insulin gene expression in the β cells of the pancreas (German et al., 1992) which also contain high levels of the neurotransmitter GABA. GABA regulates insulin secretion from the β cells via an autocrine loop (Gu et al., 1993; Sorenson et al., 1991). We find that both *lim-6* and non D-type GABAergic neurons in *C. elegans* regulate dauer arrest, which is regulated by an insulin-like neuroendocrine pathway. It is possible that *lim-6* regulates the development of the particular GABAergic neurons that in turn regulate insulin expression and secretion in *C. elegans*. For example, these GABAergic neurons may couple temperature or food or other sensory inputs to the endocrine output of dauer regulatory proteins such as the many insulin-like proteins that may converge on the single insulin-like receptor that clearly regulates dauer arrest. The synergizing effects of mutations in *lim-6* and GABA signaling on the *daf-7/TGFβ* control of dauer formation indeed mirror the synergism between insulin signaling and *daf-7/TGFβ* signaling (Ogg et al., 1997). However, the complexity of the insulin family in *C. elegans* (Duret et al., 1998) complicates the matter significantly.

Asymmetry in the *C. elegans* nervous system

The major head ganglia of *C. elegans* display an obvious overall bilateral symmetry. The majority of neural cell types have both a left and right representative that share a comparable lineage history and morphology and make similar synaptic connections (Sulston, 1983; White et al., 1986). In those cases tested, both left and right neurons are required for a full functional response of, for example, a given pair a chemosensory neurons (Bargmann and Horvitz, 1991). The asymmetric expression of *lim-6* in ASEL, but not in ASER thus represents a surprising observation. It may suggest a potential diversification of apparently symmetric sensory neurons possibly leading to a diversification in sensory function. This notion is supported by the asymmetric expression of several receptor-type guanylyl

cyclases in the ASER and ASEL neurons (Yu et al., 1997), one of which we have shown to be under control of *lim-6* activity. As the function of these receptor-type guanylyl cyclases are as yet unknown, we could not assess the physiological consequences of ectopic activation of one of these receptors. Gross defects in ASE sensory functions were not obvious in *lim-6* mutant animals (data not shown).

It will be interesting to determine how the asymmetry of *lim-6* expression is established. In vertebrates, *Lmx-1b* expression is under control of wingless signaling (Riddle et al., 1995; Vogel et al., 1995); wingless signaling in *C. elegans* is involved in determining the polarity of certain cell divisions (Sawa et al., 1996). Although we have not found an impact of the wingless-like *lin-17* gene on *lim-6* expression (data not shown), it is possible that any other of the five wingless-like genes present in the *C. elegans* genome (Ruvkun and Hobert, 1998) are involved in regulating *lim-6* expression and possibly its asymmetry. The asymmetrically expressed *lim-6* reporter gene construct is a tool for the genetic identification of mutants involved in establishing asymmetry in the nervous system.

LIM homeobox gene function in *C. elegans*: a common theme?

Homeobox genes have been shown to act at a variety of different stages of neurogenesis, including such early steps as the determination of neural identity and later steps such as neural differentiation (Manak and Scott, 1994). The analysis of 4 of the 7 LIM homeobox genes in *C. elegans* demonstrates that they are required for the terminal differentiation of the neurons that express them (Hobert et al., 1998, 1997; Way and Chalfie, 1988, 1989; this study). In many respects the neural defects caused by mutations in these genes are similar. Mutations in *lim-6*, like mutations in the *ttx-3* and *lin-11* LIM homeobox genes do not affect the generation of the neurons that express them (Hobert et al., 1998, 1997) nor do they affect several aspects of neurotransmitter choice (this study and our unpublished data). However, the *lim-6*-, *ttx-3*- and *lin-11*-expressing neurons are functionally as well as structurally defective (Hobert et al., 1998, 1997; this report). Similarly, *mec-3* is required for the structural integrity of mechanosensory neurons (Way and Chalfie, 1998). These similarities in LIM homeobox gene function suggest a common theme in the action of this class of transcriptional regulators. We speculate that each gene is required in its respective neuron to make a specific target choice and that in the absence of intact signaling partners, retrograde signaling events induce the neuron to find a signaling partner by sprouting additional processes or by inducing abnormal turns of the main axonal process. Alternatively, each gene might be required within its given neuron to directly regulate its axon outgrowth machinery. Our finding that defects in the GABA neurotransmitter synthesis do not cause axonal sprouting and the report of Jin et al. that GABAergic neurons and their postsynaptic partners show no defects in synaptic connectivity in *unc-25* mutants (Jin et al., 1999) indicate that defects in synaptic outputs are unlikely to feedback on neurite outgrowth functions. However, defects in other synaptic outputs, such as signaling by neuropeptides might be feeding back into axonal sprouting or, alternatively, defects in synaptic inputs to the GABAergic neurons, caused for example by connectivity defects, could induce axonal sprouting.

Another common feature of all *C. elegans* LIM homeobox genes studied to date is their onset of expression in postmitotic neurons and the maintenance of their expression throughout adulthood (Hobert et al., 1998, 1997; Way and Chalfie, 1989). These observations are unlikely to be a reporter gene artefact, since the expression in other tissues, such as *lim-6* in the uterus or *lin-11* in the vulva is dynamic and transient. Maintained expression of a regulatory gene throughout the life of a given cell suggests, but does not prove an involvement of the particular gene in maintenance of the differentiated features of the given cell. In the case of LIM homeobox genes it is possible that they are required for the maintenance of such neural features as synaptic connectivity.

Lastly, all of the *C. elegans* LIM homeobox genes described so far are expressed in a largely non-overlapping subsets of neurons (Hobert et al., 1998, 1997; Way and Chalfie, 1989; this study; D. H. and G. R., unpublished). The most prominent non-neural tissue that expresses *C. elegans* LIM homeobox genes is the epithelium of the somatic gonad and the vulva. Intriguingly, the LIM homeobox genes are also expressed in a complementary, non-overlapping pattern in this epithelium. *lin-11* is expressed in the vulva and cells that connect the uterus to the vulva (Freyd, 1991; Hobert et al., 1998), *lim-6* is expressed in uterine toroid cells and in spermathecal junction cells (this study), *ceh-14* is expressed in the epithelial cells of the spermatheca (T. Bürglin, personal communication) and we found another LIM homeobox gene, *lim-7*, to be expressed in the gonadal sheath cells (O. H. and G. R., unpublished). The unifying feature of all these cells is their highly polarized morphology and their engagement in complex morphogenetic events for which specific cell-cell contacts are required (Newman et al., 1996). These morphological features are shared by neurons as well. Moreover, epithelial cells and neurons utilize similar sorting and targeting mechanisms for specific cell surface molecules (Bredt, 1998; Rongo et al., 1998). It is conceivable that by regulating the expression of specific cell surface proteins, LIM homeodomain proteins in *C. elegans* are generally involved in determining the specificity of cell-cell recognition and attachment to neighboring cells both in the nervous system and in epithelial cells. The structural defects of uterine cells in *lim-6* mutants indeed point to a role at least of *lim-6* in cell recognition and adhesion.

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