Genes that guide growth cones along the C. elegans ventral nerve cord

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SUMMARY

During nervous system development, growth cone pioneering and fasciculation contribute to nerve bundle structure. Pioneer growth cones initially navigate along neuroglia to establish an axon scaffold that guides later extending growth cones. In C. elegans, the growth cone of the PVPR neuron pioneers the left ventral nerve cord bundle, providing a path for the embryonic extensions of the PVQL and AVKR growth cones. Later during larval development, the HSNL growth cone follows cues in the left ventral nerve cord bundle provided by the PVPR and PVQL axons. Here we show that mutations in the genes enu-1, fax-1, unc-3, unc-30, unc-42 and unc-115 disrupt pathfinding of growth cones along the left ventral nerve cord bundle. Our results indicate that unc-3 and unc-30 function in ventral nerve cord pioneering and that enu-1, fax-1, unc-42 and unc-115 function in recognition of the PVPR and PVQL axons by the AVKR and HSNL growth cones.

Key words: C. elegans, growth cone, pioneer, axon pathfinding, fasciculation

INTRODUCTION

Both complex behaviors and simple reflexes are mediated by circuits of appropriately connected neurons. The complexity of the nervous systems in which these connections form makes studying their development a challenging problem. This complexity is particularly bewildering when considering the vertebrate nervous system, which contains billions of neurons that project axons through complicated terrain to reach their synaptic partners. Even for simpler organisms, the problem of how neural circuits develop is formidable. For example, the adult hermaphrodite VNC consists of 302 neurons of 118 classes that make approximately 7000 synapses (White et al., 1986).

White and colleagues proposed the ‘neighborhood hypothesis’ to account for nematode synaptic connectivity (White et al., 1983, 1986). Ultrastructural analysis of the C. elegans nervous system revealed that synapses are made between adjacent axons en passant and that axons maintain their neighbors (adjacent axons) for long distances within axon bundles. Within a nerve bundle axons synapse on average with half of their neighboring axons. This level of connectivity is surprising and suggests that the ‘neighborhood’ an axon inhabits contributes significantly to its synaptic specificity. White et al. (1983, 1986) proposed that neighborhoods in C. elegans are generated by selective pathfinding of growth cones using neighborhood-specific surface molecules as recognition cues. This proposal is analogous to the ‘labeled pathway hypothesis’ proposed to account for axon pathfinding in insects and vertebrates (Raper et al., 1983; Kuwada, 1986).

Consistent with White’s model for selective pathfinding in C. elegans, two studies showed that specific axons of the ventral nerve cord (VNC) provide cues for later-extending growth cones (Durbin, 1987; Garriga et al., 1993). The adult hermaphrodite VNC consists of two parallel axon bundles that extend the length of the animal; a large bundle of approximately 40 axons on the right side is separated by a ridge of epithelium from a small bundle of 3 to 5 axons on the left side (White et al., 1976; White et al., 1986; Fig. 1). The AVG and PVPR growth cones pioneer the right and left axon bundles of the VNC, respectively. AVG and PVPR axons then provide paths that guide later-extending growth cones. For example, the PVPR axon provides a path for the extensions of the PVQL, AVKR and HSNL growth cones along the left VNC bundle.

With the goal of understanding how growth cones select specific pathways, we are studying the cellular and molecular cues that guide the HSN growth cones along the VNC. The HSNs are a pair of serotonergic motor neurons that innervate the vulval muscles and stimulate egg laying by the hermaphrodite (Trent et al., 1983; White et al., 1986). The HSNs are particularly useful for studying axon pathfinding for two reasons. First, laser microsurgery has revealed several of the cellular cues that guide the HSN growth cones (Garriga et al., 1993). Second, the HSNs are the last neurons to extend growth cones along the VNC (White et al., 1986) and are therefore a particularly sensitive indicator of VNC organization.

In order to identify genes that guide growth cones along the VNC, we screened C. elegans behavioral mutants for defects in HSN axon morphology. Here, we describe our analysis of six genes that guide growth cones along the VNC. The genes unc-3 and unc-30 are required for VNC pioneering, whereas the genes enu-1, fax-1, unc-42 and unc-115 are required for...
pathfinding of later-extending growth cones, such as those of AVK and HSN, along established nerve tracts.

MATERIALS AND METHODS

Strains and genetics

Strains of *C. elegans* were grown at 20°C and maintained as described by Brenner (1974). In addition to the standard wild-type strain (N2), strains with the following mutations were used in this work:

- LGI: unc-14(e686), unc-73(e936)
- LGII: enu-1(ev419), unc-53(e404)
- LGIII: unc-69(e587), unc-71(e541)
- LGIV: unc-30(e191), unc-30(e2327), unc-33(e204), unc-44(e362)
- LGV: egf-37(n1082dm), unc-34(e506), unc-42(e270), unc-42(e419), unc-42(e623), unc-42(gm23), unc-76(e911), unc-51(e369)
- LGX: fax-1(gm27), fax-1(gm83), lon-2(e678), unc-2(e55), unc-3(e151), unc-3(e131), unc-20(e112), unc-115(e2225), unc-115(mn490), xol-1(y9)

The genes lon-2, unc-3, unc-14, unc-20, unc-30, unc-33, unc-34, unc-42, unc-44, unc-51, unc-53, unc-69, unc-71, unc-73 and unc-76 were originally described by Brenner (1974). The egl-47(n1082dm) mutation was described by Desai and Horvitz (1989). The gene xol-1 was described by Miller et al., 1988. The gene unc-115 was originally defined by the e2225 allele (Danielle Thierry-Mieg, personal communication), and the unc-115(mn490) allele was subsequently isolated by Erik Lundquist (personal communication).

The gene enu-1 (enhancer of Unc) was defined by a mutation in the uncoordinated double mutant enu-1(ev411); yab-8(e419) by Joe Culotti (personal communication). enu-1(ev419) acts as an enhancer of the yab-8 Unc phenotype, but has no effect on movement in the absence of a yab-8 mutation (J. Culotti, personal communication; B.W. and G.G., unpublished results).

The gene fax-1 (fasciculation of axons defective) was defined by the gm27 mutation, which was identified by David Hsu during a gamma-ray mutagenesis screen because it caused Egl-c (α-tubulin) to map to the hermaphrodite Unc phenotype. The egl-47(n1082dm) mutation was described by Desai and Horvitz (1989). The fax-1(gm27) mutation was recovered from a screen of 17,000 F1 progeny. The egl-47(n1082dm) mutation was described by Desai and Horvitz (1989). The gene xol-1 was described by Miller et al., 1988. The gene unc-115 was originally defined by the e2225 allele (Danielle Thierry-Mieg, personal communication), and the unc-115(mn490) allele was subsequently isolated by Erik Lundquist (personal communication).

Indirect immunofluorescence histochemistry

For all staining procedures, animals were grown at 25°C prior to fixation. Indirect immunofluorescence histochemistry was used to stain animals for serotonin, which detects AVK axons, and FMRFamide, which detects AKR axons, using the procedure described by McIntire et al. (1992). Anti-serotonin antisera was provided by J. Steinbusch (Free University, Amsterdam) and anti-FMRFamide antisera was provided by Chris Li (Boston University). Both antisera were used at 1%.

The entire left VNC bundle was visualized with the 41A monoclonal antibody that recognizes *Drosophila* α-tubulin (Piperno and Fuller, 1985). Animals were fixed, permeabilized by the method of Finney and Ruvkun (1990), and incubated overnight at room temperature in 1% 41A antibody. Occasionally animals were not permeabilized using this procedure. In these cases the animals were placed in a Dounce homogenizer and permeabilized with three strokes of the pestle.

Double stain with anti-serotonin and anti-tubulin antibodies, animals were fixed and treated with β-mercaptoethanol as described by McIntire et al. (1992). Animals were then placed in a Dounce homogenizer and permeabilized with 15-20 strokes of the pestle.

FITC- or Texas Red-conjugated secondary antibodies were obtained from Cappel, Inc. Stained worms were viewed by immunofluorescence microscopy using a Zeiss microscope and Zeiss filters no. 487910 and no. 487915. Images were photographed using Ektachrome T160 film, scanned with a Nikon scanner to a computer graphics file, and annotated and enhanced to improve contrast using the Adobe Photoshop graphics program.

Analysis of PVQ axons using a GFP reporter

The PVQ axons were visualized in live animals that carry an *sra-6-gfp* transgene (Troemel et al., 1995). This reporter gene fusion expresses the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Chalfie et al., 1994) in PVQ and additional axons. A plasmid containing the *sra-6-gfp* fusion gene was provided by Cori Bargmann (UCSF). Both the *sra-6-gfp* and *prF4* plasmids were microinjected at concentrations of 100 μg/ml each into gonads of wild-type hermaphrodites to obtain extrachromosomal array *gmxEx71* (Mello and Fire, 1995). The *prF4* plasmid carries the dominant *rol-6(su1006) allele* that causes animals to roll (*rol* phenotype) and is used as a marker for presence of the array (Mello et al., 1991). The *gmxEx71* array was crossed into mutants with axon pathfinding defects by mating wild-type males to *gmxEx71 Rol* hermaphrodites. F1 Rol males from this cross were mated to mutants with axon pathfinding defects. Rol Unc hermaphrodites were isolated from F3 progeny to obtain strains that were homozygous for the mutation being examined and carried *gmxEx71*. The anatomy of PVQ was determined by examining larval and adult transgenic animals using a Nikon Labophot microscope and filter BV 2A-303.

Much brighter and reproducible fluorescence was achieved by immunofluorescence of *gmxEx71*-bearing wild-type and mutant animals using anti-GFP antisera (Clontech). Strains that carry the *gmxEx71* array were fixed and permeabilized with collagenase as described above for serotonin and FMRF staining. Animals were incubated in 0.1% anti-GFP antisera and visualized with FITC-conjugated secondary antibody as described by McIntire et al. (1992).

Screen for pathfinding mutants

Mutants exhibiting the Unc phenotype were stained with an anti-serotonin antisera to reveal HSN axon morphology. In wild type, the left and right HSN growth cones extend anteriorly along the ipsilateral VNC bundle to the nerve ring; anti-serotonin staining of adult hermaphrodites reveals two distinct HSN axons that extend along separate VNC bundles. In this report, we consider mutations in 19 Unc genes that cause the two HSN axons to extend together in a single fascicle. We only consider those mutants that exhibited this phenotype in over 40% of the animals. During embryogenesis, the HSN cell bodies are born in the tail and migrate anteriorly to the center
of the embryo. Because posterior displacement of the HSN cell bodies causes the HSNs axons to extend together along the VNC, we do not consider those Unc mutants with defects in both HSN migration and pathfinding.

RESULTS

Structure and development of the *C. elegans* ventral nerve cord

The major longitudinal connective of *C. elegans*, the ventral nerve cord (VNC), consists of an asymmetric pair of axon bundles that run along the ventral midline of the body wall and flank a bulge of ventral epithelium known as the hypodermal ridge. In the adult hermaphrodite, the right VNC bundle contains approximately forty axons, whereas the left bundle contains only four axons at the midbody (Fig. 1A).

Serial EM reconstruction of staged embryos has defined early developmental events that contribute to VNC structure (Durbin, 1987). Longitudinal extensions of the AVG and PVPR pioneer growth cones provide paths for the establishment of the right and left VNC bundles, respectively. From the retro-vesicular ganglion near the anterior end of the embryo, the midline neuron AVG extends the first growth cone posteriorly along the ventral epithelium to the tail. After the AVG growth cone reaches the preanal ganglion in the tail, the bilaterally symmetric PVP neurons of the preanal ganglion extend growth cones anteriorly. These growth cones first decussate at the ventral midline and then extend along contralateral sides of the ventral hypodermal ridge. At the same time, the bilaterally symmetric PVQ neurons of the lumbar ganglia in the tail extend growth cones ventrally to the preanal ganglion where they fasciculate and travel anteriorly with the PVP growth cones. Thus, the PVPR and PVQL are the first neurons to extend growth cones along the left side of the ventral hypodermal ridge. After the PVP and PVQ growth cones have reached the nerve ring, a neuropil that encircles the pharynx, each of the bilaterally symmetric AVK neurons of the retro-vesicular ganglion extends a growth cone posteriorly along the contralateral VNC bundle (see Fig. 1B for left bundle development).

The AVG and PVPR axons provide paths that guide later-extending growth cones (Durbin, 1987). Laser-operated animals that lack AVG produce a disorganized VNC. In these animals, the right bundle splits into multiple bundles, and occasionally axons normally found in the right bundle extend along the left bundle. Laser-operated animals that lack PVPR produce a VNC that lacks a left bundle, with axons normally found in the left bundle extending ectopically along the right bundle. The observation that the left VNC bundle fails to form in the absence of PVPR, indicates that the PVPR axon provides a path for other axons, including PVQ. Although both PVPR and PVQL extend growth cones at the same time, we will refer to PVPR as the left VNC bundle pioneer since it is essential for formation of this bundle.

During larval development, each growth cone of the bilaterally symmetric HSN neurons extends ventrally, enters the VNC, and extends anteriorly along the ipsilateral VNC bundle to the nerve ring. Two cell types, vulval cells and axons of the VNC, guide the HSNL axon along the VNC (Garriga et al., 1993). Vulval cells, specialized epithelial cells at the ventral midline, prevent the HSNL growth cone from extending to the right bundle. Removal of vulval cells, either genetically or by laser microsurgery, causes the HSNL growth cone to pass over axons of the left bundle and extend inappropriately along the right bundle with the HSNR growth cone.

![Diagram of the C. elegans ventral nerve cord](image)

**Fig. 1.** Development of the *C. elegans* left VNC bundle. (A) Drawing of a transverse section through the anterior portion of the adult hermaphrodite VNC. Dorsal side is up. The locations of the axons of the left VNC bundle axons and their corresponding homologous axons of the right bundle are shown. In more anterior positions, the left bundle also contains the RMEV axon, which terminates after extending a short distance posteriorly from the head. cb, motorneuron cell body. Drawing is based on an electron micrograph shown in White et al. (1976, 1986). (B) Schematic drawing of the development of the left VNC bundle. A ventral view is shown, with posterior at the bottom. The lateral position of the PVQ cell body in the lumbar ganglion and the ventral extension of its axon to the preanal ganglion are not accurately represented in these drawings. (1) During mid-embryogenesis, the PVPR and PVQL growth cones pioneer the left VNC bundle, extending anteriorly from cell bodies located in the tail. (2) Later during embryogenesis, after the PVPR and PVQL axons have reached the nerve ring in the head, the AVKR growth cone extends around the nerve ring and posteriorly along the PVPR and PVQL axons. (3) During larval development, the growth cone of the laterally positioned HSNL cell body extends ventrally, enters the left VNC bundle, and grows anteriorly along the PVPR and PVQL axons.
The PVPR and PVQL axons provide partially redundant cues that guide the HSNL growth cone along the left bundle. Killing PVPR and PVQL neurons early in the first larval stage, after the embryonic VNC has been established but before the HSN axons extend, caused the HSNL growth cone to extend aberrantly. In all laser-operated hermaphrodites, the HSNL growth cone passed over the remaining A VKR axon of the left bundle and extended inappropriately along the right bundle with the HSNR growth cone. Killing PVPR alone did not alter HSNL axon pathfinding, whereas killing PVQL alone caused the HSNL growth cone to extend along the right bundle in approximately one third of the operated animals. Therefore, cues provided by both PVQL and PVPR axons guide the HSNL growth cone along the left VNC bundle.

Although the cellular cues that guide pathfinding along the more complex right VNC bundle are poorly understood, the PVPL, PVQR, and HSNR axons are adjacent to one another in the right bundle of the adult hermaphrodite (Fig. 1A; White et al., 1986). This observation suggests that the same cues that guide the PVQL and HSNL growth cones along the left bundle may also guide the contralateral PVQR and HSNR growth cones along the right bundle.

**Nineteen genes guide the HSNL growth cone**

To identify genes that function in recognition of the PVQL and PVPR axons by the HSNL growth cone, we examined HSN axon morphology in a collection of mutants representing 102 genes that are required for normal locomotion. By staining adult hermaphrodites with an anti-serotonin antiserum to reveal HSN axon morphology, we found that mutations in the nineteen genes *enu-1, fax-1, unc-3, unc-14, unc-20, unc-30, unc-33, unc-34, unc-42, unc-44, unc-51, unc-53, unc-69, unc-71, unc-73, unc-76, unc-83, unc-84* and *unc-115* caused the HSNL axon to extend along the right instead of the left VNC bundle (Fig. 2C; Table 1; data not shown). In these mutants, the HSNL growth cone crossed the ventral midline to the right VNC bundle at variable positions along the VNC. Usually the HSNL axon crossed over to the right bundle anterior to and within about 50 μm of the vulva, but occasionally misrouting occurred just posterior to the vulva.

Mutations in the genes *unc-83* and *unc-84* disrupt vulval development, resulting in an incompletely penetrant vulvaless phenotype (Horvitz et al., 1983; Sulston and Horvitz, 1981). The HSNL axon defect of these mutants is thus indirect, reflecting the lack of vulval cells that keep the HSN axons separate in the VNC (Garriga et al., 1993).

Mutations in the genes *unc-14, unc-20, unc-33, unc-34, unc-44, unc-51, unc-53, unc-69, unc-71, unc-73* and *unc-76* also disrupt the ability of the HSN growth cones to extend anteriorly to their normal destinations. In these mutants, the HSN axons terminated prematurely in the VNC before
reaching the nerve ring (Desai et al., 1988; McIntire et al., 1992; B. W. and G. G., unpublished results). This phenotype was not observed in wild-type hermaphrodites when HS NL growth cone pathway cues were removed by laser microsurgery (Garriga et al., 1993). Therefore, the defect in the ability of the HSN axons to extend to their normal lengths suggests that these genes may function in basic aspects of growth cone motility. In this report, we will refer to a disruption in an axon's ability to extend to its full length as an axon outgrowth defect and to a disruption in growth cone guidance along a specific path as an axon pathfinding defect. Because the focus of this paper is on pathfinding, the two genes that are required for vulval development and the eleven genes that are required for HSN axon outgrowth will not be considered further.

Mutations in six genes disrupt axon pathfinding along the left VNC bundle

Mutations in the genes *enu-1*, *fax-1*, *unc-3*, *unc-30*, *unc-42* and *unc-115* alter HSNL axon pathfinding without disrupting vulval development or HSN axon outgrowth. In these mutants both HSN axons often extended anteriorly as a single fascicle within the VNC (Fig. 2), a phenotype similar to that seen in laser-operated animals that lack PVPR and PVQL (Garriga et al., 1993). These mutants did not display defects in HSN cell body position, serotonin expression, or axon outgrowth. Therefore, the HSN defects of these mutants appear to be limited to alterations in growth cone pathfinding.

Because the AVKR growth cone also extends along the PVPR and PVQL axons of the left VNC bundle, we tested whether AVKR axon pathfinding was also defective in these mutants by staining larval and adult hermaphrodites with an anti-FMRFamide antiserum that detects the AVKR axon (Fig. 3A; Schinkmann and Li, 1992). Mutations that disrupted HSNL axon pathfinding also disrupted AVKR axon pathfinding (Fig. 3; Table 1). In these mutants, the AVKR axon was either absent from the left VNC bundle or crossed from the left to the right bundle (Fig. 3B). When the left VNC bundle of

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**Table 1. Genes necessary for axon pathfinding along the ventral nerve cord**

| Genotype                        | HSNL* | AVKR† | PVQL‡ | PVPR§ | Other Phenotypes ||
|---------------------------------|-------|-------|-------|-------|-----------------|
| Wild type                       | 4(50) | 0(27) | 2(53) | 3(35) |                 |
| I. Genes necessary for ventral nerve cord pioneering |
| *unc-1(e151)*                   | 81(26) | 27(30) | 32(22) | 10(21) | Unc, defasc.    |
| *unc-30(e191)*                  | 63(37) | 24(29) | 56(57) | 27(62) | Unc             |
| *unc-30(e2327)*                 | 82(28) | 13(45) | 50(35) | 23(17) | Unc             |
| II. Genes necessary for PVQL, AVKR and HSNL growth cone pathfinding |
| *fax-1(gm27)*                   | 60(66) | 61(37) | 35(20) | 6(50)  | Unc, Fab        |
| *fax-1(gm83)*                   | 45(33) | 78(32) | 34(35) | 3(30)  | Unc, Fab        |
| *unc-42(e270)*                  | 84(50) | 94(31) | 34(50) | 31(29) | Unc, Mec        |
| *unc-42(gm23)*                  | 85(50) | 88(25) | 35(77) | 2(19)  | Unc, Mec        |
| *unc-42(e419)*                  | 64(50) | 81(27) | 23(66) | 0(23)  | Unc, Mec        |
| *unc-42(e623)*                  | 60(30) | 90(40) | 25(43) | 3(31)  | Unc, Mec        |
| III. Genes necessary for AVKR and HSNL growth cone pathfinding |
| *enu-1(e419)*                   | 63(38) | 59(39) | 5(40)  | 4(25)  | Enhances vab-8  |
| *unc-115(e2225)*                | 69(29) | 74(23) | 8(37)  | 2(45)  | Unc             |
| *unc-115(mn490)*                | 57(28) | 45(22) | 0(19)  | 0(12)  | Unc             |

The percentage of mutant animals that displayed defects in axon pathfinding are shown. The number of animals examined is indicated in parentheses. Genes have been placed into three classes based on whether they are required for AVG and PVPR pioneering of the VNC, and whether they are required for HSNL, AVKR and PVQL axon pathfinding.

*HSNL axon pathfinding was scored as defective if the HSNL axon extended along the right VNC bundle. The HSNL axon was observed by immunofluorescent staining of fixed adult hermaphrodites using anti-serotonin antiserum (see Fig. 1 and Materials and Methods).

†AVKR axon pathfinding was scored as defective if the AVKR axon was absent from the left VNC bundle or crossed from the left to the right VNC bundle. The AVKR axon was observed by immunofluorescent staining of fixed adult hermaphrodites using an anti-FMRFamide antiserum (see Fig. 2 and Materials and Methods). For *unc-3* mutants other FMRFamide-positive axons, probably VC axons, were seen in the left VNC bundle. This observation is consistent with the overall defasciculation of the right VNC bundle in *unc-3* mutants described by Durbin (1987). The presence of ectopic FMRFamide-positive axons in the left VNC bundle may have artificially lowered the penetrance of AVKR pathfinding defect.

‡PVQL axon pathfinding was scored as defective if the PVQL axon crossed to the right VNC before the retrovesicular ganglion (RVG) in adult hermaphrodites. The PVQL axon was observed by GFP fluorescence in living animals or by indirect immunofluorescence staining of fixed animals. For both detection methods, animals carried an *sra-6:gfpl27 array, which expresses GFP in PVQs and additional neurons in the head (Troemel et al., 1995). In *unc-30* and *unc-115* mutants, the PVQL axon occasionally crossed from the left to the right VNC bundle at the anterior end of the VNC at the RVG [6/28 (28%), 13/27 (45%), 25/35 (77%), 0/23 (17%) animals displayed this phenotype]. This defect is unlikely to play a major role in HSNL pathfinding defects seen in *unc-30* and *unc-115* mutants because the HSNL axon typically crosses to the right VNC bundle near the vulva in these mutants.

§PVPR axon pathfinding was scored as defective if no axons were detectable in the left VNC bundle (see text). The presence of axons in the left VNC bundle was determined by immunofluorescent staining of fixed adult hermaphrodites using the anti-α-tubulin monoclonal antibody 41A (see Fig. 3 and Materials and Methods).

¶Other phenotypes displayed by mutant animals. Fab mutants are foraging abnormal (Segalat et al., 1995). Mec mutants are mecanosensation defective (Chalfie and Sulston, 1981). Unc mutants are uncoordinated (Brenner, 1974). Mutations in *enu-1* do not cause obvious behavioral defects on their own, but do enhance the locomotion defects of *vab-8* mutants (J. Culotti, personal communication). Mutations in *unc-42* also cause unidentified FMRFamide-positive and GABAergic axons to inappropriately extend along the lateral body wall or into the nose of the animal (data not shown). Mutations in *unc-30* may cause defects in the differentiation of the DD, VD and PVP neurons (Jin et al., 1994). Mutations in *unc-3* lead to defasciculation (Defasc.) of the right VNC nerve bundle (Durbin, 1987).
these mutants lacked an AVKR axon, it presumably extended along the right bundle. However, many axons of the right bundle express FMRFamide, preventing us from identifying the misplaced AVKR axons among the other FMRFamide expressing axons.

In all of the mutants, the AVKR cell body was present, stained brightly with the antiserum, and extended an axon (data not shown). Moreover, when the AVKR axon stayed in the left VNC bundle, it extended to its normal length. Therefore, mutations in all six genes disrupt pathfinding but not outgrowth of two different growth cones that extend along the PVQL and PVPR axons of the left VNC bundle.

**Mutations in unc-3, unc-30 and unc-42 disrupt VNC pioneering**

Mutations that disrupt HSNL and AVKR axon morphology could disrupt either pioneering of the VNC or subsequent interactions between the HSN and AVK growth cones and axons of the VNC. To distinguish between these possibilities, we stained mutants with an anti-α-tubulin monoclonal antibody that stains all axons to reveal the structure of the *C. elegans* nervous system including the left and right VNC bundles. Mutations in *unc-3*, *unc-30* and *unc-42* disrupt VNC structure.

The VNC of *unc-3* mutants often appeared deranged by anti-α-tubulin staining (data not shown). This observation is consistent with EM reconstructions of *unc-3* mutants showing that the VNC is broken up into multiple smaller bundles instead of the two bundles normally present (described by Durbin, 1987). This VNC defasciculation of *unc-3* mutants is similar to that observed in laser-operated animals that lack the right bundle pioneer AVG (Durbin, 1987). Based on these observations, Durbin (1987) proposed that *unc-3* is required for AVG differentiation, AVG pioneering, or the recognition of the AVG axon by later-extending growth cones. Our results show that *unc-3* mutations also disrupt pathfinding of growth cones that extend along the left VNC bundle.

There are at least two interpretations for these observations. First, a defect in AVG pioneering could indirectly disrupt pathfinding along the left bundle. For example, the presence of right VNC bundle axons in the left bundle could repel HSNL and AVKR growth cones from the left VNC bundle. Alternatively, *unc-3* could function directly in pathfinding of growth cones that normally extend along the left VNC bundle. Two results are consistent with the hypothesis that *unc-3* functions in cells other than AVG. First, laser-operated animals that lack AVG display subtle defects in locomotion (Durbin, 1987), whereas *unc-3* mutants are severely uncoordinated. Second, mosaic analysis indicates that *unc-3* function is required in motor neurons for normal locomotion (Herman, 1987).

Mutations in *unc-30* and *unc-42* disrupt pioneering of the left VNC bundle. No left VNC bundle axons were detectable by anti-α-tubulin staining in 10% and 27% of *unc-30*(e2327) and *unc-30*(e191) animals, respectively (Fig. 4D; Table 1). Similarly, no left bundle axons were detectable in 31% and 21% of *unc-42*(e270) and *unc-42*(gm23) animals, respectively (Table 1). These observations suggest that PVPR occasionally fails to pioneer the left VNC bundle of *unc-30* and certain *unc-42* mutants. Dosage experiments indicate that the PVPR pioneering defect of *unc-42*(e270) and *unc-42*(gm23) mutants result from altered gene activity, suggesting that *unc-42* does not normally function in PVPR pioneering (see Discussion).

The penetrance of the PVPR pathfinding defect, however, is not high enough to account for the penetrance of the HSNL pathfinding defect, which varies between 63% and 85% for these *unc-30* and *unc-42* mutants. Two additional observations suggest that the *unc-42* HSN pathfinding defect is not always caused by defects in PVPR pioneering. First, double staining of *unc-42*(e270) mutants with both anti-tubulin and anti-serotonin antibodies identified animals in which the HSNL
axon crossed to the right VNC bundle despite the presence of axons in the left VNC bundle (Fig. 5). Second, unc-42(e419) and unc-42(e623) mutants are normal for PVPR pioneering, but display severe defects in HSN axon pathfinding. In unc-30 and unc-42 mutants, therefore, factors other than absence of the PVPR axon in the left VNC bundle contribute to the HSNL pathfinding defect.

The unc-30 gene encodes a homeodomain protein that is expressed in PVPR but not in other neurons that extend axons along the left VNC bundle (Jin et al., 1994), suggesting that pathfinding defects of unc-30 mutants arise, at least in part, as a consequence of PVPR differentiation defects.

Mutations in enu-1, fax-1, unc-42 and unc-115 disrupt growth cone-target recognition in the left VNC bundle

In contrast to unc-3 and unc-30 mutants, at least one axon was present in the left VNC bundle of all enu-1, fax-1 and unc-115 mutants and of certain unc-42 mutants, indicating that PVPR pioneering was normal (Fig. 4C; Table 1). These results suggest that these genes function in recognition of the PVPR and PVQL axons by the AVKR and HSNL growth cones.

Mutations in fax-1, unc-3, unc-30 and unc-42 disrupt PVQL axon pathfinding

Mutations in the genes fax-1, unc-3, unc-30 and unc-42 also disrupt PVQL axon morphology, which can be visualized in animals that express GFP from an sra-6 promoter (Troemel et al., 1995; see Materials and Methods). In these mutants, the PVQL axon occasionally extended with the PVQR axon along the right VNC bundle (Fig. 6; Table 1), or crossed from the left to right bundle at various positions between the pre-anal ganglion in the tail and the retro-vesicular ganglion in the head. No defects in the expression of the sra-6-gfp transgene, position of the PVQ cell bodies, or PVQ axon outgrowth were observed. These observations indicate that these genes function in recognition of the PVPR axon by the PVQL growth cone. The penetrance of the PVQL pathfinding defect [e.g., 35% for fax-1(gm27)] caused by mutations in each gene is not sufficient, however, to account for the HSNL [e.g., 60% for fax-1(gm27)] or AVKR pathfinding defects [e.g., 60% for fax-1(gm27)]. Because the HSNL growth cone uses both PVPR and PVQL axons for pathfinding, the HSNL growth cone of fax-1, unc-3, unc-30 and unc-42 mutants must occasionally fail to recognize both PVPR and PVQL axons.

**Fig. 5.** Double staining of wild-type and unc-42(e270) mutant adult hermaphrodites with anti-serotonin and anti-α-tubulin antibodies. Ventral views, anterior is to the left. Immunofluorescence photomicrographs of adult hermaphrodites stained with an anti-serotonin antiserum (A,C) to detect the HSN axons and the 41A monoclonal antibody (B,D) to detect all axons. Anti-serotonin staining was visualized using an FITC-conjugated goat anti-rabbit IgG secondary antibody, while 41A staining was visualized using a Texas Red-conjugated goat anti-mouse IgG secondary antibody. The same focal plane of a wild-type hermaphrodite is shown in A and B, and the same focal plane of an unc-42(e270) hermaphrodite is shown in C and D. The HSNL axon of the unc-42(e270) mutant extends past axons of the left VNC bundle to join the HSNR axon in the right bundle. Large arrows in A, B and D point to the left VNC bundle, small arrows in A-D point to the right VNC bundle. The large arrow in B points to the HSNL axon entering the right VNC bundle. Arrowheads point to the vulva. Scale bar, 10 μm.

**Fig. 6.** PVQL axon morphology in wild-type and fax-1 hermaphrodites. Ventral views, anterior is to the left. (A) Schematic drawing showing PVQL axon morphology of a wild-type adult hermaphrodite. The boxed area is the portion of the animal shown in B and C. (B,C) Immunofluorescence photomicrographs of wild-type and fax-1 (gm83) adult hermaphrodites that carry an sra-6-gfp transgene (gmEx71). Animals were stained with anti-GFP antiserum. The body is twisted due to the presence of the rol-6 transgene. Arrows point to the PVQL axon. The arrowhead points to the right VNC bundle at the vulva. Note that in C the PVQL axon is in the left VNC bundle in the posterior portions of the animal, but crosses to the right VNC bundle just posterior to the vulva. Scale bar, 10 μm.
In contrast to \textit{fax-1, unc-3, unc-30} and \textit{unc-42} mutants, the PVQL axon was always present in the left VNC bundle of \textit{enu-1} and \textit{unc-115} mutants. Thus, \textit{enu-1} and \textit{unc-115} mutations do not disrupt interactions between the PVQL growth cone and PVPR axon, but do disrupt interactions between the growth cones of HSNL and AVKR and the axons of PVPR and PVQL.

**DISCUSSION**

The observation that multiple axons that traverse the left VNC bundle are affected in \textit{enu-1, fax-1, unc-3, unc-30, unc-42} and \textit{unc-115} mutants indicates that pathfinding is perturbed. AVKR and HS NL are generated from different lineages (Sulston et al., 1983), express different neurotransmitters (Desai et al., 1988; Schinkmann and Li, 1992), extend axons at different times, and synapse with different partners (White et al., 1986). The only feature shared by these two neurons is that their growth cones extend along the PVPR and PVQL axons of the left VNC bundle. Because mutations in these six genes affect pathfinding by both neurons, the defects are likely to reflect a failure in recognition of the pre-existing axons by the AVKR and HSNL growth cones.

**unc-30 functions in PVPR differentiation**

The pathfinding defects of \textit{unc-30} mutants appear to be caused by a PVP differentiation defect. The penetrance of the PVPR and AVKR pathfinding defects are similar in \textit{unc-30} mutants (Table 1), consistent with the hypothesis that the AVKR growth cone extends along the right bundle when PVPR fails to pioneer the left bundle. The PVQL and HSNL pathfinding defects observed in \textit{unc-30} mutants could be caused by PVP differentiation defects. The gene \textit{unc-30} encodes a homeo-domain protein that is expressed in PVP, but not in HSN or PVQ (Jin et al., 1994). Beside regulating genes involved in PVPR pioneering, \textit{unc-30} could also regulate genes that encode PVQL and HSNL growth cone guidance cues expressed on the PVPR axon. Lack of such cues could contribute to the highly pententrant PVQL and HSNL axon pathfinding defects of \textit{unc-30} mutants.

**fax-1, unc-42, enu-1 and unc-115 function in axon recognition**

Mutations in \textit{fax-1, enu-1} and \textit{unc-115} disrupted HSNL and AVKR pathfinding, but not PVPR pioneering (Table 1). We propose that these genes function in interactions between the AVKR and HSNL growth cones and the PVPR and PVQL axons of the left VNC bundle. In addition, mutations in \textit{fax-1} disrupted PVQL pathfinding, suggesting that \textit{fax-1} guides the PVQL growth cone along the PVPR axon. Thus, \textit{fax-1} appears to function in PVQL, AVKR and PVQL pathfinding, whereas \textit{enu-1} and \textit{unc-115} appear to act more specifically in AVKR and HSNL pathfinding.

Pathfinding defects displayed by \textit{unc-42} mutants, however, depended on the allele examined (Table 1). At least one axon of the left VNC bundle was present in \textit{unc-42(e419)} and \textit{unc-42(e623)} mutants, suggesting that PVPR pioneering was normal. By contrast, the left VNC was occasionally missing in \textit{unc-42(e270)} and \textit{unc-42(gm23)} mutants, suggesting that PVPR pioneering was defective. The \textit{e270} and \textit{gm23} alleles appear to alter \textit{unc-42} function to cause a PVPR pioneering defect since both mutations are semidominant; 10-15% of the HSNL axons extend along the right VNC in hermaphrodites heterozygous for \textit{e270} or \textit{gm23}, whereas HSNL axons were normal in hermaphrodites heterozygous for \textit{e419} or hemizygous for a deficiency of the \textit{unc-42} locus (R. B. and G. G., unpublished results). These results suggest that \textit{unc-42} does not normally function in PVPR pioneering.

In addition to the PVQL and PVPR axons, vulval cells also guide the HSNL growth cone along the left VNC bundle (Garriga et al., 1993). Mutations that prevent the formation of the vulva, such as mutations in \textit{unc-83} and \textit{unc-84}, cause the HSNL growth cone to extend along the left VNC bundle. This raises the possibility that mutations in \textit{enu-1, fax-1, unc-42} or \textit{unc-115} could disrupt interactions between the HSNL growth cone and vulval cells. No defects in vulval development, however, were observed in these mutants. More significantly, the AVKR and PVQL growth cones extend during embryogenesis, long before the vulva is formed. Therefore, defects in vulval development cannot account for the AVKR and PVQL pathfinding defects seen in these mutants. Consistent with this interpretation, mutations in \textit{unc-83} and \textit{unc-84} disrupt HSNL but not AVKR pathfinding (data not shown).

**The mechanism of axon pathfinding in the left VNC bundle**

The neighborhood hypothesis of White et al. (1983) requires that growth cones recognize and discriminate among several axons. An attractive possibility is that axons that grow out early in the development of an axon bundle express molecules on their surface that ‘label’ a pathway (Raper et al., 1983, 1984). Later extending growth cones would express receptors on their surface that interact with specific label molecules. Receptors would distinguish among different axons by recognizing the different labels that they express. For example, the HSNL growth cone recognizes the PVPR and PVQL axons, but not the AVKR axon. Thus, growth cone-axon recognition may involve the expression of specific receptor and label molecules on the surface of growth cones and axons, respectively.

The genes \textit{enu-1, fax-1, unc-42 and unc-115} could play one of two roles in growth cone-axon recognition. First, the genes could participate directly in growth cone recognition of axons by encoding labels, receptors, or signal transduction molecules. Several genes appear to control pathfinding along established axon bundles directly. In particular, neural cell adhesion molecules containing immunoglobulin (Ig) and fibronectin type III (FnIII) repeats, receptor tyrosine kinases and receptor tyrosine phosphatases have been implicated in regulating growth cone-axon interactions. In \textit{Drosophila}, for example, the Ig/FnIII cell adhesion molecule Fasciclin II functions in axon bundling (Lin et al., 1994), and tyrosine kinases and phosphatases regulate pathfinding (Callahan et al., 1995; Desai et al., 1996; Krueger et al., 1996). Vertebrate molecules have also been implicated in growth cone pathfinding along axon bundles. For example, perturbations of the Ig/FnIII cell adhesion molecule Ng-CAM with anti-Ng-CAM antibodies disrupts bundling of commissural axons as they extend longitudinally in chick explants (Stoeckl and Landmesser, 1995).

Second, the genes could participate in pathfinding indirectly, encoding molecules that produce or activate a critical component of the label-receptor interaction. For example, \textit{unc-30}, encodes a Hox gene that is likely to regulate other genes...
that function in growth cone pioneering. For the other genes, distinguishing between these possibilities awaits molecular analysis.

**Function outside of the left VNC bundle**

With the exception of *enu-1*, mutations in these six genes cause neural deficits that cannot be accounted for by the VNC pathfinding defects described in this report. Mutations in the genes *fax-1*, *unc-3*, *unc-30*, *unc-42* and *unc-115* cause Unc (uncoordinated, abnormal locomotion) phenotypes, indicating that these mutations disrupt the circuitry that controls locomotion. These mutations could disrupt pathfinding of axons that control locomotion. Alternatively, they could disrupt aspects of neuronal development other than pathfinding. The *unc-30* mutations disrupt GABAergic motor neuron differentiation, including GABA expression, to produce the Unc phenotype (McIntire et al., 1993; Jin et al., 1994).

In addition, mutations in *unc-42* cause a Mex (neurosensorily-defective, no response to light touch; Chalfie and Sulston, 1981; Chalfie et al., 1985) phenotype and mutations in *fax-1* cause a Fab (forming abnormal, increased head movement while feeding; Segalat et al., 1995) phenotype (Table 1). These phenotypes could be caused by defects in pathfinding of axons used to construct the neural circuits required for mechanosensation and foraging.

Finally, mutations in genes *fax-1*, *unc-3*, *unc-30*, *unc-42* and *unc-115* disrupt the regulation of egg laying by hermaphrodites (B. W. and G. G., unpublished results). Wild-type hermaphrodites normally stop laying eggs for one to two hours when removed from food, whereas these mutants continue to lay eggs in the absence of food. This egg-laying constitutive phenotype is not caused by rerouting of the left HSN axon to the right VNC *per se* because *enu-1* mutants or animals that lack PVPR and PVQL are not egg-laying constitutive (data not shown).

**Evolution of the nematode VNC**

Although all nematode VNCs studied to date are asymmetric, extant nematodes are thought to have evolved from progenitors with symmetric VNCs (reviewed by Chitwood and Chitwood, 1950). The lack of nematode VNC symmetry can be explained by VNC function. Two types of neurons contribute axons to the VNC. Motorneurons that control locomotion innervate ventral muscles via VNC synapses and dorsal muscles by sending commissures dorsally. Interneurons of the head and tail either innervate VNC motorneurons to control movement or use the VNC as a conduit to reach the other end of the animal. Except the head, which can move in all directions, nematodes flex their body along their dorsal-ventral axis, obviating the need for bilaterally symmetric motor control. In the context of the neighborhood hypothesis, axons that fasciculate with one another may have shifted from the left to right bundle in groups during evolution. The remaining axons of the *C. elegans* left VNC bundle may represent a vestige of an ancestral organism that required a symmetric nerve cord.

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