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The *fax-1* nuclear hormone receptor regulates axon pathfinding and neurotransmitter expression

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SUMMARY

Specification of neuron identity requires the activation of a number of discrete developmental programs. Among these is pathway selection by growth cones: in order for a neuron's growth cone to respond appropriately to guidance cues presented by other cells or the extracellular matrix, the neuron must express genes to mediate the response. The *fax-1* gene of *C. elegans* is required for pathfinding of axons that extend along the ventral nerve cord. We show that *fax-1* is also required for pathfinding of axons in the nerve ring, the largest nerve bundle in the nematode, and for normal expression of FMRFamide-like neurotransmitters in the AVK interneurons. The *fax-1* gene encodes a member of the superfamily of nuclear hormone receptors and has a DNA-binding domain related to the human PNR and *Drosophila* Tailless proteins. We observe *fax-1* expression in embryonic neurons, including the AVK interneurons, just prior to axon extension, but after neurogenesis. These data suggest that *fax-1* coordinately regulates the transcription of genes that function in the selection of axon pathways, neurotransmitter expression and, perhaps, other aspects of the specification of neuron identity.

Key words: Axon, Pathfinding, Nuclear hormone receptor, Transcription factor, FMRFamide, *tailless*, Tlx, PNR, Neuron, *Caenorhabditis elegans*

INTRODUCTION

The development of the nervous system requires the generation of basic neuronal cell types (neurogenesis) and the subsequent specification of a wide variety of unique neuron identities. Individual neurons must express the proper spectrum of neurotransmitters, the correct molecules required for transduction of synaptic stimuli, and direct appropriate axon pathfinding and synaptic specificity. Genes that regulate neurogenesis and nervous system patterning have been identified and studied in some detail (reviewed by Tanabe and Jessell, 1996), but relatively little is known about the specification of discrete neuronal cell types (Lewin, 1994). A complete understanding of neuron development demands knowledge of the mechanisms by which the unique properties of individual neurons are specified.

One important aspect of neuron identity is selection of axon pathways. Growth cones navigate to their targets using molecular cues presented on cell and axon surfaces and the extracellular matrix (reviewed by Culotti, 1994; Tessier-Lavigne and Goodman, 1996). The appropriate response to these cues presumes expression of the appropriate receptors on the growth cone. For example, ectopic expression of the putative netrin receptor UNC-5 causes growth cones to make inappropriate pathfinding decisions (Hamelin et al., 1993). Thus, expression of the correct receptors for axon pathfinding is a significant aspect of a particular neuron's identity.

The specification of neuron identities is thought to be mediated, at least in part, by transcription factors that modulate the expression of effector genes that function directly in specifying axon pathways and other aspects of neuron identity (reviewed by Daston and Koester, 1996; Goulding, 1998). Considerable attention has been focused on the LIM homeodomain transcription factors of both vertebrates and invertebrates. Examples of LIM proteins that have been shown to play significant roles in specifying particular pathways for growth cones include the *Isl1, Isl2, Lim1, Lim3* and *Lhx4* genes of vertebrates (Tsuchida et al., 1994; Sharma et al., 1998) and the *islet*, *apterous*, *mec-3* and *lin-11* genes of invertebrates (Way and Chalfie, 1988; Lundgren et al., 1995; Hobert et al., 1998; Thor et al., 1999).

We are studying the mechanisms by which neurons select axon pathways in *C. elegans*, and have identified six genes that are required for pathfinding of growth cones along the ventral nerve cord (VNC; Wightman et al., 1997). Among these genes is *fax-1*, named for its defective *f*asciculation of *a*xons phenotype. In *fax-1* mutants, the growth cones of the AVKR, HSNL and PVQL neurons fail to follow their normal pathway. Because these growth cones all depend on the pre-existing PVPR axon (Garriga et al., 1993), *fax-1* appears to be required

for recognition of pre-existing axons by later-extending growth cones.

In order to understand the function of *fax-1* in directing axon pathfinding, we examined pathfinding by the AVKR growth cone and its contralateral homolog, AVKL, and found that both growth cones make navigational errors in the nerve ring as well as the VNC. In the course of this analysis, we discovered that *fax-1* is also required for normal expression of a neuropeptide precursor protein in both AVK neurons. Molecular cloning revealed that *fax-1* encodes a nuclear hormone receptor with a DNA-binding domain related to *Drosophila* and vertebrate members of the superfamily, suggesting that it is likely to regulate the transcription of other genes. We observed expression of *fax-1* reporter constructs in embryonic neurons just prior to axon extension, but after neurogenesis, consistent with a role for *fax-1* in pathway selection. Among the neurons that express *fax-1* are the AVK neurons, suggesting that *fax-1* may function in these neurons to regulate pathway selection and neurotransmitter expression. Taken together, these results suggest that *fax-1* regulates the specification of a unique neuronal identity.

MATERIALS AND METHODS

Strains and genetics

Strains of *C. elegans* were maintained at 20°C, unless otherwise indicated, as described by Brenner (1974). Genotypes of strains used in this study are: N2 (Wild type), NG27 *fax-1(gm27)*, NG83 *fax-1(gm83)*, MU1085 *bwIs2* and NY1043 *ynEx43*. Except for the last two strains, all mutations are on the left arm of LG X. The NY1043 strain was generously provided by Christine Li (Boston University). *bwIs2* was created by integrating *ynEx43* by irradiation of NY1043 animals at a dosage of 5400 Rad (Mello et al., 1991).

Analysis of axon anatomy and FMRFamide expression

The anatomy of the AVK axons was examined using a reporter transgene that fuses a neuron-specific promoter to the green fluorescent protein (GFP) of *Aequorea victoria* (Chalfie et al., 1994). The *bwIs2* integrated *flp-1::gfp* transgene, which expresses GFP in the AVK neurons and no other cells, was crossed into *fax-1(gm27)* and *fax-1(gm83)* mutant backgrounds. The *bwIs2* transgene causes animals that carry it to roll in circles (Rol) due to presence of a *rol-6(su1006)* dominant allele in the transgene (Mello et al., 1991). Wild-type males were mated to *bwIs2* hermaphrodites to generate Rol males, which were in turn mated to *fax-1(–)* hermaphrodites. GFP fluorescence was detected directly in live larvae and adults grown at 25°C and immobilized in 25 mM levamisole (Sigma) using a Nikon Labophot microscope and Chroma FITC-LP filter or an Olympus BX60 microscope and FITC filter. Alternatively, animals were stained for indirect immunofluorescence using 0.1% anti-GFP antiserum (Clontech), as described previously (Wightman et al., 1997). FMRFamide expression in the AVK neurons was examined by indirect immunofluorescence using rabbit anti-FMRFamide antisera, as described previously (Schinkmann and Li, 1992).

Molecular cloning of *fax-1*

The *fax-1* gene was mapped genetically to a position between *unc-20* and *unc-78* on the left arm of the X chromosome (Fig. 4A; data not shown). Duplications of portions of this region have been mapped physically using an in situ hybridization technique (Albertson, 1993). These data indicate that the duplications *mnDp33* and *yDp14* do not include YAC clones Y46A5 and Y60B6, respectively. Because both *mnDp33* and *yDp14* include *fax-1* (data not shown), we determined that the *fax-1* gene lies to the right of Y46A5 and to the left of Y60B6.

We identified an allele-specific polymorphism associated with the *fax-1(gm27)* mutation in the region defined by the duplication mapping breakpoints. Southern blot analysis of genomic DNA prepared from *fax-1(gm27)* animals identified a deletion that removes all DNA corresponding to cosmid F56E3 and a portion of cosmids C15C7 and F40F4 (summarized in Fig. 4). These data indicate that the *gm27* mutation deletes *fax-1* and several flanking genes located on overlapping sequenced cosmid clones C15C7, F56E3, F28B4 and F40F4.

We performed standard germline-mediated transformation rescue experiments to identify cosmid clones that contain the *fax-1* gene (Mello et al., 1991). Subclones of the rescuing F56E3 cosmid were created using standard techniques (Fig. 4C; Sambrook et al., 1989). In each experiment, cosmid clones and plasmid subclones were microinjected into wild-type or *fax-1(gm83)* hermaphrodite gonads at a concentration of 10-100 μg/ml with co-transforming pRF4 plasmid at a concentration of 100 μg/ml using a Nikon UD or Zeiss Axiovert microscope system. The pRF4 plasmid causes the dominant Rol phenotype that allows identification and recovery of transformed animals. Transgenic lines were maintained as extrachromosomal arrays. Mutations in *fax-1* cause an Uncoordinated (Unc) phenotype characterized by awkward forward movement and more normal backward movement. Extrachromosomal arrays were assayed for their ability to rescue the Unc phenotype by examining the behavior of Rol animals that carry the array in a *fax-1(gm83)* background. We scored Rol animals as rescued if they could roll forward smoothly. Rescue of AVKR axon pathfinding defects was assayed by indirect immunofluorescence staining of transgenic lines using anti-FMRFamide antiserum (Schinkmann and Li, 1992; Wightman et al., 1997). Rescuing lines had normal AVKR axons present in the left bundle in greater than 90% of Rol animals, while non-rescuing lines had AVKR axons present in the left bundle in less than 10% of Rol animals, similar to *fax-1(gm83)* without an extrachromosomal array.

Isolation and analysis of cDNA clones

We isolated cDNA clones from wild-type and *fax-1(gm83)* total RNA by PCR amplification. Total RNA was prepared from mixed stage animals by grinding frozen animals with a mortar, treatment with proteinase K, multiple phenol/chloroform extractions and multiple rounds of precipitation in 2 M LiCl. cDNA was synthesized from total RNA using AMV reverse transcriptase and oligo(dT) primer (Boehringer). *fax-1* cDNA was amplified with Taq polymerase (Promega) or Vent polymerase (New England Biolabs) using 5′ and 3′ primers designed from the cosmid F56E3 sequence (accession number U41536). 5′ oligonucleotides used for cDNA amplification had the sequences: 5′-GGGAATTCATACACCTTTGCTCTTTCCTCCTCC-3′ and 5′-GGGAATTCAACACTGGCGTCTGCGTTGGCTG-3′. 3′ oligonucleotides had the sequences: 5′-GGGAATTCACGTTTCAT-GAAGATGGTGATCG-3′ and 5′-CCGAATTCGGTGGGATACAC-ATGTAAAC-3′. Oligonucleotides were synthesized by the University of California DNA synthesis facility and Research Genetics, and PCR products were subcloned into pGEM-T or pBluescript KS+ vectors. We isolated five independent cDNA clones from wild type and four independent cDNA clones from *fax-1(gm83)*. cDNA clones were sequenced using the Silver Sequence system (Promega) and the University of California DNA sequencing facility. All cDNA clones that we obtained had the same exon-intron structure. The *fax-1* transcript predicted from cDNAs differs at the 5′ and 3′ end from the F56E3.4 gene predicted by Genefinder, thus the FAX-1 protein predicted from our cDNAs differs at the N and C terminus from the predicted F56E3.4 protein. Attempts to amplify cDNA using the Genefinder-predicted 5′ and 3′ ends and oligonucleotides complementary to the *trans*-spliced leaders SL1 and SL2 were unsuccessful. We compared the FAX-1 amino acid sequence to database sequences using the BLAST utility (Altschul et al., 1997) at NCBI and performed alignments with other nuclear hormone receptors using MEGALIGN (Lasergene).

Construction and analysis of *fax-1::gfp* translational fusions

We constructed translational fusions of *fax-1* to the coding region for GFP in order to determine the spatial and temporal pattern of *fax-1* expression. Vectors containing GFP (S65C) were generously provided by Andrew Fire (Carnegie Institution). pFX1G1 was created by cutting a 9.2 kb *Sac*II-*Hpa*I subclone from cosmid F56E3 (pF56SH9) with *Sph*I and *Eag*I and ligating it to an *Sph*I-*Eag*I fragment containing *gfp* from vector pPD 95.75. pFX1G2 was created by the same strategy as for pFX1G1, except a 6.7 kb *Hpa*I subclone from F56E3 was used (pF56Hp6). Both of these constructs include all sequences 5′ to the *fax-1* gene that are required for rescue plus the first three introns, but eliminate portions of the coding region, the last three introns, and approximately 200 bp immediately 3′ to the gene.

We introduced *fax-1::gfp* translational fusions into wild-type animals using germline-mediated transformation as described above. GFP expression was assayed by direct visualization or immunofluorescence. We identified cells that express *fax-1::gfp* on the basis of cell morphology, position and axonal anatomy (White et al., 1986).

Relative quantitative RT-PCR

In order to determine the amount of *flp-1* RNA present in *fax-1* mutants, as compared to wild type, we performed relative quantitative reverse transcription PCR using a commercial system (Ambion). We prepared RNA from wild-type and *fax-1* mutants, as described above, and used primers complementary to the *flp-1* gene, and 18S rRNA primers to normalize each sample. Reverse transcription and amplification were performed as described by the manufacturer. Primers for amplification of *flp-1* cDNA had the sequences 5′-CACACCGGGAGCCACTTCAGACTTT-3′ and 5′-ATCGAAGTT-GTCGAATGAGCGTCCG-3′. Results from five independent experiments were analyzed using Sigma ScanPro and a two-tailed *t*-test.

RESULTS

fax-1 is required for AVK axon pathfinding in the nerve ring and ventral nerve cord

Mutations in *fax-1* cause defects in pathfinding by the AVKR, HSNL and PVQL growth cones (Wightman et al., 1997). These growth cones normally extend along the left bundle of the ventral nerve cord (VNC; Fig. 1; White et al., 1986). In *fax-1* mutants, the HSNL and PVQL growth cones extend inappropriately along the larger right bundle of the VNC. The previous study also showed that the AVKR axon was absent from the left VNC bundle, but the methodology employed for visualizing the AVKR axon did not allow determination of the trajectory followed by the AVKR axon when it was outside of the left VNC bundle. Moreover, we could not examine pathfinding by AVKR's contralateral homolog, AVKL.

To address this issue, we utilized a *flp-1::gfp* transgene to visualize the axons of AVKR and AVKL in *fax-1* mutants. The *flp-1* gene encodes a precursor protein for FMRFamide-like neurotransmitter peptides (Nelson et al., 1998a). The *flp-1::gfp* transgene used in this study expresses GFP in both AVK neurons, but no other cells (Fig. 2; Nelson et al., 1998b). The cell bodies of the AVK neurons are located symmetrically on the left (AVKL) and right (AVKR) sides of the ventral portion of the head (White et al., 1986). Each AVK neuron extends an axon anteriorly into the ventral cord and around the nerve ring to the contralateral side (Figs 1, 2A). Here, the axon exits the nerve ring and extends posteriorly along the opposite side of the VNC until terminating in the preanal ganglion located in the tail of the animal. Thus, the AVKR axon extends along the left VNC bundle and the AVKL axon extends along the right VNC bundle.

In *fax-1* mutants, we observed defects in pathfinding by AVKR and AVKL axons (Fig. 2B,C). Both AVKR and AVKL axons exited the nerve ring at inappropriate locations in *fax-1* mutants. These misrouted AVK axons frequently followed the dorsal nerve cord or a lateral nerve bundle posteriorly into the middle of the body before terminating. Occasionally, misrouted AVK axons exited from ventral portions of the nerve ring and followed a less-directed route into the nose of the animal (<3%, data not shown). Some AVKR axons circumnavigated the nerve ring, but once in the VNC extended along the right VNC bundle instead of the left bundle, and a few AVKL axons extended along the left VNC bundle instead of the right bundle. Therefore, *fax-1* is required for normal pathfinding in the nerve ring and VNC by both AVK growth cones.

fax-1 is required for normal expression of a peptide neurotransmitter precursor gene

In addition to the axon pathfinding defects described above, expression of the *flp-1::gfp* reporter was altered in *fax-1* mutants. In a wild-type background, almost all AVK neurons expressed the *flp-1::gfp* reporter at detectable levels, while in *fax-1* mutants only 65-76% of AVK neurons expressed the *flp-1::gfp* reporter at detectable levels (Table 1). Expression of *flp-1::gfp* in either AVKL or AVKR was equally likely to be missing. Some *fax-1* mutant AVK neurons expressed detectable *gfp* fluorescence, but at reduced level as compared to wild type (data not shown). In AVK neurons that did not express *flp-1::gfp* at detectable levels, we could not examine axon morphology, so we do not know whether axon pathfinding was normal in these neurons. Therefore, the actual penetrance of AVK axon pathfinding defects could be higher than that shown in Fig. 2C.

Similar results were obtained when we examined FMRFamide immunoreactivity in the AVK neurons (Fig. 3,

Table 1. Expression of FMRF-related products in wild-type and *fax-1* mutant AVK neurons

	Percentage of AVK neurons (*n*)		
	Wild-type	*fax-1 (gm83)*	*fax-1 (gm27)*
Express *flp-1::gfp* fluorescence	100 (40)	65 (62)	76 (58)
Express FMRFamide immunoreactivity	98 (40)	70 (56)	76 (42)

Percentages indicate the frequency of AVK neurons that display detectable GFP fluorescence from the *flp-1::gfp* transgene or detectable staining in immunofluorescence experiments using anti-FMRFamide antisera.
n, number of neurons examined.

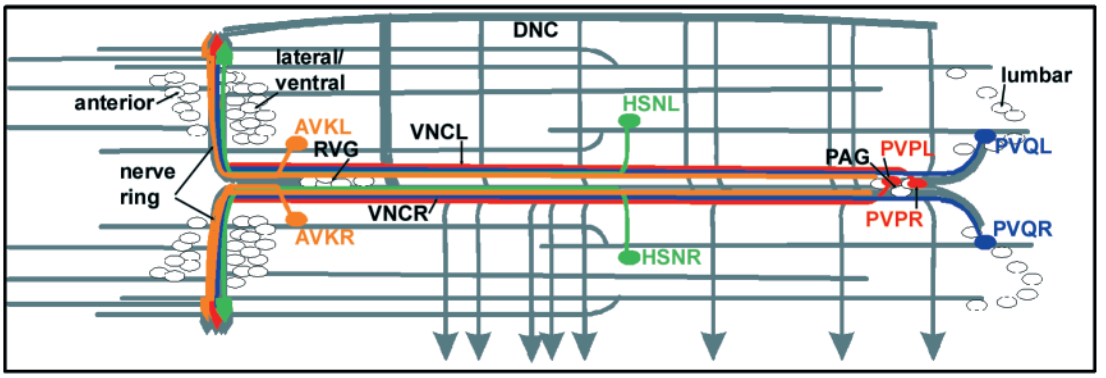
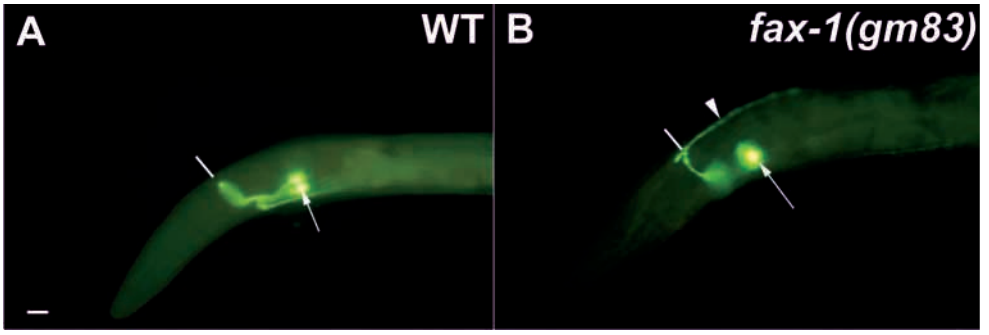


Fig. 1. Neuroanatomy of *Caenorhabditis elegans.* Schematic shows the major axon pathways and ganglia present in an adult hermaphrodite. A cylindrical projection of the entire body wall viewed from the ventral side is shown. The mid-dorsal bundles are at the top. Top to bottom in the schematic is left side to right side of the animal. Left to right in the schematic is anterior to posterior. The major circumferential bundle, the nerve ring, is labeled, as are the major longitudinal bundles, the dorsal nerve cord (DNC) and the two bundles of the ventral nerve cord (VNCL and VNCR). The major ganglia, including the anterior, lateral/ventral, retrovesicular (RVG), preanal (PAG) and lumbar ganglia are depicted schematically by open ovals. The number of neurons depicted in each ganglion is not an exact representation of the number or position of neurons located there. The anatomy of some of the neurons discussed in this study and their axons is depicted in color.

Table 1); however, *C. elegans* has multiple *flp* genes (Nelson et al., 1998a), so we do not know whether the reduction in FMRFamide immunoreactivity is due to changes in *flp-1* expression levels or changes in the expression of other *flp* genes as well. A neuronal nucleus was present at the AVK position when FMRFamide expression was not detected by immunofluorescence, suggesting that, even when AVK neurons fail to express FMRFamide, the neurons are still present and normally positioned (Fig. 3C,D). In addition, the reduction of FMRFamide levels in *fax-1* mutants is not a necessary consequence of the AVK axon pathfinding defects, as mutations in other genes that cause AVKR axon pathfinding defects similar to those observed in *fax-1* mutants, such as *unc-42* and *unc-115*, do not display detectable reductions in FMRFamide immunoreactivity (Wightman et al., 1997; B.W., unpublished data). Therefore, *fax-1* is partially required, directly or indirectly, for regulation of *flp-1* and possibly other *flp* genes.

Consistent with these results, examination of *flp-1* RNA levels in *fax-1* mutants revealed a reduction in steady-state *flp-1* RNA. We used relative quantitative RT-PCR to compare the amount of *flp-1* RNA in *fax-1* mutants to wild-type. *fax-*



C

AVK axon pathfinding		Percentage of axons: Wild-type	*fax-1 (gm83)*	*fax-1 (gm27)*
AVKR	L	98	46	63
	R	0	14	5
		1	22	21
		1	19	12
	n =	67	59	43
AVKL	L	100	68	74
	R	0	8	9
		0	20	14
		0	5	2
	n =	70	65	43

Fig. 2. AVK axon defects in *fax-1* mutants. Immunohistochemical staining of GFP expression in AVK axons of wild-type and *fax-1* mutant adult hermaphrodites. Left side views: anterior is to the left, dorsal is up. All animals carry the *flp-1::gfp* transgene that expresses GFP in the cytoplasm of the bilaterally symmetric AVK neurons. Arrows designate the AVKL cell body. Straight lines designate the position of the nerve ring. (A) Wild-type animal showing circumnavigation of the nerve ring by AVK axons. Scale bar, 10 μm. (B) *fax-1(gm83)* mutant showing pathfinding error by the AVKL axon. The axon exits the nerve ring and projects along the DNC (arrowhead). (C) Summary of AVK axon defects. Drawings at left show ventral views of axon anatomy of AVKR and AVKL neurons in the head. n, number of AVK neurons examined for each genotype.

1(gm83) and *fax-1(gm27)* mutants displayed *flp-1* RNA levels that were 0.61 times ($P<0.05$) and 0.49 times ($P<0.01$) the level detected in wild-type, respectively (data not shown).

fax-1 encodes a member of the nuclear hormone receptor superfamily

To understand the mechanism of *fax-1* function in axon pathfinding, we cloned the gene using a standard transformation rescue approach (Fig. 4). A 9.2 kb *Sac*II-*Hpa*I subclone derived from cosmid F56E3 (pF56SH9) was sufficient to rescue the *fax-1* Uncoordinated (Unc) phenotype and the AVKR axon pathfinding defects of a *fax-1* mutant (Fig. 4C). This subclone includes a predicted gene (F56E3.4) that shows sequence similarity to the superfamily of nuclear hormone receptor zinc-finger transcription factors. Deletion of the region of pF56SH9 predicted to encode the DNA-binding domain (DBD) and most of the ligand-binding domain (LBD) resulted in a loss of all *fax-1* rescuing activity (pSH9ΔSp), indicating that this nuclear receptor is the *fax-1* gene. *fax-1* cDNAs were isolated by PCR amplification. The largest open-reading frame predicted from analysis of cDNA clones encodes a 419 amino acid protein that is similar to other members of the nuclear hormone receptor superfamily (Fig. 5). Therefore, FAX-1 is likely to regulate the transcription of other genes.

The predicted FAX-1 DBD is 86% and 71% identical to the human photoreceptor-specific nuclear receptor (PNR) and *Drosophila melanogaster* terminal-class receptor Tailless (Tll), respectively (Fig. 5B,C; Pignoni et al., 1990; Kobayashi et al., 1999). FAX-1 is also similar to the PNR and Tll proteins in the T/A box located immediately C-terminal to the zinc-finger region, which has been implicated in dimerization and DNA binding (Wilson et al., 1992). In this domain, FAX-1 is 66% and 67% identical to PNR and Tll, respectively, but is more diverged from most other nuclear receptors. Two other regions of the DBD, the P box and D box, have been shown to play significant roles in recognition of specific DNA sequences (Schwabe et al., 1993). FAX-1 is identical to PNR and similar to Tll in the P box. Finally, most nuclear receptors have a 5 amino acid D box in the second zinc-finger, whereas Tll has a 7 amino acid D box and FAX-1 and PNR have a 6 amino acid D box. These observations suggest that FAX-1 is a member of a conserved subfamily of nuclear receptors and may bind to a DNA sequence related to the Tll binding site (Hoch et al., 1992). FAX-1 is considerably more diverged from PNR, Tll and all other known nuclear receptors in the LBD (less than 25% identical; data not shown).

Both existing *fax-1* mutations are likely to eliminate *fax-1* function. Southern blot analysis of genomic DNA from *fax-1 (gm27)* mutants demonstrated that this mutation is associated with a deletion that entirely eliminates *fax-1* and several flanking predicted genes (Fig. 3A; Materials and Methods). cDNA and genomic clones of the *fax-1* gene isolated from *fax-1 (gm83)* mutants identified a C to T point mutation in the *fax-1* DBD that converts an arginine codon into a stop codon (Fig. 5A). Therefore, if FAX-1 protein is produced in *fax-1(gm83)* mutants, it is likely to be lacking the second zinc-finger, T/A box and entire LBD, suggesting that it is a null mutation.

fax-1::gfp translational fusion genes are expressed in neurons, including the AVKs

We constructed a *fax-1::gfp* translational fusion gene (pFX1G1) to address where and when the *fax-1* gene is expressed (Fig. 6). Transgenic animals bearing pFX1G1 had high levels of GFP fluorescence or immunoreactivity in embryonic and postembryonic neurons (Fig. 7). We first detected *fax-1::gfp* expression in embryos prior to elongation (approximately 350 minutes of development). At this time, most neurons have been generated, but have not yet extended axons. Axon extension begins prior to 480 minutes during the initial stages of the elongation of the embryo (Durbin, 1987). By approximately 400 minutes, we observed strong *fax-1::gfp* expression in as many as 20 neurons in the embryonic head and 1-2 neurons in the embryonic tail. GFP immunoreactivity was present in the cytoplasm, axons and nuclei of cells. Axons of neurons that express *fax-1::gfp* embryonically were observed in the process of outgrowth (Fig. 7A). Therefore, as judged by *fax-1::gfp* expression, the *fax-1* gene is likely to be expressed in neurons at a time appropriate for regulation of axon pathfinding.

In transgenic animals carrying pFX1G1, we observed *fax-1::gfp* expression in 20 neurons postembryonically, through the adult stage (Figs 6, 7B,C). The position of these neurons indicates that most or all of them are among the 22 neurons that express *fax-1::gfp* embryonically. These cells include both

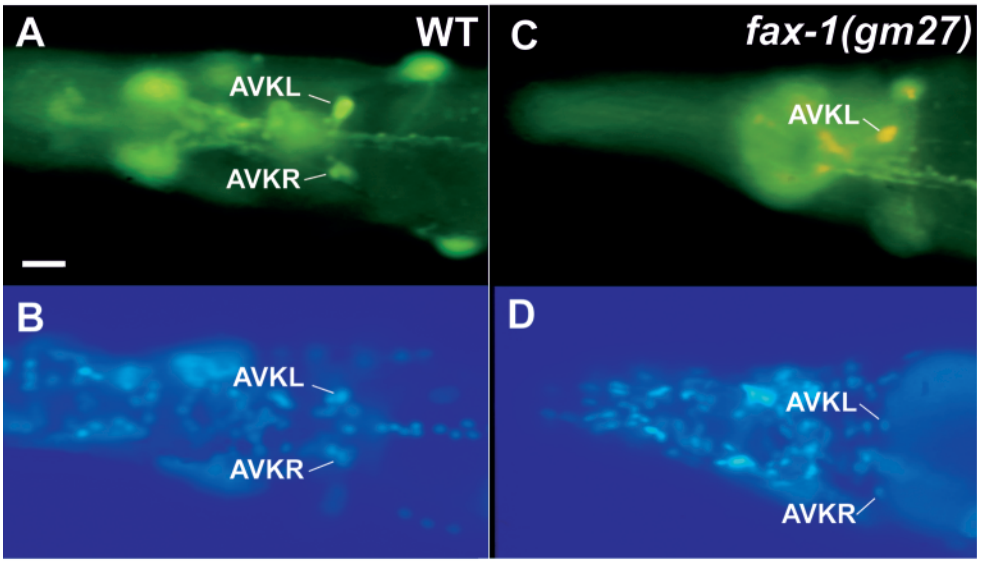


Fig. 3. FMRFamide-related expression in *fax-1* mutants. Immunohistochemical staining of FMRFamide-related products in the AVK neurons (labeled) of wild-type (A,B) and *fax-1(gm27)* mutant (C,D) adult hermaphrodites. Ventral views, anterior to left. (A,C) FMRFamide immunoreactivity; (B,D) nuclei of the same animals detected by DAPI staining. Scale bar, 10 μm.

AVKR and AVKL (Fig. 7C), suggesting that *fax-1* may function in the AVK neurons to regulate pathfinding, as opposed to functioning in another neuron on which AVK depends for pathfinding. We did not observe expression of *fax-1::gfp* in either of the HSN or PVQ neurons, or in the PVPR neuron at any stage of development.

We identified *fax-1::gfp* expression in several other neurons and two non-neuronal cell types in transgenic animals carrying pFX1G1. These include the pairs of CEPD and URX sensory neurons, three pharyngeal neurons (M1, MI and probably M5), two pairs of ring interneurons (including the RIC pair), five neurons in the retrovesicular ganglion (including SABD and the pair of SABV neurons), a single neuron in the preanal ganglion (either PVPL or PVT) and a single neuron in the dorsorectal ganglion of the tail (probably DVA). *fax-1* expression and function in these neurons will be addressed in a future study. We also observed incompletely penetrant *fax-1::gfp* expression in a few additional neurons that we did not identify, and in the non-neuronal dorsal rectal cell and distal tip cells of the somatic gonad.

fax-1 expression in AVK correlates with rescue of AVK axon pathfinding defects

The pF56SH9 subclone rescued both the AVKR pathfinding and Unc phenotypes of *fax-1* mutants. In contrast, a 6.7 kb *Hpa*I subclone (pF56Hp6) that is missing 2.5 kb of 5′ sequence flanking the *fax-1* coding region rescues the *fax-1* Unc phenotype fully, but does not rescue the AVKR pathfinding defects (Fig. 4C). This observation suggested that regulatory sites located in the 2.5 kb of 5′-flanking region that is missing from the 6.7 kb subclone may be required for *fax-1* function in AVKR pathfinding. We constructed a *fax-1::gfp* reporter construct that is missing the 2.5 kb of 5′-flanking sequence (pFX1G2) to address the possibility that *fax-1::gfp* expression in some neurons required this region (Fig. 6). Transgenic animals bearing the smaller pFX1G2 *fax-1::gfp* reporter expressed GFP in most of the 20 postembryonic cells observed for the related pFX1G1 fusion, with the exception of the AVK neurons, URX neurons, a pair of ring interneurons and the neurons of the preanal and dorsorectal ganglia (Fig. 6). These data suggest that *fax-1* expression in one or more of these neurons is required for guidance of AVK growth cones. An appealing possibility is that *fax-1* functions cell autonomously in the AVK neurons to direct pathfinding by AVK growth cones.

DISCUSSION

fax-1 regulates pathfinding by growth cones

The pathfinding defects observed in *fax-1* mutants suggest that growth cones are unable to recognize the cues they normally follow in the VNC and nerve ring. The HSNL and PVQL growth cones extend along the right VNC bundle instead of the left VNC bundle in *fax-1* mutants (Wightman et al., 1997). Laser ablation studies have shown that pathfinding by the HSNL growth cone requires the presence of the PVQL and PVPR axons (Garriga et al., 1993), while pathfinding by the PVQL growth cone requires the presence of the PVPR axon (Durbin, 1987). These data suggest that molecular cues are produced by the pre-existing axons (PVPR and PVQL), and that later-extending axons (AVKR and HSNL) respond to these cues via receptor molecules that allow them to distinguish among various cues. Because pathfinding by the PVPR pioneer axon is normal in *fax-1* mutants (Wightman et al., 1997; B.W., unpublished results), we proposed that *fax-1* may function in the recognition of PVPR and/or PVQL axons by later-extending growth cones.

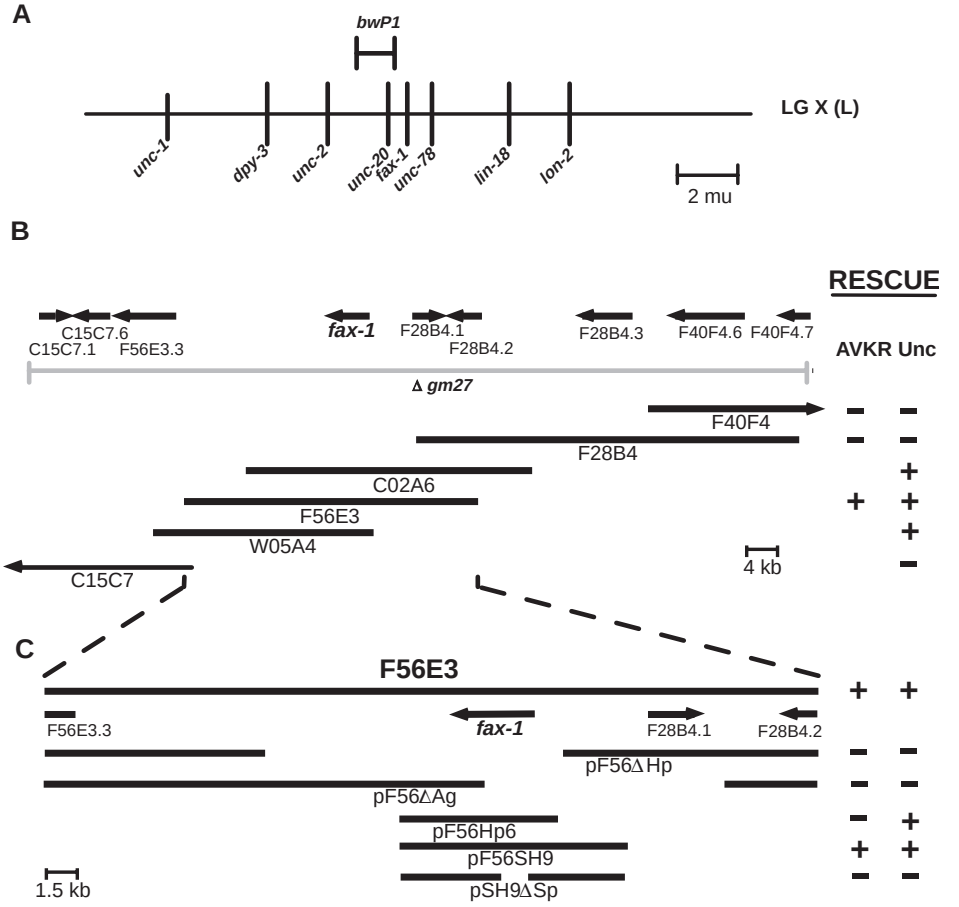


Fig. 4. *fax-1* cloning. (A) Genetic map of a portion of the left arm of the X chromosome. (B) Physical map in the *fax-1* region. Arrows indicate the positions of predicted genes from the *C. elegans* genome sequencing effort. The region deleted by the *gm27* mutation is shown by the gray bar. DNA included in cosmid clones is shown below. Right-hand columns indicate transformation rescue data for each genomic clone in a *fax-1(gm83)* mutant background. The AVKR column summarizes results for rescue of AVKR pathfinding defects. The Unc column summarizes results for rescue of the movement defect. + denotes rescue of mutant phenotype, – denotes failure to rescue. Where no symbol is shown, the experiment was not performed. (C) Subclones derived from cosmid F56E3.

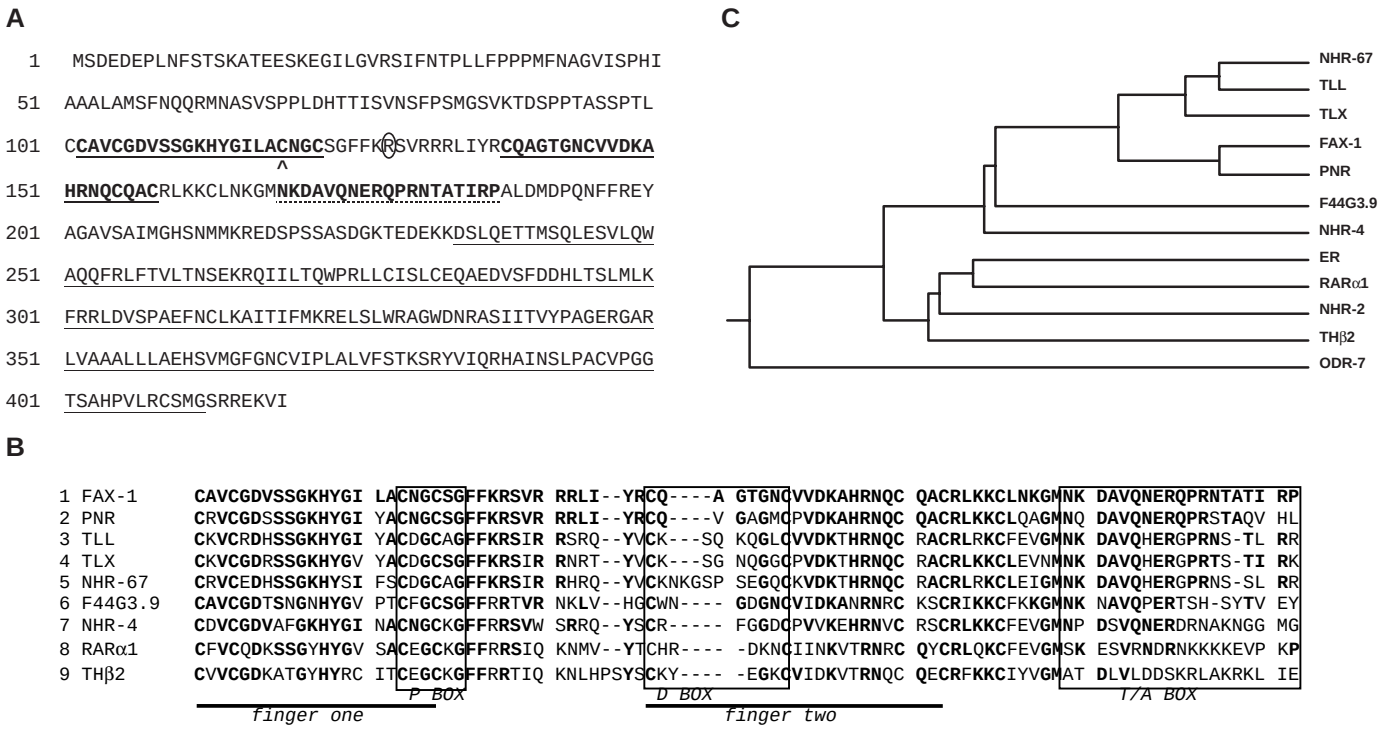


Fig. 5. *fax-1* sequence. (A) Predicted FAX-1 amino acid sequence. Bold with solid underline indicates the two zinc fingers of the DBD. Dashed underline indicates the T/A box. Plain text with solid underline indicates the predicted LBD. The C terminus of the LBD is approximate because the FAX-1 amino acid sequence diverges significantly from other nuclear hormone receptors in this region. The circled Arg residue is converted into a stop codon by the *fax-1(gm83)* mutation. Carat indicates the positions at which GFP was fused to create pFX1G1 and pFX1G2. (B) Alignment of FAX-1 and selected nuclear hormone receptor DBD's. Amino acid residues that are identical between FAX-1 and other proteins are bolded. (C) Dendogram of FAX-1 and selected nuclear hormone receptors illustrating the relationship among the *tailless*-class receptors at the uppermost portion of the tree. Accession numbers for sequences used are: human PNR (AF121129), *D. melanogaster* Tll (P18102), chicken Tlx (S48074), *C. elegans* NHR-67 (Z73103), *C. elegans* F44G3.9 (Z83109), *C. elegans* NHR-4 (AF083223), human retinoic acid receptor alpha 1 (RARα1; P10276), human thyroid hormone receptor beta 2 (Thβ2; P37243), *C. elegans* NHR-2 (Q10902), *C. elegans* ODR-7 (P41933), chicken estrogen receptor (ER; P50240). The accession number for the *fax-1* sequence reported here is AF176087.

In addition to VNC pathfinding defects, both AVK neurons display defects in pathfinding in the nerve ring of *fax-1* mutants (Fig. 2). This is significant because both AVK axons fasciculate with different axons in the nerve ring as compared to the VNC. The AVKR axon is most closely associated with the PVPR and RMEV axons in the left VNC bundle, while the AVKL axon is most closely associated with the DVA and PDE axons in the right VNC bundle (White et al., 1986). In the nerve ring, both AVK axons are closely associated with each other, and with the AVE, DVB, DVC, RIG, RIM, RIS and RMF axons. While it is not known for certain which axons the AVK growth cones follow in the nerve ring or VNC, these observations suggest that the two AVK growth cones respond to different axons in the two VNC bundles, and to different axons in the nerve ring as compared to the VNC. Therefore, *fax-1* may be required for AVK growth cones to respond to one or more cues from different pre-existing axons.

Expression of *fax-1::gfp* reporters in the AVK neurons just prior to axon extension is consistent with the idea that *fax-1* regulates pathway choice in the AVK growth cones. However, pathfinding defects by HSNL and PVQL growth cones are not as easily explained by the *fax-1::gfp* expression pattern. We did not observe *fax-1::gfp* expression in the HSNL, PVQL or PVPR neurons, so there is no evidence to support cell-autonomous *fax-1* function in any of these cells. The HSNL and PVQL pathfinding defects are unlikely to be consequences of the AVK pathfinding defects: the HSNL growth cone navigates along the left VNC bundle normally in the absence of the AVKR axon (Garriga et al., 1993), and the PVQL axon extends along the left VNC bundle prior to the AVKR axon (Durbin, 1987). One possibility is that the HSNL and PVQL pathfinding defects could be consequences of a *fax-1* requirement in another cell type. For example, *fax-1* function in PVPL, PVT, or DVA could be required for the PVPR neuron to express the appropriate pathway signaling molecules on which the later-extending HSNL and PVQL growth cones depend. Alternatively, pathfinding defects by right VNC bundle axons that we have not examined may lead to the presence of ectopic axons in the left VNC bundle of *fax-1* mutants that cause the HSNL and PVQL growth cones to be repelled from their normal pathway.

Phenotypic analysis suggests that *fax-1* is likely to function in other neurons in addition to the AVKs. Mutations in *fax-1* cause animals to display Uncoordinated (Unc) movement. Because the AVKR, HSNL and PVQL do not appear to play a role in locomotion, this observation suggests that *fax-1* is also required for the development or function of other neurons.

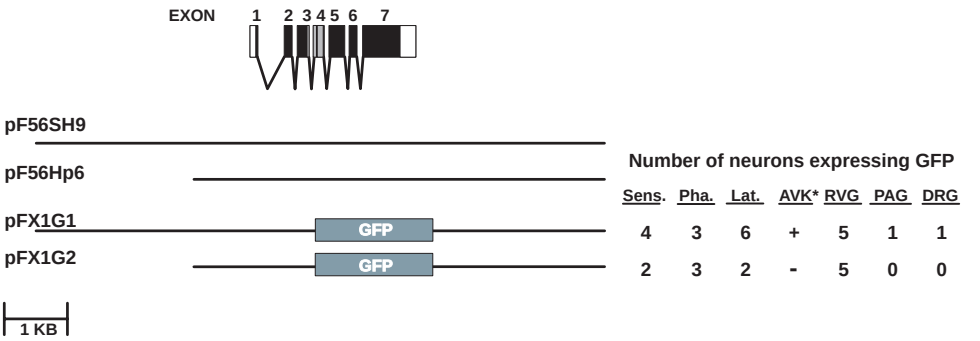


Fig. 6. *fax-1::gfp* reporter constructs used in this study. Schematic at left diagrams DNA included in each construct. For reference, the regions included in rescuing genomic subclones pF56SH9 and pF56Hp6 are shown at top. Table at right summarizes expression data for each *gfp* reporter. Sens., sensory neurons, including CEPDR/L and URXR/L; Pha., pharyngeal neurons, including M1 and MI; Lat., lateral and ventral ganglion neurons; RVG, retrovesicular ganglion neurons, including SABD and SABVR/L; PAG, preanal ganglion neuron; DRG, dorsorectal ganglion neuron. *AVK indicates whether the AVKR/L neurons are among the lateral and anterior neurons that express GFP.

These may include some of the neurons that express *fax-1::gfp* (Figs 6, 7).

FAX-1 may regulate the transcription of genes that function directly in neuron identity

Because *fax-1* encodes a predicted member of the superfamily of nuclear hormone receptor transcription factors (Fig. 5; reviewed by Mangelsdorf et al., 1995), it is likely to function as a regulator of the transcription of other genes. There are several precedents for transcriptional regulation of axon pathway selection: *unc-30* and *unc-42* in *C. elegans* (Jin et al., 1994; Wightman et al., 1997; Baran et al., 1999), and *eagle* and *apterous* in *Drosophila* (Lundgren et al., 1995; Dittrich et al., 1997). In addition, nuclear hormone receptors in particular have been shown to have roles in nervous system development in a variety of organisms. For example, the *staggerer* nuclear receptor of mice has been shown to be required for Purkinge cell generation and identity (Hamilton et al., 1996), and the *unc-55* nuclear receptor of *C. elegans* regulates synaptic specificity (Zhou and Walthall, 1998).

Among the best-studied nuclear receptors that function in nervous system development are the *tailless* gene of *Drosophila (tll)* and its vertebrate orthologs, the *tlx* genes. In addition to being required for anteroposterior pattern formation in the *Drosophila* embryo, *tll* also functions in the generation, survival and/or differentiation of embryonic CNS neurons (Strecker et al., 1988). Deletion of the mouse *tlx* gene leads to significant reduction of the limbic system and increased aggressiveness (Monaghan et al., 1997).

The function of the human PNR receptor related is not known, but expression of this protein appears restricted to photoreceptor cells of the retina (Kobayashi et al., 1999). In light of the common function in nervous system development, the strong sequence similarity among *fax-1*, PNR and *tll* is intriguing. However, the amino acid relationships among these proteins is limited to the DBD (Fig. 5B), suggesting that functional conservation may be limited to DNA-binding specificity. Tll binds the sequence AAGTCA, either as monomers or dimers, which is distinct from those bound by other nuclear receptors with known specificities (Hoch et al., 1992; Yu et al., 1994; Mangelsdorf et al., 1995). While it is possible that FAX-1 may recognize Tll binding sites, a few key amino acids that are known to be directly involved in contacting base-pairs in other nuclear receptors are not identical in FAX-1 and Tll (see Results), leaving open the

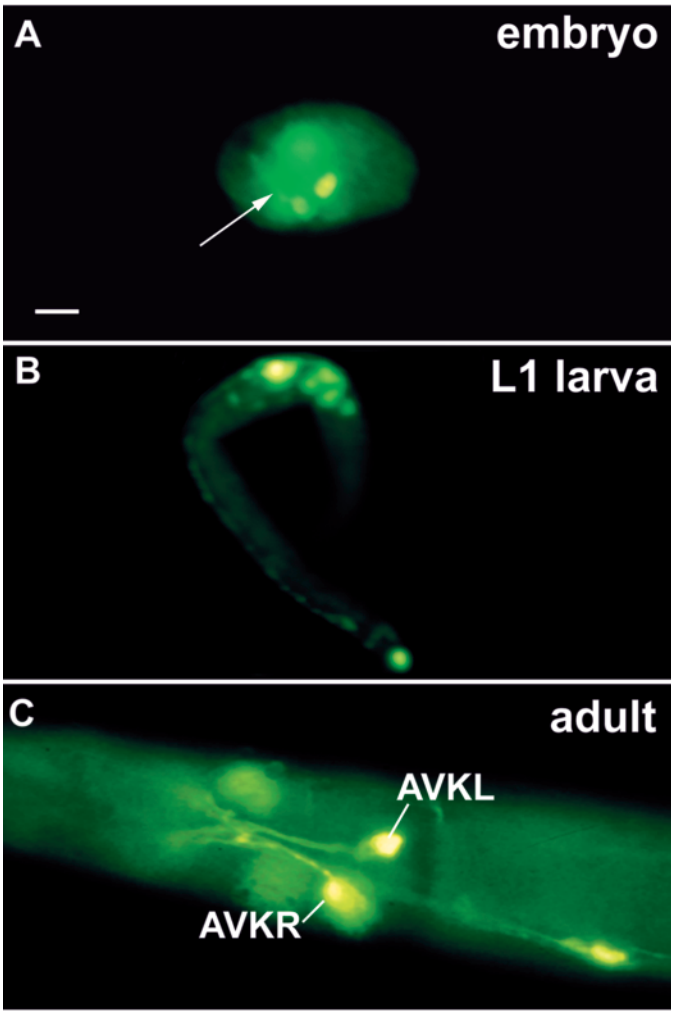


Fig. 7. Expression of *fax-1::gfp* reporter constructs in wild-type animals. Immunohistochemical staining of GFP. (A) Embryonic expression at approximately 350 minutes of development from the pFX1G1 construct. Left lateral view. Arrow identifies an axon projecting from a neuronal cell body. (B) Expression in an L1 larva from the pFX1G1 construct. Left lateral view. Neuron cell bodies in the anterior and ventral ganglia are visible at top just out of the plane of focus, as well as expression in the preanal and dorsorectal ganglia. (C) Expression in the AVKR and AVKL interneurons of an adult hermaphrodite from the pFX1G1 construct. Ventral view. Neurons in the retrovesicular ganglion are visible at lower right. Scale bar, 10 μm.

possibility that FAX-1 may recognize a distinct DNA sequence, perhaps one related to the AAGTCA site of Tll. PNR has been shown to bind to dimeric AAGTCA, but not monomeric AAGTCA sites in vitro (Kobayashi et al., 1999).

An important goal for future research on *fax-1* will be the identification of downstream regulated genes, as some of these could include direct mediators of axon pathfinding. One possible target for *fax-1* function is the *flp-1* gene. The observation that *flp-1::gfp* expression is altered in *fax-1* mutants suggests that *fax-1* may be partially required for *flp-1* transcription. Consistent with this possibility, both *flp-1* and *fax-1* mutants display hyperforaging behavior and wander off bacterial lawns (Wightman et al., 1997; Nelson et al., 1998b; D. Brightbill, G. Sarver and B. W., unpublished results). Thus, some Fax-1 phenotypes may result from changes in FMRFamide-related neurotransmitter levels. *fax-1* regulation of *flp-1* could be direct or indirect, and a biochemical study of *fax-1* function should help resolve this issue. There are two potential nuclear receptor binding sites immediately upstream of the *flp-1* gene that have the sequence TCGTCA, but there are none that match the AAGTCA site of Tll.

Aspects of neuron identity are coordinately regulated

The observation that mutations in *fax-1* disrupt both neurotransmitter expression and axon pathfinding in a single cell type (AVK) suggests that axon pathway selection and neurotransmitter expression may be coordinately regulated. Similar observations have been made in *Drosophila:* the *eagle* gene encodes a zinc finger protein that regulates both axon pathfinding and expression of the neurotransmitter serotonin, and the *apterous* gene encodes a LIM protein that regulates axon pathfinding and FMRFamide expression in the ventral nerve cord (Dittrich et al., 1997; Benveniste et al., 1998).

Some regulators of neuronal identity have been identified that transform, or partially transform, a neuron into another type of neuron with concomitant changes in axon pathfinding and other properties. For example, mutations in *mec-3* and *unc-4* cause conversion of neurons into different types of neurons (Way and Chalfie, 1988; Miller and Niemeyer, 1995). In these cases, the affected neurons appear to be transformed into their sister cells. For example, mutations in the *mec-3* gene, which encodes a LIM homeodomain protein, cause defects in axon pathfinding by the ALM neuron (Way and Chalfie, 1988). However, the pathway chosen by the ALM growth cone is that of its sister cell, the BDU neuron, suggesting that the ALM neuron's identity has been changed to BDU. This does not appear to be the case in the AVK neurons of *fax-1* mutants. The growth cones of the AVK neurons variably select either dorsal or lateral pathways, suggesting a more general 'confusion' concerning pathway choice rather than a reprogramming to a different route. In addition, the sister cells of the AVK neurons are the excretory gland cells (Sulston et al., 1983). Transformation of the AVK neurons into this non-neuronal cell type would be expected to have much more dramatic consequences for AVK morphology than are observed in *fax-1* mutants. Therefore, it does not appear that *fax-1* functions in directing the AVK neurons to adopt a different identity than their sister cells.

fax-1 probably does not function in neurogenesis either. We have not observed changes in the numbers of neurons that we have examined (e.g. AVK, HSN, PVP, PVQ, etc.) in *fax-1* mutants. Moreover, we do not observe expression of *fax-1::gfp* in embryos until after the relevant neurons are generated.

Taken together, these observations suggest that *fax-1* functions after neurons are generated to specify discrete aspects of cell identity. Among these aspects are pathway selection by growth cones and neurotransmitter expression. The observation that both pathway selection and neurotransmitter expression in the AVK neurons require *fax-1* suggests a model for neuron development in which different components of specific neuron identity are coordinately regulated, perhaps by a highly combinatorial mechanism. Given the precedents for coordinate control of axon pathfinding and neurotransmitter expression, this model may explain how individual neuronal types are specified throughout the animal kingdom.

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