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Cell interactions control the direction of outgrowth, branching and fasciculation of the HSN axons of *Caenorhabditis elegans*

Gian Garriga*, Chand Desai† and H. Robert Horvitz

Howard Hughes Medical Institute, Department of Biology, Room 56-623, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

*Author for correspondence at present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA
†Present address: Department of Biology, California Institute of Technology, Pasadena, CA 91125, USA

SUMMARY

The two serotonergic HSN motor neurons of the nematode *Caenorhabditis elegans* innervate the vulval muscles and stimulate egg laying by hermaphrodites. By analyzing mutant and laser-operated animals, we find that both epithelial cells of the developing vulva and axons of the ventral nerve cord are required for HSN axonal guidance. Vulval precursor cells help guide the growth cone of the emerging HSN axon to the ventral nerve cord. Vulval cells also cause the two HSN axons to join the ventral nerve cord in two separate fascicles and to defasciculate from the ventral nerve cord and branch at the vulva. The axons of either the PVP or PVQ neurons are also necessary for the HSN axons to run in two separate fascicles within the ventral nerve cord. Our observations indicate that the outgrowth of the HSN axon is controlled in multiple ways by both neuronal and nonneuronal cells.

Key words: *C. elegans*, axonal outgrowth, axonal branching, fasciculation

INTRODUCTION

A major goal in developmental neurobiology is to understand how neurites navigate to find their synaptic partners. What types of cells guide neurites to their targets? What is the molecular nature of the interactions between extending growth cones and the cells with which they interact?

The nematode *Caenorhabditis elegans* has been used in two different ways to study axonal guidance. One approach has been genetic. By analyzing the morphologies of individual axons in mutants with behavioral deficits, investigators have identified genes that are required for axonal outgrowth (Hedgecock et al., 1985, 1987, 1990; Desai et al., 1988; Siddiqui and Culotti, 1991; McIntire et al., 1992). An alternate approach has been to define the cell interactions required for axonal outgrowth by removing specific cells and asking if the growth of particular axons is affected. In this way, Durbin (1987) showed that the AVG and PVP cells act as pioneer neurons in the development of the ventral nerve cord, the major longitudinal nerve bundle of the animal. In two additional studies, the cellular requirements for axonal branching were investigated. First, the BDU axon was shown to guide or stabilize the branch of the AVM touch neuron (Walthall and Chalfie, 1988). Second, epithelial cells of the vulva were found to be required for the branching of the VC neurons (Li and Chalfie, 1990).

We have been studying axonal outgrowth of the *C. elegans* HSN neurons, 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). We have identified ten genes required for the outgrowth of the HSN axons (Desai et al., 1988; McIntire et al., 1992). Some of these genes are needed for pioneering HSN outgrowth along epithelial cells, while others are needed for HSN outgrowth along other axons within the ventral nerve cord. All ten of these genes are also required for the outgrowth of the axons of other neurons. In principle, these genes could act either within the neurons altered in axonal outgrowth or within other cells that interact with these neurons. Genetic mosaic analysis suggested that one of these genes, *unc-6*, is required in epithelial cells for the outgrowth of motor neurons across these cells (cited by Hedgecock et al., 1990). Although genetic mosaic analysis should also be useful in defining the sites of action of many of the other genes required for axonal outgrowth, this type of study will be more difficult for genes required for growth of axons in nerve bundles, where the number of potential interacting cells is large. Knowledge of which cells are involved in HSN axonal guidance should help identify the cells in which these genes function.

In this paper, we identify cell interactions that are required for HSN axonal guidance. By analyzing HSN axonal morphology in animals missing specific cells, we find that both vulval cells and neurons that contribute axons to the ventral nerve cord are required for normal HSN axonal guidance. Vulval cells, which are specialized types

of epithelial cells, are needed for specifying the initial direction of HSN axonal outgrowth, for HSN axonal branching and for two distinct aspects of HSN axonal fasciculation. The axons of the PVP and PVQ neurons are also needed for normal fasciculation of the HSN axons. We propose that interactions between the HSN growth cones, the PVP and PVQ axons and vulval cells are required for normal pathfinding by the HSN axons.

MATERIALS AND METHODS

Strains and genetics

Strains were grown at 20°C unless otherwise stated and were maintained as described by Brenner (1974). Most of the mutant strains used in this work were described by Greenwald et al. (1983), Trent et al. (1983), Ferguson and Horvitz (1985), Hedgecock et al. (1985), Desai et al. (1988), Manser and Wood (1990) and Thomas et al. (1990). The weak *lin-39* allele *709* was described by Fixsen et al. (1985), and the stronger *n1490* and *n1760* alleles were isolated and characterized by S. Clark (personal communication), as was *lin-2(n1602)*. The *sem-2(n1343)* allele was isolated and characterized by M. Stern. This paper uses standard *C. elegans* genetic nomenclature (Horvitz et al., 1979).

In addition to the standard wild-type strain (N2), strains with the following mutations were used in this work.

LGI: *lin-10(n1390)*, *lin-11(n382* and *n389)*, *mig-1(e1787* and *n1354)*, *sem-2(n1343)*.

LGII: *clr-1(e1745*ts*)*, *egl-27(n170)*, *egl-43(n997)*, *lin-7(e1413)*.

LGIII: *dig-1(n1321)*, *lin-12(n137*sd, *n302*sd and *n302*sd n865), *lin-39*(*n709*, *n1490* and *n1760*), *mig-10(ct41)* and *unc-32(e189)*.

LGIV: *egl-18(n162 and n474)*, *egl-20(n585)*, *lin-1(n1777)*, *lin-3(e1417)*.

LGX: *egl-15(n484)*, *lin-2(n1602)*, *lin-15(n765ts)*.

lin-39(n709) double mutants were constructed by crossing *lin-39(n709)*/+ heterozygous males with hermaphrodites from a strain containing the second mutation. Potential doubly heterozygous animals were picked; from those animals segregating progeny with ectopic cell deaths in the VNC (the VC neurons variably undergo programmed cell death in *lin-39(n709)* mutants, and this phenotype can be scored using Nomarski optics; Fixsen et al., 1985), animals homozygous for *lin-39(n709)* were isolated. Animals homozygous for the second mutation were isolated from the progeny of these animals. Similarly, *mig-1; lin-15* double mutants were constructed by crossing *mig-1* homozygous males with *lin-15* hermaphrodites. Non-Lin doubly heterozygous animals were picked from the F_1 progeny, and Lin F_2 animals were then isolated. From the F3 progeny, Mig animals were identified by their displaced HSNs using Nomarski optics.

Indirect immunofluorescence histochemistry and microscopy

Indirect immunofluorescence histochemistry was used to stain animals for serotonin, essentially using the procedure described by McIntire et al. (1992). Adult hermaphrodites were fixed for 24 hours at 4°C in PBS (10.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 4.4 mM KCl, pH 7.2), 4% paraformaldehyde fixative. After being washed three to four times in PBS, the worms were rocked gently overnight at 37°C in a solution of 5% β-mercaptoethanol, 1% Triton X-100 in 0.125 M Tris-HCl, pH 6.9. The worms were washed three more times in PBS, and 20-30 μl of worms were shaken vigorously in 0.4 ml of 100 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$ and 1,000-2,000 units/ml collagenase type IV (Sigma, St. Louis, MO). After 20-50% of the worms were fragmented (30 minutes to 3 hours), the worms were washed with PBS three to four times and incubated overnight at room temperature in 20 μl of 1% rabbit anti-serotonin antiserum (provided by J. Steinbusch, Free University, Amsterdam, The Netherlands) in PBS, 1% Triton X-100, 1 mM sodium azide. The worms were then washed three times in PBS and incubated for 2 hours at 37°C in 20 μl of 5% FITC-conjugated goat anti-rabbit antiserum (Cappel Inc., West Chester, PA) in PBS, 1% Triton X-100, 1 mM sodium azide. The worms were then washed three times in PBS and incubated for 2 hours at 37°C in 20 μl of 5% FITC-conjugated rabbit anti-goat antiserum in PBS, 1% Triton X-100, 1 mM sodium azide. The worms were then washed three times in PBS. 5 μl of stained worms were mixed with 5 μl of 1 mg/ml p-phenylenediamine in 10% PBS, pH 8.0, and 90% glycerol and viewed either by standard fluorescence microscopy (illuminated with a mercury vapor lamp, and viewed through an FITC filter; Zeiss 48705, excitation range 395-440 Å) or by confocal microscopy (Bio-Rad MRC-500 scanning confocal microscope; illuminated with an argon ion laser, excitation 488 Å).

Double staining animals with both anti-serotonin and anti-FMRFamide antisera to visualize the defasciculation of the HSN axons at the vulva was done using either of two procedures. One approach was to follow the protocol described above for anti-serotonin staining with the addition of a 5% rabbit anti-FMRFamide antiserum (provided by C. Li, Boston University, Boston, MA) with the anti-serotonin antiserum during the primary antisera incubation step. An alternative approach was to permeabilize the animals using a Dounce homogenizer, as described by Li and Chalfie (1990). Following the douncing, all antibody incubation steps were carried out as described above. Permeabilization by douncing resulted in slightly increased staining but higher background levels. Double staining usually was preformed in double mutants that carried the mutation *lin-39(n709)*. Since the only cell bodies in the VNC that obviously stain with the anti-FMRFamide antiserum are those of the VC neurons, *lin-39(n709)* animals missing all six VC neurons were identified as those animals with no stained cell bodies in the VNC.

Procedures for using Nomarski optics microscopy to observe living animals were described by Sulston and Horvitz (1977).

Laser killing of cells and staining of operated animals with anti-serotonin antiserum

Nuclei were destroyed as described by Avery and Horvitz (1987) in early L1 larvae shortly after hatching. The Pn.a or Pn.p cells were killed later during the L1 stage shortly after the P cells had divided.Verification of the kills were made the next day using Nomarski optics, except for the kills of the somatic gonad precursor cells Z1 and Z4, which were confirmed by the resulting sterility. Once mature, laser-operated animals were placed in microtiter wells containing M9 buffer (Brenner, 1974) and chilled on ice for 20 minutes. The M9 was then replaced with ice cold 4% paraformaldehyde fixative in PBS and fixed overnight at 4°C. The worms were washed three times with PBS and incubated overnight at 37°C in 100 μl of a solution of 5% β-mercaptoethanol, 1% Triton X-100 in 0.125 M Tris-HCl, pH 6.9. The worms were washed three times in PBS and incubated for 1-3 hours in 100 μl of 100 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$ and 1,000 units/ml collagenase type IV (Sigma, St. Louis, MO). The worms were then washed with PBS three to four times. Two precautions were taken to reduce the background of nonspecific staining that resulted when staining small numbers of worms. First, before incubating the worms in the primary antiserum, 20-30 μl of worm shards were added to the microtiter wells. Because of their small sizes, these pieces of worms are easily distinguished from intact laser-operated worms. The worm shards were prepared by exhaustively digesting worms that had been fixed and treated with β-mercaptoethanol with collagenase (see above) until only

small fragments of worms remained. Second, the primary, secondary and tertiary antisera used in these experiments were previously used in the staining of bulk quantities of worms as described above. These antisera were pooled from staining experiments and centrifuged to remove residual worms. The supernatants were then stored at 4°C. The worms and worm shards were incubated overnight at room temperature in 10 μl of 1% rabbit anti-serotonin antiserum in PBS, 1% Triton X-100, 1 mM sodium azide. The worms were then washed three times in PBS and incubated for 2 hours at 37°C in 10 μl of 5% FITC-conjugated goat anti-rabbit antiserum in PBS, 1% Triton X-100, 1 mM sodium azide. The worms were then washed three times in PBS and incubated for 2 hours at 37°C in 10 μl of 5% FITC-conjugated rabbit anti-goat antiserum in PBS, 1% Triton X-100, 1 mM sodium azide. As the worm shards were lost in the PBS washes, new shards were added. The worms were then washed three times in PBS. 5 μl of stained worms were mixed with 5 μl of 1 mg/ml p-phenylenediamine in 10% PBS, pH 8.0, and 90% glycerol and viewed by fluorescence microscopy as above.

We found that growing animals at 25°C instead of 20°C helped increase the intensity of anti-serotonin staining. For the cell killing experiments in which antisera staining tended to be marginal, animals were raised at 25°C, except in the case of *lin-15(n765*ts*)* animals, which were grown at 20°C. *lin-15(n765*ts*)* animals are sick and stain poorly at 25°C.

We also attempted to determine HSN axonal morphology in animals missing their germline gonadal cells. These experiments were attempted either by killing, in newly hatched L1 stage animals Z2 and Z3, the germline precursor cells (Kimble and Hirsh, 1979), or by killing, in the embryo P4, the precusor to Z2 and Z3 (Sulston et al., 1983). In these experiments, the HSNs either failed to stain or stained poorly with anti-serotonin antisera, making it impossible to reliably score HSN axonal morphology. We do not know the reason for this poor HSN staining.

Use of the *clr-1* mutant to visualize HSN axons

To observe the ventral growth of the HSN axons, we used the temperature-sensitive mutant *clr-1(e1745*ts; Hedgecock et al., 1990). The boundaries of cells and axons can be visualized when *clr-1(e1745*ts*)* animals are grown at the nonpermissive temperature of 25°C. To view HSN axonal morphology, we shifted *clr-1(e1745*ts*)* animals grown at 15°C to 25°C for 4 hours and then scored HSN axonal morphology in these animals using Nomarski optics.

RESULTS

The *C. elegans* egg-laying system

The components of the *C. elegans* egg-laying system (White, 1988) are illustrated in Fig. 1. Eggs are stored in the uterus and are laid through the vulva upon contraction of the 16 uterine and vulval muscle cells. Eight uterine muscle cells (not shown) run circumferentially around the uterus and contract to squeeze eggs from the uterus. Eight vulval muscle cells (only four are shown) attach to the vulva and contract to open the vulva and allow eggs to pass. Gap junctions interconnect the vulval and uterine muscle cells and presumably cause all 16 muscle cells to contract synchronously.

Two classes of neurons synapse directly with the vulval muscles: the HSNs and the VCs (White et al., 1986). The cell bodies of the two HSN neurons are located on either lateral side of the animal just posterior to the vulva. (Fig. 1 shows only the left HSN cell body and its axon.) The HSN neurons are required for normal egg laying: hermaphrodites missing or with functionally impaired HSNs are egg-laying defective and bloat transiently with late-stage embryos (Sulston and Horvitz, 1981; Trent et al., 1983; Desai et al., 1988; Desai and Horvitz, 1989). The cell bodies of the six VC neurons are located along the ventral side of the hermaphrodite. (Fig. 1 shows the cell bodies of only the fourth and fifth VC neurons.) The VCs have yet to be assigned a role in egg laying, since hermaphrodites that are missing VCs appear to lay eggs normally (G.G., C. Trent and H.R.H., unpublished observations).

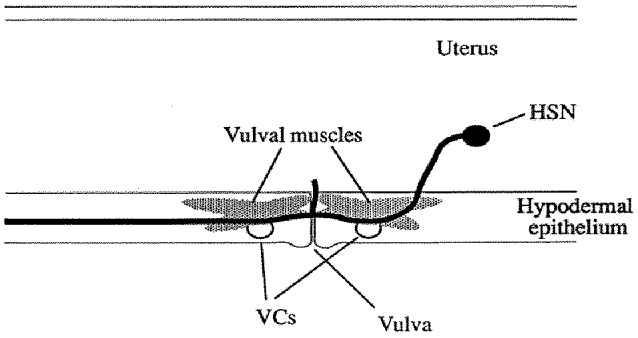


Fig. 1. The *C. elegans* egg-laying system. Schematic drawing of the components of the egg-laying system, showing the positions of the vulva, uterus, four of the eight vulval muscle cells, one of the two HSN neurons and the cell bodies of two of the six VC neurons, VC4 (anterior) and VC5 (posterior). The VC neurons, vulval muscle cells and HSN axons are adjacent to a ridge of the ventral hypodermal epithelium that runs along the ventral side of the animal. The HSN axon runs anteriorly in the ventral nerve cord, which is not shown. The HSN cell bodies and ventral-directed axons are adjacent to the uterus. Anterior is to the left, and ventral is down.

Wild-type HSN axonal outgrowth

Fig. 2A illustrates four aspects of HSN axonal outgrowth which will be the focus of this study: (1) ventral growth of the HSN axon to the ventral nerve cord (VNC), (2) growth of the left and right HSN axons anteriorly along other axons in the left and right bundles of the VNC, respectively, (3) defasciculation of the HSN axons from other axons of the VNC at the vulva, and (4) branching of the HSN axon at the vulva. Below we describe in more detail these aspects of HSN axonal outgrowth and the techniques used to analyze them.

As we described previously (Desai et al., 1988), the mature HSN can be visualized in the adult hermaphrodite by staining animals with antiserum to serotonin (Fig. 2B). A single axon emerges from each HSN cell body and extends to the ventral nerve cord, which consists of two parallel bundles of axons that extend the length of the animal. The axons from the left and right HSNs enter the left and right bundles of the VNC, respectively. Once in the VNC, each HSN axon turns anteriorly and skirts the vulva, at which point it forms varicosities and, usually, a small branch. The synapses with the egg-laying muscles are made at the varicosities and along the branch (White et al.,

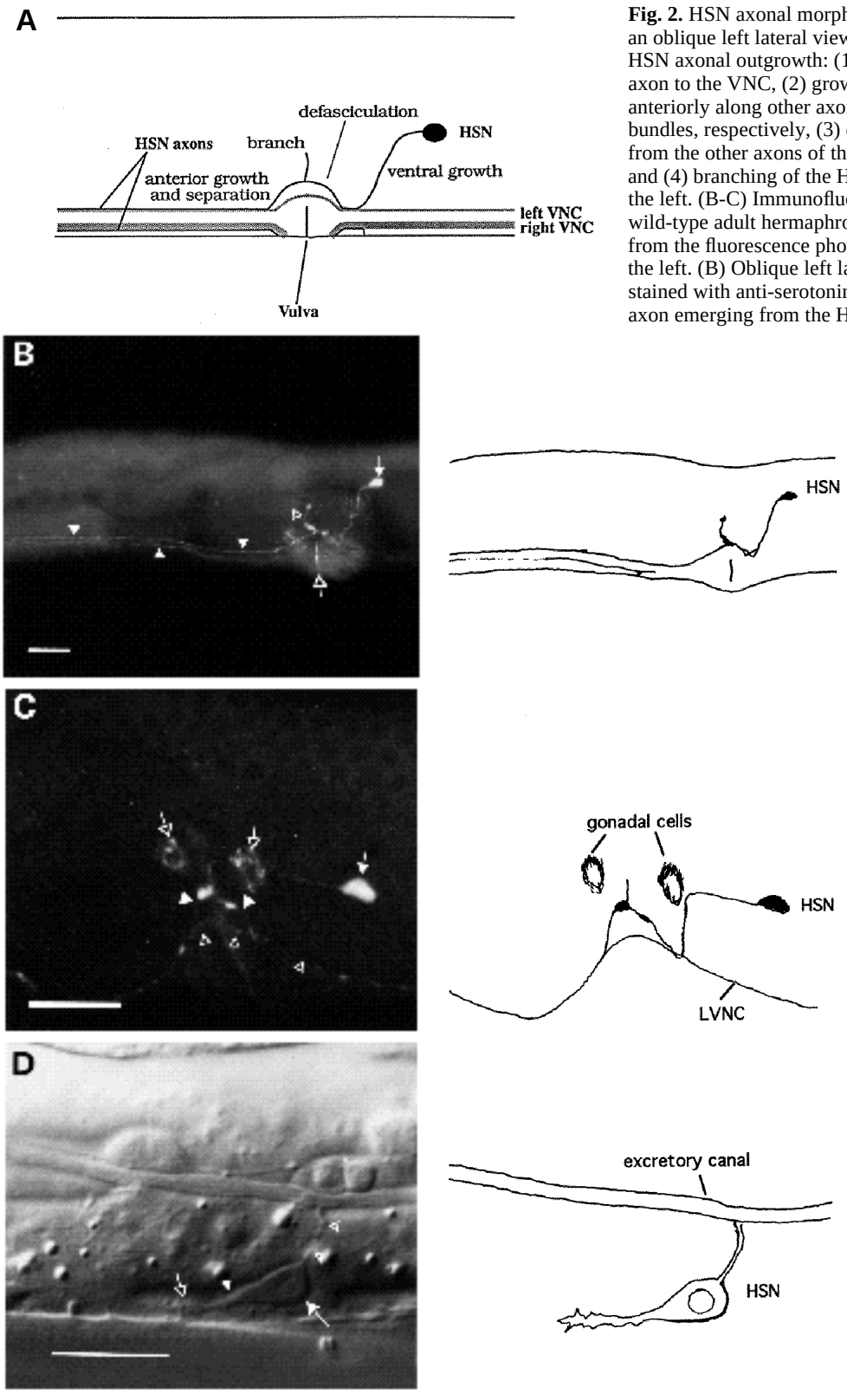


Fig. 2. HSN axonal morphology. (A) Schematic drawing of an oblique left lateral view showing the different aspects of HSN axonal outgrowth: (1) ventral growth of the left HSN axon to the VNC, (2) growth of the left and right HSN axons anteriorly along other axons within the left and right VNC bundles, respectively, (3) defasciculation of the HSN axon from the other axons of the VNC in the region of the vulva, and (4) branching of the HSN axon at the vulva. Anterior is to the left. (B-C) Immunofluorescence photomicrographs of wild-type adult hermaphrodites (left) and tracing of the HSN from the fluorescence photomicrographs (right). Anterior is to the left. (B) Oblique left lateral view of a wild-type animal stained with anti-serotonin antiserum showing the left HSN axon emerging from the HSN cell body (solid arrow) and extending ventrally into the ventral nerve cord (VNC). After entering the VNC, the HSN axon skirts the vulval opening (open arrow). The HSN axons (solid arrowheads; the right HSN axon can be seen running into the right side of the VNC) continue to extend along opposite sides of the VNC anteriorly into the head, where they enter the nerve ring (not shown). In the region of the vulva, each HSN axon forms varicosities and a small branch (open arrowhead). The bright spots in the region of the HSN cell body and the vulva are not dependent on anti-serotonin immunoreactivity; they do not depend on the presence of the anti-serotonin antiserum and have been seen with the secondary antisera alone. This photomicrograph is from Desai et al. (1988). (C) Confocal microscopic image of a left lateral view of a *lin-39(n709)* animal that lacked all six VC neurons stained with anti-serotonin and anti-FMRFamide antisera showing the separation of the left HSN axon (stained with the anti-serotonin antiserum, solid arrowheads) from the VNC (the AVK axon in the left bundle of the VNC is stained with the anti-FMRFamide antiserum, open arrowheads). The left HSN axon emerges from the HSN cell body (solid arrow), grows anteriorly a short distance before growing ventrally to the VNC at the position of the gonadal cell. Open arrows indicate two of four gonadal cells that stain with anti-FMRFamide antiserum (Li and Chalfie, 1990). (D) Nomarski photomicrograph of a left lateral view of a *clr-1* L2 stage animal that had been shifted from the permissive temperature of 15°C to the restrictive temperature of 25°C for 4 hours (left) and a tracing of the HSN from the Nomarski photomicrograph (right). This view shows both a dorsal neurite (open arrowheads) and a ventral neurite (solid arrowhead) emerging from the HSN cell body (solid arrow). A possible HSN growth cone is indicated by the open arrow. *C. elegans* growth cones have been described by Durbin (1987). Scale bars, 20 μm in B and C, 10 μm in D.

1986). Each HSN axon continues anteriorly on its respective side of the VNC to the head, where it enters the nerve ring, a dense neuropil that runs circumferentially around the pharynx (not shown).

The HSN and the VC axons separate from the other processes of the VNC at the vulva and run in a distinct fascicle just dorsal to the main VNC bundle (White et al., 1986; J. White, personal communication; C.D., unpublished results). Originally observed in electron micrographs of serial sections, this defasciculation of the HSN axons from other axons in the VNC can also be seen and analyzed using the light microscope by staining the HSNs with an anti-serotonin antiserum and staining the VNC with an anti-FMRFamide antiserum (Fig. 2C). Anti-FMRFamide antiserum recognizes the AVK axon on the left side of the VNC and several axons on the right side of the VNC (Li and Chalfie, 1990; Schinkmann and Li, 1992). However, anti-FMRFamide antiserum also recognizes the VC neurons, which defasciculate along with the HSNs. Thus, to analyze HSN defasciculation we had to develop a method in which the axons of the HSNs would not be confused with those of the VCs. We accomplished this objective by eliminating the VCs using the *lin-39* mutation *n709*, which results in a variable loss of the VCs (Ellis, 1985; Fixsen et al., 1985; Li and Chalfie, 1990). Fig. 2C shows a *lin-39(n709)* animal that lacked all six VC neurons stained with both anti-serotonin and anti-FMRFamide antisera. Near the vulva, the left HSN axon can be seen to be separated from the left VNC bundle, which is labeled by the AVK axon. We find that it is where the HSN axons are separated from the main VNC fascicle that they form larger varicosities and branch. The HSN axonal morphology seen in *lin-39(n709)* animals is the same as the morphology of wild-type animals, determined by electron microscopy.

Because only the adult HSN contains detectable serotonin (Desai et al., 1988), staining with anti-serotonin antiserum allows an analysis of HSN axonal morphology only after the axons have completed outgrowth. Based upon these anti-serotonin staining patterns, we have inferred the temporal sequences of events that lead to the final morphologies of the HSNs in wild-type and mutant animals (Desai et al., 1988; McIntire et al., 1992). In particular, we would not have observed dynamic aspects of axonal outgrowth, such as probing by and retraction of the HSN axons.

Using a method developed by Hedgecock et al. (1990), we now can observe HSN morphologies during the early stages of HSN axonal outgrowth. The boundaries of cells and axons can be visualized when animals carrying the temperature-sensitive mutation *clr-1(e1745*ts*)* are viewed using Nomarski optics after growth at the restrictive temperature (Hedgecock et al., 1990). Fig. 2D shows a photomicrograph of a late second larval (L2) stage *clr-1* animal. The HSN can be seen to extend two neurites, which are typical of the HSNs in L2 or third larval (L3) stage animals (some late L2 stage animals do not appear to have yet extended HSN axons). One neurite extends dorsally, terminating at the excretory canal. This neurite presumably would later retract or rearrange, since such morphology is not seen in older animals. We do not know if this dorsal extension plays a role in HSN development. A second neurite extends anteriorly and ventrally (see below). Unfortunately, once near the VNC, the HSN axon cannot be distinguished from the other axons in this bundle.

Thus, the HSN axons begin to grow during the L2 or L3 stage. These axons apparently do not terminate growth until after the late fourth larval (L4) stage, since they have not reached the nerve ring by this stage (White et al., 1986). The relatively long period of more than 20 hours of HSN outgrowth in the VNC indicates either that the HSN axons grow quite slowly (approximately 10-15 μm/hour) or that there is a period when the HSN axons slow or arrest their outgrowth.

Search for cell interactions involved in HSN axonal outgrowth

To identify cell interactions involved in HSN axonal guidance, we focused on cell types that we considered likely to contact the extending HSN axons: cells of the egg-laying system and neurons that contribute axons to the VNC. These cells were removed either by mutation or by killing with a laser microbeam, and the mutant or operated adult animals were stained with anti-serotonin antisera to reveal HSN axonal morphology. The HSN axons initially extend along a hypodermal syncytium that constitutes the epithelial covering of the animal; the syncytial nature of this epithelium has prevented us from directly testing its role in HSN axonal outgrowth. We scored animals for HSN axonal outgrowth ventrally to the VNC, for the separate outgrowth of the two HSN axons on opposite sides of the VNC, for HSN axonal branching and for HSN axonal outgrowth anteriorly along the VNC. Cells required to guide the HSN axons ventrally were also removed in a *clr-1* mutant background, and the early stages of HSN axonal outgrowth were examined directly in these animals. In addition, to score defasciculation of the HSN axons from the VNC at the vulva, we constructed double mutants carrying a mutation that removes a specific cell that we wished to test for a role in HSN defasciculation and the *lin-39(n709)* mutation, which removes the VC neurons; we stained these double mutants with anti-serotonin and anti-FMRFamide antisera (see above).

Vulva development

The vulval cells or their precursors play key roles in HSN axonal guidance (see below). To describe how we analyzed this function of the vulval cells, we will first briefly summarize aspects of what is known about how different types of vulval cells develop. A more detailed summary of vulval development has been recently published (Horvitz and Sternberg, 1991).

Six epithelial cells (P3.p-P8.p) located along the ventral side of the animal have the potential to generate cells of the vulva. The fates of these Pn.p cells can be distinguished by the numbers and cell types of the progeny they generate. The fate of each of these cells depends on its proximity to a signal emanating from the anchor cell of the somatic gonad, with the closest cell (P6.p) adopting the primary fate, the next two distal cells (P5.p and P7.p) adopting the secondary fate and the most distal cells (P3.p, P4.p and P8.p) adopting the tertiary fate. The vulva is formed by the 22 descendants of P5.p-P7.p, the cells that adopt the primary and secondary fates. Cells that adopt the tertiary fate

generate nonvulval progeny. In the absence of the signal from the somatic gonad, all six of these Pn.p cells adopt a tertiary fate. No vulval cells are formed, and the animal is said to be vulvaless. The Pn.p cells divide in the L3 stage, and vulval morphogenesis occurs during the L4 stage.

The vulval cells or their precursors are required for HSN axonal guidance

Vulvaless animals, generated either by mutation or by killing cells required for vulva formation with a laser microbeam, exhibit four types of HSN axonal defects: in the initial direction of HSN axonal outgrowth, in positioning within the VNC, in defasciculation and in branching of the HSN axons at the vulva. Each of these defects is described below.

(1) Directed growth of the HSN axons

In vulvaless animals, the HSN axons sometimes fail to grow ventrally and instead extend anteriorly along a lateral pathway, eventually entering the nerve ring, their normal destination (for phenotype see Fig. 3A; Table 1). This failure to enter the VNC occurs much more frequently in vulvaless animals generated by eliminating the somatic gonad or the Pn.p cells by laser microsurgery (23-25%) than in vulvaless animals generated by mutations in the *lin-2, lin-3, lin-7* and *lin-10* genes (0-1%). We suspect this difference reflects the fact that some Pn.p cells retain partial vulval cell identity in these mutants (vulvaless animals can generate some vulval cells but still not generate a functional vulva; Ferguson and Horvitz, 1985). Consistent with this interpretation, the HSN axons also fail to enter the VNC more frequently in *lin(n300), lin-12(n302*sd*), lin-39(n1490)* and *lin-39(n1760)* vulvaless mutants (8-22%); these mutants only infrequently generate vulval cells (Ferguson and Horvitz, 1985; S. Clark, personal communication). (In contrast to the weak *lin-39* mutant *n709*, which is variably missing VC neurons and has a vulva, the stronger *lin-39* mutants *n1490* and *n1760* lack all six VC neurons and are vulvaless; Li and Chalfie, 1990; S. Clark, personal communication.) This defect in HSN axonal morphology is a consequence of a defect in the initial direction of HSN axonal outgrowth, since we have directly observed the HSN axons extend aberrantly during the L3 stage in a *clr-1; lin-39(n1760)* vulvaless double mutant (20%, *n*=15). The abnormally extending HSN axons of *clr-1; lin-39* animals grow along the canal-associated fascicle, which normally contains the processes of the ALA, BDU, CAN and PVD neurons and the excretory canal (White et al., 1986). In addition, in those vulvaless animals in which ventral outgrowth occurs, the HSN axons often extend anteriorly or posteriorly a short distance before growing ventrally and entering the VNC (see Fig. 3C).

Vulval cells also appear to play a minor role in the direction of HSN axonal outgrowth within the ventral nerve cord. In vulvaless animals, the HSN axons can enter the VNC normally and then grow posteriorly to the tail or grow a short distance posteriorly in the VNC before reversing direction and growing anteriorly (see left HSN axon in Fig. 3B). However, this defect occurs infrequently. For example, only 5% of the HSN axons that enter the VNC in *lin-39(n1760)* mutants grow to the tail.

(2) Positioning of the left HSN axon within the VNC

In vulvaless mutants, the HSN axons usually fasciculate with one another in the VNC instead of growing separately on either side of the VNC (Fig. 3B, Table 1). In these animals, the left HSN axon grows across the left nerve bundle of the VNC and enters the right nerve bundle of the VNC, where it runs with the right HSN axon to the nerve ring (see Fig. 3C). At the level of resolution of immunocytochemical staining, the two HSN axons appear to run as a single fascicle that separates only infrequently.

(3) HSN axonal defasciculation

In vulvaless animals, the HSN axons fail to defasciculate from the VNC bundle at the vulva. This failure to defasciculate was suggested by the relatively direct path the HSN axons take once they enter the VNC in vulvaless mutants (see Fig. 3B) and has been confirmed by double staining of the *lin-39(n1490)* vulvaless and VC-deficient mutant with both anti-FMRFamide and anti-serotonin antisera (Fig. 3C, Table 2).

(4) HSN axonal branching

In vulvaless animals, the HSN axons fail to branch (Table 1, Fig. 3B). Moreover, extra HSN branches are occasionally seen at the positions of ectopic vulval protrusions in the multivulva mutants *lin-1* and *lin-15* (Li and Chalfie, 1990; Fig. 4A and unpublished results). These results suggest that vulval cells determine the position of the HSN branch. Vulval cells are also required for branching of the axons of the VC neurons, the other class of neurons that innervate the egg-laying muscles (Li and Chalfie, 1990).

Primary vulval cells or their precursors are required for HSN axonal outgrowth

To determine which types of vulval epithelial cells are required for HSN axonal outgrowth, we analyzed HSN axonal morphology in mutants that produced only secondary or only primary vulval cells. This analysis suggests that primary vulval cells play the major role in HSN axonal guidance. The dominant *lin-12(n137*sd) mutant, which generates secondary but not primary vulval cells (Greenwald et al., 1983), was defective in ventral-directed growth of the HSN axons, in the positioning of the left HSN axon within the VNC, in defasciculation of the HSN axons from the VNC and in HSN branching (Fig. 3A, Tables 1, 2). By contrast, the recessive *lin-12(n302 n865)* mutant, which generates primary but not secondary vulval cells (Greenwald et al., 1983), had normal HSN axonal morphology (Fig. 4B, Table 2). These results strongly suggest that the primary vulval cells or their precursors control many aspects of HSN axonal guidance. An alternative interpretation of these results is that the *lin-12* gene functions in the HSNs and the HSN axonal defects of dominant *lin-12* mutants is caused by intrinsic defects in the HSNs rather than by defects in vulval cells.

There might also be some influence of the secondary vulval cells on HSN axonal outgrowth. In *lin-12(n137*sd*)* mutants, the HSN axons entered the VNC and then grew posteriorly to the tail more frequently than in vulvaless mutants. Of those HSN axons that entered the VNC in *lin-*

*12(n137*sd*)* animals, 21% extended to the tail (Fig. 2D), as opposed to only 5% in the *lin-39(n1760)* vulvaless mutant. This increased frequency of posterior-directed outgrowth in *lin-12(n137*sd*)* mutants might be a consequence of the absence of primary vulval cells combined with the presence of ectopic secondary cells posterior to where the HSN axons enter the VNC. If so, growing HSN axons within the VNC might be attracted toward secondary as well as primary vulval cells (see below).

Since the HSN axons begin to grow ventrally during the L2 and early L3 stages, which is before the vulval cells divide, it appears that vulval precursor cell P6.p is required for the ventral-directed outgrowth of the HSN axons. However, it is not known when the HSN axons grow anteriorly within the VNC, defasciculate from the VNC or branch, so either the Pn.p cells or their progeny could influence these aspects of HSN axonal outgrowth.

Proximity of the HSN cell body to vulval cells is important for HSN axonal guidance

The HSN neurons are generated in the tail of the embryo and then migrate anteriorly to their final positions near the middle of the body (Sulston et al., 1983). Mutations in several genes result in the HSNs either failing to migrate or migrating only partially, leading to posterior displacement of the HSN cell bodies (Desai et al., 1988; see Fig. 5). All of these mutants with posteriorly displaced HSNs display defects in HSN axonal morphology that are in many ways similar to those seen in vulvaless animals (Table 3). First, the axons of the displaced HSNs often extend anteriorly or posteriorly a short distance before entering the VNC. However, these HSN axons never extend laterally all the way to the head as in vulvaless mutants: laterally extending axons of posteriorly displaced HSNs invariably enter the VNC at the vulva, presumably in response to P6.p or its

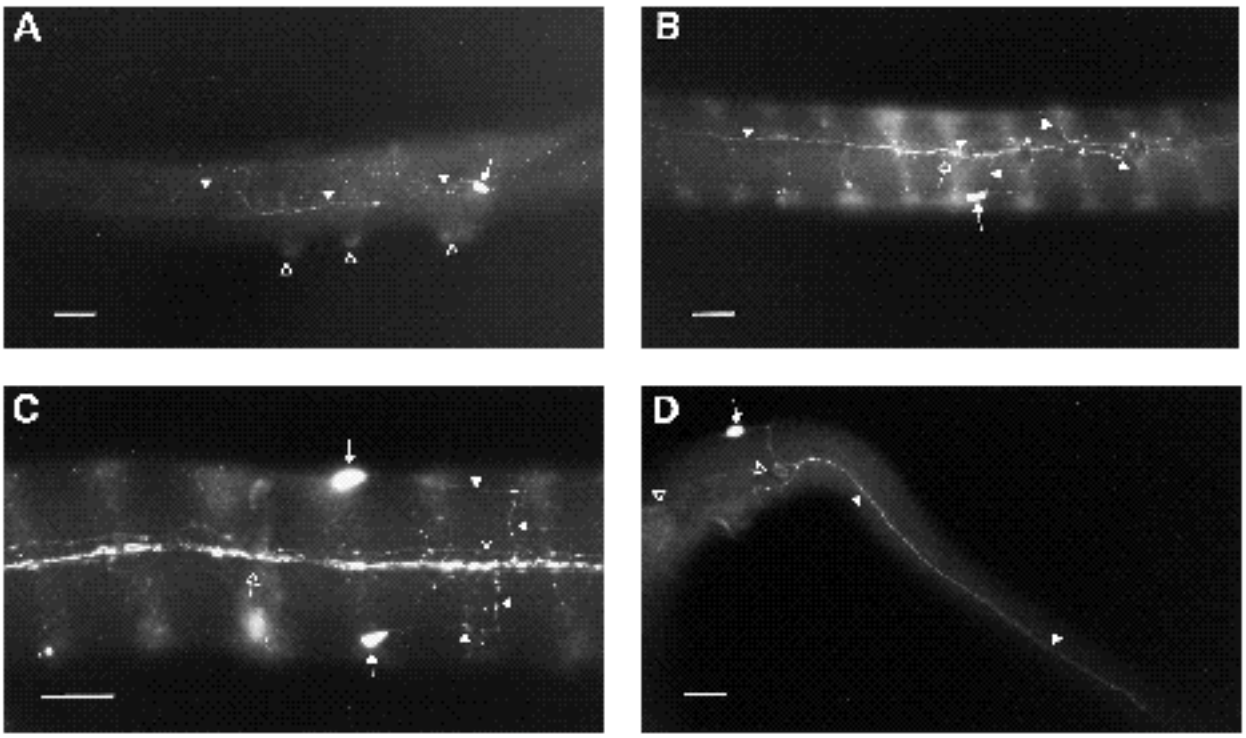


Fig. 3. Spectrum of HSN axonal guidance defects seen in animals missing primary vulval cells. (A-D) Immunofluorescence photomicrographs of mutant adult hermaphrodites. Anterior is to the left. (A) Left lateral view of a *lin-12(n137*sd*)* multivulva animal (containing only secondary vulval cells) stained with an anti-serotonin antiserum. The left HSN axon (closed arrowheads) extends abnormally along the lateral hypodermal epithelium. Note the abnormal loop of the HSN axon. The solid arrow indicates the HSN cell body. The open arrowheads indicate the positions of the vulval protrusions formed from ectopic secondary vulval cells. (B) Ventral view of a *lin-10* animal stained with anti-serotonin antiserum. The HSN axons (closed arrowheads) enter the VNC and then extend anteriorly together in a single fascicle. The left HSN axon first extends posteriorly on the left side of the VNC, then crosses to the right side of the VNC and extends anteriorly. The right HSN cell body is indicated by the solid arrow. The left HSN cell body is out of the plane of focus. Note that these HSN axons fail to branch. The position where the vulva would normally be is indicated by an open arrow. (C) Ventral view of a *lin-39(n1490)* animal stained with both anti-serotonin and anti-FMRFamide antisera. The axons (solid arrowheads) of both HSNs extend posteriorly and then ventrally. Note the left HSN axon crosses the left VNC bundle (marked by the AVK axon, open arrowhead). The HSN cell bodies are indicated by the solid arrows. The position where the vulva would normally be is indicated by an open arrow. No axons separate from the main VNC bundle, indicating that the HSN axons do not defasciculate in this region. (D) Ventral view of a *lin-12(n137*sd*)* animal stained with anti-serotonin antiserum. Both HSN axons (closed arrowheads) enter the VNC but grow posteriorly to the tail as a single fascicle. The solid arrow indicates the left HSN cell body. The right HSN cell body is out of the plane of focus. The open arrowheads indicate the positions of the vulval protrusions formed from ectopic secondary vulval cells. Scale bars, 20 μm.

descendants. As in the vulvaless mutants, the HSN axons can enter the VNC and grow posteriorly to the tail (e.g. 2% of the time in *mig-1(n1354)* mutants), which is again consistent with the vulval cells or their precursors playing a minor role in establishing the direction of HSN axonal extension in the VNC. Second, the HSN axons are positioned abnormally in the migration mutants: the left HSN axon passes over the left nerve bundle of the VNC, enters the right bundle and runs alongside the right HSN axon. Finally, the HSN axons fail to branch in the migration mutants. Unlike vulvaless animals, the HSN axons defasciculate at the vulva in the HSN migration mutant *mig-1(n1354)* (Table 2).

The severity of the defects in HSN migration and in HSN axonal morphology are generally correlated. For example, mutant strains with relatively severe defects in HSN migration tend to have higher frequencies of HSN axonal defects (Table 3). There is also a correlation between the severities of the HSN migration and axonal defects within individuals from a given mutant strain. For example, as shown in Fig. 5 for *mig-1(n1354)* animals, 77% of the HSN axons are normally positioned within the VNC and 80% of the HSN axons branch when the HSN cell bodies are found near or at their normal positions (located more than 70% of the distance from the anus to the vulva); by contrast, 13% of the HSN axons are normally positioned within the VNC and 28% of the HSN axons branch when the HSN cell bodies are displaced more posteriorly (located less than 70% of the distance from the anus to the vulva).

One interpretation of the existence of both migration and axon defects in these mutants is that the genes they define are separately involved in the two processes of HSN cell

Table 1. Vulval cells are required for normal HSN axonal morphology[a]

	Cells missing	Ventral-directed outgrowth[b]	Separation within VNC[c]	branching[d]
Wild type (N2)	none	100% (84)	100% (42)	80% (73)
Mutants				
lin-2	vulval[e]	99% (118)	28% (50)	2% (108)
lin-3	vulval	100% (26)	70% (13)	13% (23)
lin-7	vulval	100% (10)	40% (5)	20% (10)
lin-10	vulval	99% (98)	8% (48)	2% (97)
lin(n300)[f]	vulval	78% (103)	31% (32)	7% (82)
*lin-12(n302*sd)[g]	vulval	90% (21)	50% (6)	8% (13)
lin-39(n1490)[h]	vulval, VC neurons	92% (74)	20% (25)	13% (56)
lin-39(n1760)	vulval, VC neurons	83% (70)	5% (22)	0% (58)
lin-12(n137sd)	1° vulval	80% (72)	19% (16)	10% (59)
*lin-12(n302*sd *n865)*[i]	2° vulval	100% (13)	100% (6)	92% (13)
lin-11(n382)[j]	abnormal 2°	100% (27)	92% (12)	4% (27)
lin-11(n389)	abnormal 2°	100% (18)	100% (9)	6% (17
Cells killed				
Z1, Z4 (wild type)[k]	somatic gonad, vulval	77% (13)	34% (3)	0% (10)
P3-P8 (wild type)[l]	vulval, VNC neurons	75% (8)	50% (2)	0% (5)

[a]HSN axons were scored after staining animals with anti-serotonin antisera as described in Materials and Methods. The HSN axons that entered the VNC and then grew posteriorly to the tail were not considered in the results presented in this Table. Numbers in parentheses indicate the number of HSN axons scored.

[b]HSN axons were scored as either growing to the ventral nerve cord (VNC) or growing laterally. Only lateral axons that ran all the way to the head were scored as being defective in ventral-directed growth. HSN axons that ran posteriorly or anteriorly short distances but entered the VNC were not scored as abnormal for ventral-directed outgrowth, since the HSN axons in the wild-type sometimes grow in this way, but to a lesser extent. Lateral-growing HSN axons usually entered the VNC near the pharynx, presumably following the commisure of the deirids, sensory organs of the head (White et al., 1986).

[c]HSN axons were scored as either running in the two separate nerve bundles of the VNC or as running together in bundle on the right side. A low percentage (5%) of the left HSN axons in wild-type animals crossed to the right side of the VNC far anterior to the region of the vulva. We scored these axons or comparable axons in mutants as being separate in the VNC, since where this switch occurred vulval cells were unlikely to influence HSN axonal outgrowth.

[d]HSN axons were scored for branching in the region of the vulva. The number of HSNs scored for branching are somewhat lower than those those scored for ventral-directed outgrowth, because it was occasionally impossible to determine if an HSN axon had branched. The positioning of some animals obscured viewing the HSN axons at the vulva.

[e]The vulvaless mutants *lin*, *lin-3*, *lin-7*, *lin-10*, *lin(n300)*, *lin-12(n302*sd*)* and *lin-39* are variably missing vulval cells. For those mutant strains in which not all individuals are vulvaless, we scored only animals without any apparent vulva.

[f]The *n300* mutation, which is associated with the breakpoint of the reciprocal translocation *nT1*, causes a vulvaless phenotype (Ferguson and Horvitz, 1985).

[g]*n301* is a weak semidominant *lin-12* allele that causes a vulvaless (Greenwald et al., 1983).

[h]*n1490* and *n1760* are strong alleles of *lin-39* that cause an absence of all six VC neurons and also result in a vulvaless phenotype (S. Clark, personal communication; C. Li, personal communication).

[i]Homozygous *lin-12(n302*sd *n865)* animals were identified as sterile adults with a large protruding vulva derived from the balanced strain *unc-32(e189) lin-12(n302*sd)/*lin-12(n302*sd *n865)*.

[j]*lin-11* animals exhibit an abnormal lineage of the 2° vulval cells (Ferguson et al., 1987).

[k]Z1 and Z4 are the precursors to the somatic gonad (Kimble and Hirsh, 1979).

[l]P3-P8 are the precursor cells that generate both the cells of the vulval equivalence group (P3.p-P8.p) and the neuroblasts (P3.a-P8.a) that divide to produce several types of VNC motor neurons, including the VCs (Sulston and Horvitz, 1977).

migration and HSN axonal outgrowth. Alternatively, these genes could be required primarily for HSN migration, with the HSN axonal defects occurring as a secondary consequence of the abnormal positions of the HSN cell bodies. To distinguish between these two possibilities, we constructed a double mutant containing mutations in the *mig-1* and *lin-15* genes. In this double mutant, ectopic vulval cells are positioned near the displaced HSN cell bodies, allowing us to determine if the HSN axonal defects of the *mig-1* mutant are suppressed by the nearby vulval cells. Indeed, both the fasciculation and branching defects were suppressed, with the HSN axon branching ectopically at the nearby vulval protrusion (Fig. 5). Thus, the HSN axonal outgrowth defects of the HSN-migration mutants are a secondary consequence of the abnormal positions of the HSN cell bodies. In other words, it is not the correct position of the HSN cell body *per se* that is required for normal HSN axonal outgrowth, but rather it is the proximity of the HSN cell bodies to the vulval cells that is important.

Animals defective in the gene *dig-1* have the same HSN axonal defects as the HSN-migration mutants (Table 3). In *dig-1* animals, the gonad is anteriorly displaced so that the vulva develops in a more anterior position than it would normally; the HSN cell bodies are in their normal positions (Thomas et al., 1990). The existence of HSN axonal defects in *dig-1* animals further supports the hypothesis that it is the relative positions of the HSN cell bodies and the vulva rather than the absolute positions of the HSNs that are important for normal HSN axonal guidance.

Table 2. Defasciculation of the HSN axons from the ventral nerve cord

	Cells missing	Defasciculation[a]
lin-39(n709)	VC neurons	10/11
sem-2; *lin-39(709)*	Egg-laying muscles; VC neurons	9/10
lin-39(n1490)[b]	Vulval; VC neurons	0/15
*lin-12(n137*sd)[c]	1° vulval	0/16
P3.a-P8.a, P3.p-P5.p, P7.p-P8.p (wild type)[d]	2° vulval VC neurons	4/5
lin-11(n382)[e]	Abnormal 2° vulval	0/14
mig-1(n1354); *lin-39(n709)*	HSN migration defective; VC neurons	8/10

[a]Defasciculation refers to the normal separation at the vulva of the HSN axons from the other axons in the VNC. Defasciculation was usually scored in the background of the mutation *lin-39(n709)*, which results in a variable loss of VC neurons (see Results and Materials and Methods).

[b]*lin-39(n1490)* is a strong *lin-39* allele that results in a vulvaless phenotype and a lack of all six VC neurons (S. Clark, personal communication; C. Li, personal communication).

[c]It was not necessary to construct the *lin-39; lin-12(n137)* double mutant, since the VC axons fail to defasciculate from the VNC in *lin-12(n137*sd*)* animals (G. G., unpublished results).

[d]Since *lin-39* and *lin-12* are linked, we generated animals missing VC neurons and containing only primary vulval cells by killing the neuroblast cells P3.a-P8.a, which generate the VCs, and all of the vulval precursor cells except P6.p, which generates the primary vulval cells.

[e]It was not necessary to construct the *lin-11; lin-39* double mutant, since the VC axons fail to defasciculate from the VNC in *lin-11* animals.

The defects in ventral growth and VNC positioning of the HSN-migration and *dig-1* mutants seem likely to reflect the lack of vulval cells in the vicinity of the HSN axons as they begin to extend. However, the basis of the branching defect in these mutants is less obvious, since the HSN axons eventually grow past the vulval cells, and even appear to respond to cues for defasciculation at the vulva (Table 2). Perhaps the HSN axons are capable of branching only for a short distance or for a short period of time after their initial outgrowth.

The *lin-11* gene is required for HSN branching and fasciculation

While analyzing the effects of abnormalities in vulval development on HSN axonal outgrowth, we discovered that

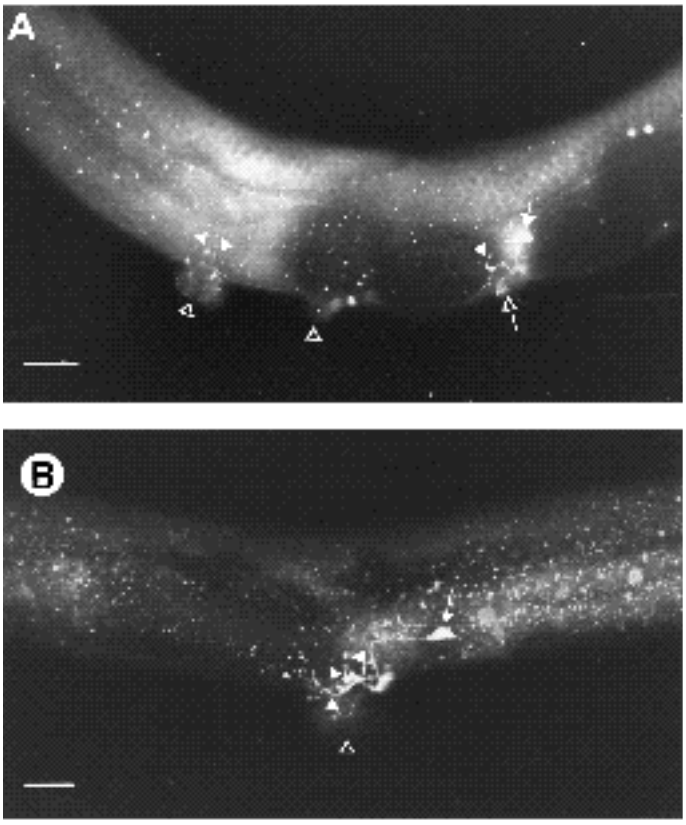


Fig. 4. Normal HSN axonal guidance requires primary vulval cells. Immunofluorescence photomicrographs of mutant adult hermaphrodites stained with an anti-serotonin antiserum. Anterior is to the left. (A) Left lateral view of a *lin-15* animal grown at 20°C. The HSN axon forms branches (solid arrowheads) at the vulva and at an extra vulva-like protrusion anterior to the normal vulva. The solid arrow indicates the left HSN cell body. The open arrow indicates the position of the vulva. The open arrowheads indicate the positions of extopic vulval protrusions formed from both primary and secondary vulval cells. (B) Left lateral view of a *lin-12(n302*sd *n865)* animal showing apparently normal HSN axonal morphology. The positions of three branches are indicated by the solid arrowheads. (The HSN axons in wild-type animals occasionally have more than one branch.) The solid arrow indicates the HSN cell body. The position of the abnormal vulva consisting of only primary cells is indicated by the open arrowhead. Scale bars, 20 μm.

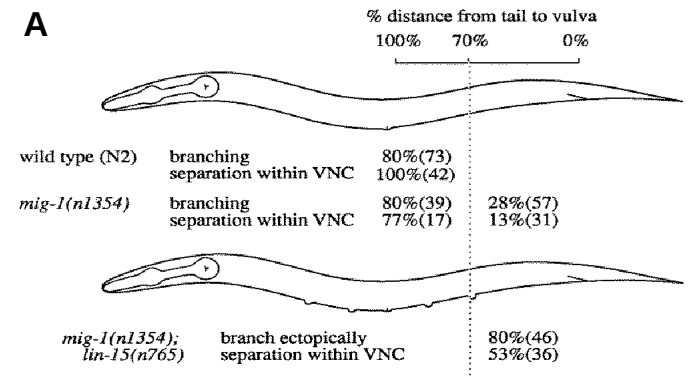
lin-11 mutants are defective in HSN branching and HSN defasciculation at the vulva (Fig. 6, Tables 1, 2). The axons of the VC neurons also fail to branch and defasciculate at the vulva in *lin-11* animals (C. Li, personal communication; G.G., unpublished results). In *lin-11* animals, the fates of the secondary vulval cells are altered, but the fates of the primary vulval cells are not obviously abnormal (Ferguson et al., 1987). Since *lin-12(n302 n865)* mutants, which lack secondary vulval cells, appear to be normal in HSN branching and defasciculation (see above), the HSN abnormalities in *lin-11* animals were unexpected. Perhaps it is the presence of abnormal secondary vulval cells (as opposed to the absence of normal secondary cells) that is responsible for these HSN and VC outgrowth defects in *lin-11* animals. Alternatively, *lin-11* could affect another cell type involved in HSN and VC outgrowth, such as the primary vulval cells or the HSNs and VCs themselves.

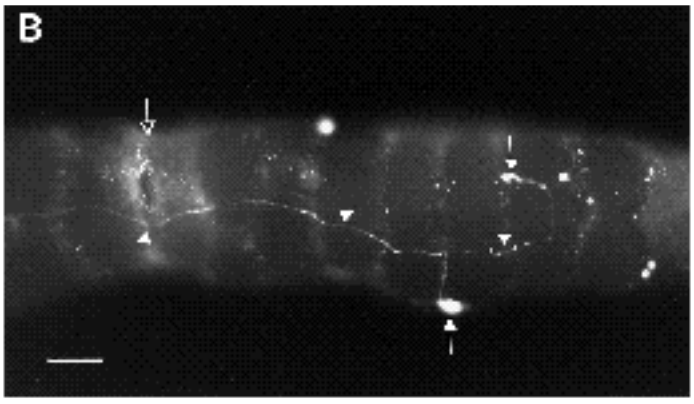
It is interesting that *lin-11* is required for HSN branching and defasciculation at the vulva but not for ventral- or anterior-directed outgrowth or for positioning of the left HSN within the left bundle within the VNC. That the *lin-11* mutations affect only some aspects of HSN axonal outgrowth suggests that *lin-11* acts differently from the other genes required for vulval development and is consistent with the hypothesis that *lin-11* functions in cells other than the primary vulval cells or in only a subset of these cells.

The egg-laying muscles, the VC neurons and cells of the somatic gonad are not required for HSN axonal guidance

The egg-laying muscles, the VC neurons and cells of the somatic gonad do not appear to play a role in HSN axonal outgrowth. HSN axonal morphology appeared normal both in mutants with displaced (*egl-15*) or missing (*sem-2*) egg-laying muscles (Trent et al., 1983; Stern and Horvitz, 1991; M. Stern, personal communication), and in animals in which the precursor to the egg-laying muscles (M cell; Sulston and Horvitz, 1977) had been killed with a laser microbeam (Tables 2, 4). Similarly, the VC neurons, the other class of neurons that innervate the egg-laying muscles and branch at the vulva, also are not necessary for normal HSN axonal morphology. First, HSN axonal morphology is normal in *lin-39(n709)* animals missing all six VC neurons (Fig. 2C). Second, animals in which the neuroblasts that give rise to the VCs were killed with a laser microbeam still formed apparently normal HSN axons (Table 4).

In addition, the somatic (non-germline) cells of the gonad do not appear to be required for normal HSN axonal morphology. We could not test the direct involvement of these cells simply by killing the somatic gonad precursor cells Z1 and Z4 (Kimble and Hirsh, 1979) in wild-type animals,





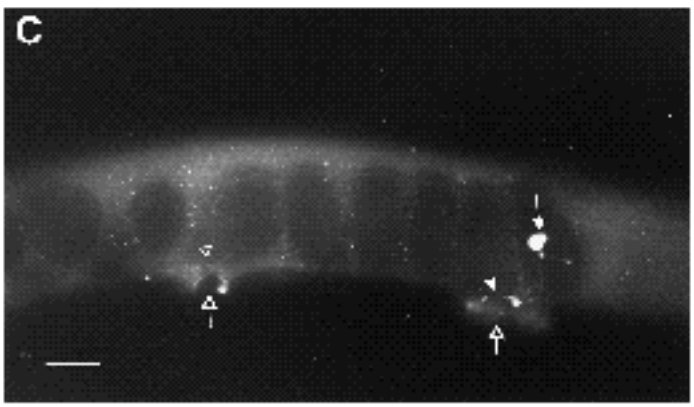


Fig. 5. The HSN axonal outgrowth defects of the displaced HSNs in *mig-1* mutants are suppressed by ectopic vulval cells in *mig-1; lin-15* double mutants. (A) Schematic diagram illustrating effects of the mispositioning of the HSN cell body on HSN axonal branching and fasciculation. The position of the HSN cell body relative to the anus and the vulva was measured in adult animals after staining with anti-serotonin antiserum. In wild-type and *mig-1* animals, whether the left HSN axon ran within the left VNC nerve bundle (separately from the right HSN axon) or within the right VNC bundle and the branching of the HSN axons at the vulva were scored. In *mig-1; lin-15* animals, the positions of the HSN cell bodies, whether the left HSN axon ran within the left VNC nerve bundle (separately from the right HSN axon) or within the right VNC bundle and the branching of the HSN axons at a nearby ectopic vulva-like protrusion were scored.
(B,C) Immunofluorescence photomicrographs of mutant adult hermaphrodites stained with anti-serotonin antiserum. Anterior is to the left. Scale bars, 20 μm. (B) Ventral view of a *mig-1* animal. The HSN axons (solid arrowheads) from posteriorly displaced HSN cell bodies (solid arrows) enter the VNC and fasciculate within the right nerve bundle of the VNC. Note that the HSN axons fail to branch at the vulva (open arrow). (C) Left lateral view of a *mig-1; lin-15* animal. The left HSN axon from a posteriorly displaced HSN cell body forms a branch (solid arrowhead) at the posterior ectopic vulva-like protrusion (right open arrow), but fails to branch (open arrowhead) at the vulva (left open arrow). The HSN cell body is indicated by the solid arrow.

since the somatic gonad signals the vulval precursor cells to develop as vulval cells and thus is indirectly needed for HSN axonal outgrowth (see above). To circumvent this problem, we killed the somatic gonad precursor cells in a *lin-15* mutant (Table 4). Because the vulval cells develop independently of the gonadal signal in *lin-15* mutants, *lin-15* animals missing a somatic gonad still generate vulval cells (Ferguson et al., 1987). In these animals, the HSN axons extended to the VNC and branched in the absence of the somatic gonad. For technical reasons, we could not determine if the HSN axons fasciculated abnormally in the VNC (see Table 4 legend). We cannot absolutely exclude a role for the somatic gonad in HSN axonal outgrowth from this experiment, since it is conceivable that *lin-15* causes gonad-independent HSN axonal outgrowth just as it causes gonad-independent vulval development.

Our methods would not have detected subtle changes in HSN morphology, such as defects in the extent or shape of the HSN branch or alterations in the positions of the HSN axons relative to other axons within the VNC. Thus, it remains possible that the egg-laying muscles, VC neurons or cells of the somatic gonad play a role in these more subtle aspects of HSN axonal guidance.

The PVP and PVQ axons are required for the HSN axons to fasciculate normally within the ventral nerve cord

The *C. elegans* VNC is bilaterally asymmetric, with a large bundle of axons on the right side (50-60 axons) separated by a ridge of ventral hypodermal epithelium from a small bundle of axons on the left side (3-5 axons; Fig. 7A; White et al., 1976, 1986). In the region of the vulva in the left bundle of the VNC there are three axons besides that of the left HSN, those of the AVKR, PVPR and PVQL neurons. The cell bodies of the two AVK neurons, AVKL and AVKR, are located in the ventral ganglion of the head, and the cell bodies of the two PVP neurons, PVPL and PVPR, are located in the pre-anal ganglion of the tail. Both the AVK and PVP neurons send axons to the side of the VNC contralateral to their cell bodies. For example, the AVKR cell body on the right side of the head has an axon on the left side of the VNC. The cell bodies of the two PVQ neurons, PVQL and PVQR, are located in the lumbar ganglia of the tail and each sends an axon to the ipsilateral side of the VNC. To determine if axons within the VNC are

Table 3. Severity of HSN mispositioning correlates with severity of HSN axonal outgrowth defects[a]

Strain	HSN cell body position[b]	Separation within VNC[c]	Branching[d]
egl-43	12% (90)	1% (68)	1-2% (90)
egl-18(n475)	56% (44)	27% (23)	11-26% (44)
mig-10	57% (44)	9% (22)	7-11% (44)
mig-1(n1354)	67% (96)	35% (48)	45-53% (96)
egl-20	68%(64)	23% (35)	37-49% (64)
mig-1(e1787)	71% (103)	27% (52)	27-39% (103)
egl-27	72% (82)	51% (39)	40-47% (82)
egl-18(n162)	73% (47)	71% (24)	53-64% (47)
wild type (N2)	96% (73)	100% (42)	80% (73)
dig-1	78% (58)[e]	22% (31)	26-31% (58)

[a]The numbers in parentheses indicate the number of HSN axons scored.

[b]The average position of the HSN cell body is reported as the ratio of the distance between the anus and the HSN cell body and the distance between the anus and the vulva, times 100.

[c]% of animals in which the two HSN axons ran in the two separate bundles of the VNC.

[d]% HSN axons that branch is given as a range, because in these mutants the left HSN axon often crossed to the right side of the VNC to run with the right HSN axon. If a branch was visible from the two axons that ran together, we could not determine whether one or both HSN axons branched. The lower number in the range assumes only one of the axons branched, while the higher number assumes both branched.

[e]The HSN cell body position in *dig-1(n1321)* animals reflects an abnormality in the position of the vulva rather than an abnormality in the position of the HSN cell body. Since the distance between the anus and vulva is larger in *dig-1* than in migrationmutants, the value of 78% cell body position for *dig-1* animals reflects a larger absolute distance between the HSN cell body and the vulva than it would for a mutant in which the HSN cell body was displaced posteriorly.

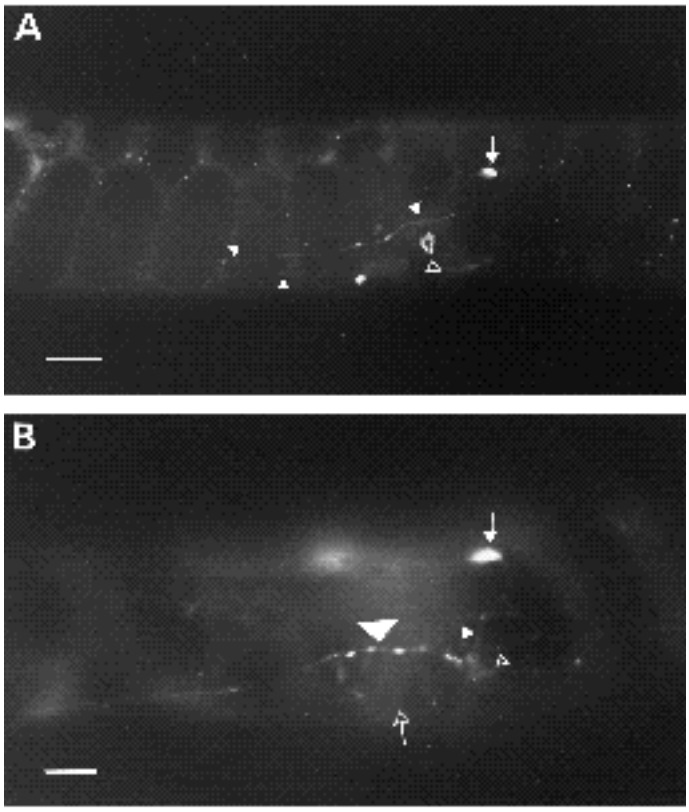


Fig. 6. *lin-11* mutants are defective in HSN defasciculation and branching at the vulva. Immunofluorescence photomicrographs of *lin-11* adult hermaphrodites. Anterior is to the left. (A) Oblique left lateral view of a *lin-11(n389)* animal stained with an anti-serotonin antiserum. The left HSN axon (arrowheads) extends to the VNC but fails to form varicosities or to branch. The HSN cell body is indicated by the solid arrow. The position of the abnormal vulva is indicated by the open arrow. (B) Left lateral view of a *lin-11(n382)* animal stained with both anti-serotonin and anti-FMRFamide antisera. The left HSN axon (stained with the anti-serotonin antiserum, small solid arrowhead) extends to the VNC and joins the VNC (the AVK axon in the left VNC is stained with the anti-FMRFamide antiserum, open arrowheads) but fails to defasciculate. The position of the adjacent AVK and HSN axons at the vulva is indicated by the large arrowhead. The HSN cell body is indicated by the solid arrow. The position of the abnormal vulva is indicated with an arrow. Scale bars, 20 μm.

Table 4. The egg-laying muscles, VC neurons and cells of the somatic gonad are not required for normal HSN axonal morphology

	Cells missing	Ventral-directed growth[a]	Separation within VNC[a]	Branching[a]
wild type (N2)	none	100% (84)	100% (42)	80% (73)
Mutants				
egl-15	egg-laying muscles[b]	100% (50)	100% (25)	59% (44)
sem-2	egg-laying muscles[c]	100% (94)	100% (47)	69% (83)
Cells killed				
M (wild type)[d]	egg-laying muscles	100% (25)	100% (10)	80% (25)
Pn.a (wild type)[e]	VC neurons	100% (20)	100% (10)	80% (20)
Z1,Z4 (*lin-15(n765)*)[f]	somatic gonad, multivulva	100% (11)	n.d.	83% (7)

[a]See Table 1 legend.

[b]The precursors to the egg-laying muscles, which normally migrate anteriorly to their final positions during the L3 stage, are defective in this migration in *egl-15* animals (Trent et al., 1983; Stern and Horvitz, 1991). As a consequence, the egg-laying muscles are displaced posteriorly.

[c]The precursors to the egg-laying muscles are transformed into body wall muscles in sem-2 animals (M. Stern, personal communication).

[d]The M cell is a mesoblast that generates the egg-laying muscles, some body wall muscles and coelomocytes (which are of unknown function) (Sulston and Horvitz, 1977).

[e]The Pn.a cells are 12 neuroblasts that divide postembryonically to generate ventral nerve cord motor neurons (Sulston and Horvitz, 1977). The VC neurons are generated from P3.a-P8.a. In these experiments, theeight cells P2.a to P9.a were killed in five animals, and the six cells P3.a to P8.a were killed in five other animals.

[f]Z1 and Z4 are the precursors to the somatic gonad (Kimble and Hirsh, 1979). These cells were killed in 11 newly hatched L1 stage *lin-15* animals. Because of poor staining, perhaps as a consequence of the general sickness of this strain, we were able to score only a subset of the HSN axons (11/22 for ventral-directed outgrowth, 7/22 for branching, 0/22 for growth within the VNC).

required for extension of the left HSN axon along the left nerve bundle of the VNC, we killed AVKR, PVPR and PVQL, let the animals grow to become adults and stained them with an anti-serotonin antiserum. When all three of these neurons were killed, the left HSN axon crossed the hypodermal ridge and extended along the right bundle of the VNC with the right HSN axon, as in vulvaless animals (Fig. 7B, Table 5). Thus, the left HSN axon is not capable of pioneering the left side of the VNC. Electron micrographs of the VNC of animals in which the AVKR, PVPR and PVQL neurons were killed in newly hatched animals confirmed that the left side of the VNC was absent by the late L2 and early L3 stages, the time when the HSN axons begin to extend (E. Hartwieg and G.G., unpublished results).

To determine which of these three axons are required for growth of the left HSN axon on the left side of the VNC, we killed neurons individually and in pairwise combinations (Table 5). In experiments in which a single neuron was killed, only killing of the PVQL neuron had any obvious effect on HSN axonal morphology: the left HSN axon extended along the right bundle of the VNC with the right HSN axon in two out of seven operated animals. However, in all animals missing both PVPR and PVQL, the left HSN axon extended along the right bundle of the VNC. These results suggest that the PVP and PVQ axons play partially redundant roles in guiding the outgrowth of the left HSN axon along the left VNC fascicle, perhaps by expressing similar molecules on their surface that are recognized by the HSN growth cone. These results also suggest that the left HSN growth cone does not interact with the left AVK axon.

When we killed the PVPL and PVQR neurons, which contribute axons to the right side of the VNC, we saw no obvious HSN axonal defects. In particular, the right HSN axon did not run in the left bundle of the VNC. Presumably, the right HSN axon can recognize one of the many axons on the right side of the VNC. We note that the apparently normal morphology of the right HSN axon in animals missing the PVPL and PVQR axons could be misleading. Our procedures would not have detected changes in the precise positioning of the HSN axon within the VNC, and it is possible that the right HSN axon fasciculated in a different region of the VNC in these animals. When both PVP

Table 5. Ventral nerve cord interactions required for normal HSN axonal fasciculation

Cells killed			Separation within VNC[a]
Left side of ventral nerve cord[b]			
AVK	PVP	PVQ	0/5
AVK			7/7
	PVP		9/9
		PVQ	5/7
AVK	PVP		7/7
AVK		PVQ	4/6
	PVP	PVQ	0/7
Right side of ventral nerve cord[c]			
	PVP	PVQ	4/4
Both sides of ventral nerve cord[d]			
	PVP	PVQ	0/9

[a]The HSN axons normally run separately on either side of the VNC. Removing all other axons from the left nerve bundle of the VNC resulted in the left HSN axon's crossing to the right side of the VNC. Although the left HSN axon sometimes crossed to the right side of the VNC posterior to the vulva (5/25), usually it crossed anterior to the vulva (20/25), as shown in Fig. 6. Of the 20 left HSN axons that crossed to the right side of the VNC anterior to the vulva, two turned posteriorly and continued within the VNC to the tail.

[b]The AVKR, PVPR and PVQL neurons were killed in these experiments.

[c]The PVPL and PVQR neurons were killed in these experiments.

[d]The PVPL, PVPR, PVQL and PVQR neurons were killed in these experiments.

neurons and both PVQ neurons were killed, the left HSN axon extended along the right bundle of the VNC and ran with the right HSN axon.

DISCUSSION

By analyzing HSN morphology in both mutants and laser-operated animals, we have discovered that many aspects of HSN axonal outgrowth are controlled by interactions with other cells. Epithelial cells of the vulva, in particular the primary vulval cells or precursors that generates these cells, play a major role. Our experiments, summarized in Table 6, lead to the models for HSN axonal outgrowth illustrated in Fig. 8. First, the vulval precursor cell P6.p attracts the growth cone of each HSN, helping its axon to extend ventrally to the VNC (Fig. 8A). There the HSN growth cones interact with other axons, including those of PVP and PVQ, to determine which of the two fascicles each will join (Fig. 8B). The primary vulval cells or their precursors help direct the left HSN axon to the left VNC fascicle. Each HSN axon then elongates anteriorly for a short distance, after which interactions with the primary vulval cells or their precursors cause it to defasciculate from the VNC in the region of the developing vulva (Fig. 8C), where it will branch, form varicosities and make synapses with the vulval muscles. Developing vulval cells also induce or stabilize HSN axonal branching at the vulva (Fig. 8D). The HSN axons return to the VNC and fasciculate with the axons of the PVP and PVQ neurons, which guide them anteriorly toward the head. We discuss these steps in more detail below.

Table 6. Summary of effects of cell interactions on HSN axonal guidance

Cells missing	Ventral-directed growth	Separation within ventral nerve cord	Defasciculation from ventral nerve cord	Branching
All vulval cells	−	−	−	−
1° vulval cells	−	−	−	−
2° vulval cells	+	+	+	+
PVP and PVQ	+	−	n.d.	+

n.d., not determined.

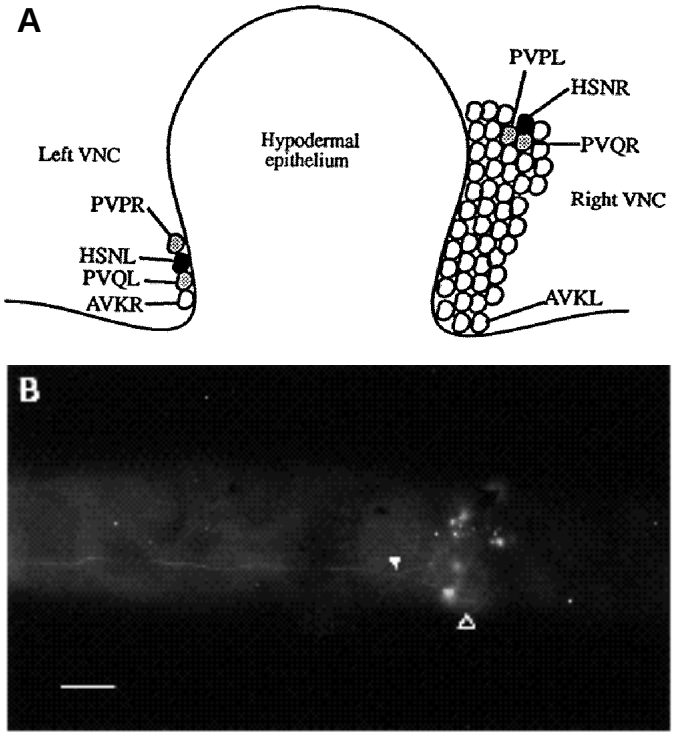


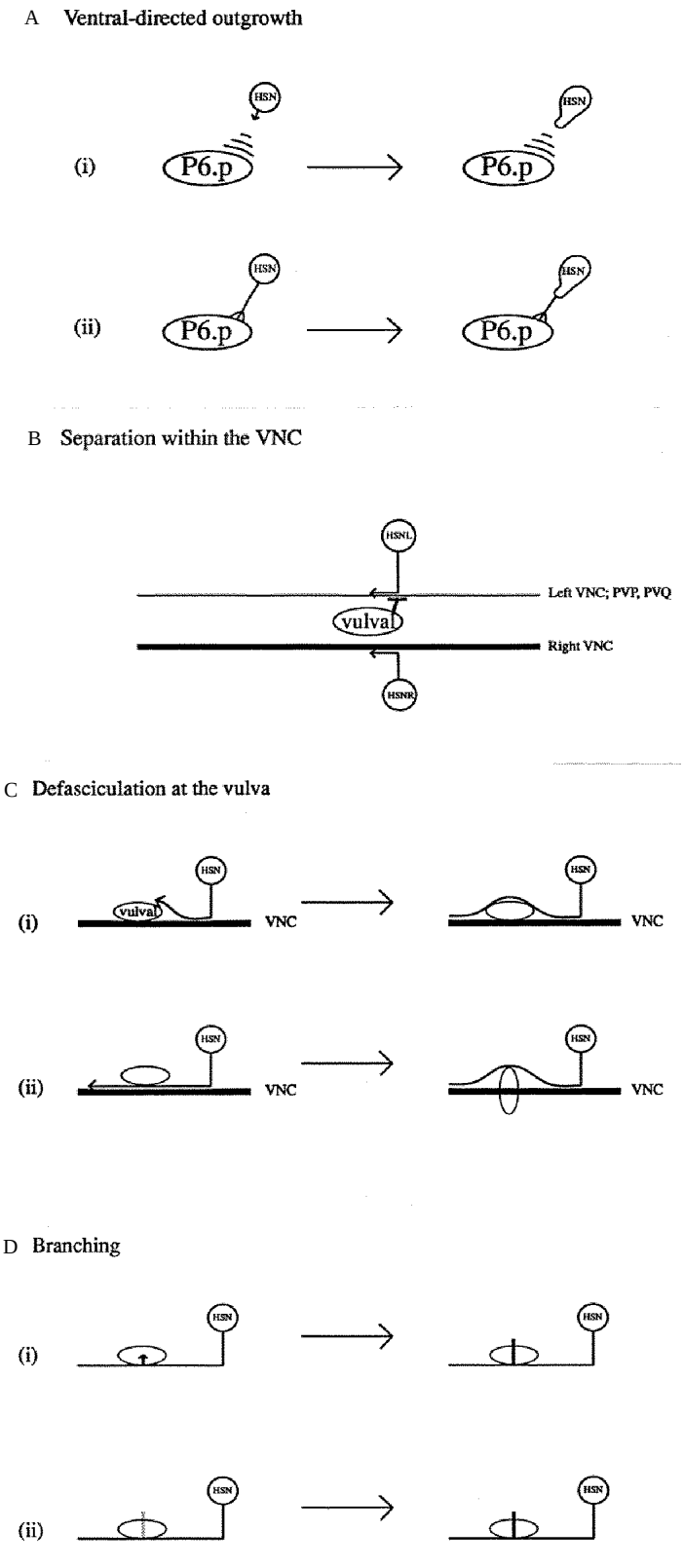
Fig. 7. Removing the PVPR and PVQL axons results in the HSNL axon crossing the hypodermal epithelium and entering the right VNC fascicle. (A) Schematic diagram of a cross-section of a normal VNC, somewhat anterior to the vulva (adapted from White et al. 1986). The large bundle of axons on the right side is separated by a hypodermal epithelial ridge from the small bundle of axons on the left. Note that on both sides of the VNC the HSN axon runs adjacent to the PVP and PVQ axons, whereas the AVK axon, the only other axon present at this position within the left bundle, is located far from the HSN axon on the right side. There is some variability of the positions of these axons along the VNC; for example, the HSNL axon can be dorsal to PVPR axon. (B) Immunofluorescence photomicrograph of a ventral view of an adult wild-type (N2) hermaphrodite in which the PVPR and PVQL neurons were killed by laser microsurgery. The PVPR and PVQL neurons contribute axons to the left side of the VNC. Anterior is to the left. The left HSN axon crosses to the right side of the VNC (position of crossing indicated by solid arrowhead) and runs with the right HSN axon. The position of the vulva is indicated by the open arrowhead. Both HSN cell bodies are out of the plane of focus. Scale bar, 20 μm.

A vulval precursor cell helps direct the HSN axons to the VNC at the proper position

In wild-type animals, ventral guidance cues for the HSN growth cones appear to compete with another guidance system provided by laterally positioned processes: if the ventral guidance system is perturbed, the HSN axons extend along the lateral processes. The vulval precursor cell P6.p, which constitutes a part of the ventral epithelial suface of the animal (J. White, personal communication) and gives rise to the primary vulval cells (Sulston and Horvitz, 1977), provides a significant but minor component of this ventral guidance system. Since the HSN axons grow across lateral epithelial cells and are not in obvious contact with the vulval precursor cells (which are located along the ventral side of the animal), P6.p could be producing a diffusible signal that attracts the HSN growth cone (Fig. 8A). Alternatively, HSN filopodia, not visible in the light microscope, might contact P6.p and draw the HSN axons toward the VNC. Both chemotropic guidance (Lumsden and Davies, 1983, 1986; Tessier-Lavigne et al., 1988; Heffner et al., 1990) and contact between filopodia and 'guidepost cells' (Bentley and Keshishian, 1982; Taghert et al., 1982; Bentley and Caudy, 1983) are known to direct neurite outgrowth.

The major component of the ventral guidance system for HSN-axonal outgrowth is provided by the genes *unc-6* and *unc-40*, two of three genes required for the circumferential outgrowth of many axons (Desai et al., 1988; Hedgecock et al., 1990; McIntire et al., 1992). Hedgecock et al. (1990) proposed that these genes encode products involved in the

interaction between the axonal growth cones and the epithelium over which they extend. If so, the vulval cell P6.p and the lateral epithelium might act together to define the ventral direction of initial HSN outgrowth. Alternatively, *unc-6* and *unc-40* might act within P6.p to control the outgrowth of the HSN axons. However, defects in ventral-directed HSN outgrowth are much more frequent in *unc-6* and *unc-40* mutants (only 10% of the HSN axons extend ventrally in the *unc-6(e7)* mutant; Desai et al., 1988; McIntire et al., 1992) than in vulvaless animals (about 75% of the HSN axons extend ventrally in laser-generated vulvaless animals; Table 1). Thus, *unc-6* and *unc-40* are unlikely to act only within P6.p to control ventral-directed outgrowth of the HSN axon, although they still could act both within P6.p and other cells, including those of the lateral epithelium. Our attempts to resolve whether vulval cells and the *unc-6* gene product act in the same process (e.g., P6.p secretes the *unc-6* protein) or in separate processes for ventral-directed growth of the HSN axons by analyzing HSN morphology in *unc-6* mutants that lack vulval cells resulted in an ambiguous finding: the ventral-growth defect was slightly enhanced in these animals relative to the *unc-6* mutants, but still incomplete.



Vulval cells are required for defasciculation of the HSN axons at the vulva

In animals that fail to generate the primary vulval cells, the HSN axons do not defasciculate from the VNC at the vulva. Because our light microscopic techniques do not permit visualization of the HSN axons at the time they are growing along the VNC, we do not know when this defasciculation normally occurs. Thus, either the primary vulval cells themselves or one or more of their precursor cells could be required for defasciculation. Similarly, we do not know if defasciculation occurs at the time of HSN axonal ougrowth or later, after the axon has passed the region of the vulva. As illustrated in Fig. 8C, vulval cells could directly affect HSN axonal outgrowth in this region by defining the paths along which the HSN axons grow. Alternatively, vulval cells could separate the HSN axons from most of the other VNC axons after the HSN axons have extended beyond the vulval cells. During the L4 stage, the vulval cells undergo a complex set of rearrangements to generate the vulva (Sulston and Horvitz, 1977; J. White, personal communication),

Fig. 8. Models for the interactions between the HSN axons and the vulval cells and PVP and PVQ axons. Anterior is to the left. (A) Ventral-directed outgrowth. Lateral view of the HSN and P6.p. The primary vulval precursor P6.p could either (i) emit a diffusible signal that attracts the HSN growth cone or (ii) contact filopodia from the HSN growth cone, drawing the HSN axons to the VNC. (B) Separation within the VNC. Ventral view of the HSN axons, VNC and vulval cells. The left HSN axon requires P6.p or its descendants as well as the PVP and PVQ axons to fasciculate normally along the left VNC nerve bundle. P6.p might inhibit the HSN growth cone from crossing to the right VNC bundle. (C) Defasciculation at the vulva. Lateral view of the HSN and vulval cells. P6.p or its descendants could cause defasciculation of the HSN axon from other axons in the VNC bundle either (i) by providing an alternative pathway for HSN axonal outgrowth or (ii) by separating the HSN axons from the VNC bundle axons after the HSN axons have already grown along the axons in the VNC, possibly as a result of vulval morphogenesis in the L4 stage (Sulston and Horvitz, 1977). (D) Branching. Lateral view of the HSN and vulval cells. P6.p or its descendants could either (i) induce HSN branching or (ii) stabilize the HSN branch once it has formed.

and vulval morphogenesis might well be responsible for this defasciculation.

Vulval cells are required for branching of the HSN axons

Either the primary vulval cells or their precursors are required for the HSN axons to branch. Since we have been unable to use light microscopy to study the HSN axons as they are growing after they enter the VNC, we do not know when or how the HSN axons form branches. In other organisms, branches can be formed by bifurcation of an extending growth cone (Fujisawa, 1987; Raper et al., 1983a) or by sprouting either from just behind the growth cone (Harris et al., 1987) or from the side of a fully extended axon (O'Leary and Terashima, 1988). Thus, the HSN branch might form either as the HSN axon begins to grow anteriorly in the VNC or after the HSN axon has grown beyond this region. The vulval cells or their precursors could induce branching, provide a permissive substrate for growth of the branch or stabilize the branch once it has formed (Fig. 8D).

Studies of neurite branching in other organisms have often found that growing branches lack obvious growth cones (Fujisawa, 1987; Harris et al., 1987; O'Leary and Terashima, 1988), which suggests a mechanistic difference between most forms of axonal elongation and branching. In this context, it is interesting to note that mutations in many of the genes (*unc-6*, *unc-14*, *unc-33*, *unc-34*, *unc-40*, *unc-44*, *unc-51*, *unc-71*, *unc-73* and *unc-76*) required in general for axonal outgrowth in *C. elegans* (Desai et al., 1988; Hedgecock et al., 1985, 1990; McIntire et al., 1992) do not lead to obvious defects in HSN branching (G. G., unpublished observations).

The HSN branch might not be required for HSN function

Our observations suggest that branching is not an absolute requirement for HSN function. Assuming our immunocytochemical methods can detect all HSN branches, some of the HSN migration mutants that are missing HSN branches lay eggs normally. For example, approximately 25% of *mig-1* animals are missing branches of both HSNs, but only 5% of *mig-1* animals are egg-laying defective (Desai et al., 1988). Similarly, of the 45 *egl-43* animals that we could score for HSN branching, only one contained an HSN branch; however, 10% of *egl-43* animals are egg-laying competent. These results might explain why we have not found any mutants that are specifically affected in HSN branching in our collection of egg-laying defective mutants (Trent et al., 1983; Desai and Horvitz, 1989; C.D. and G.G., unpublished results).

The active role played by *C. elegans* muscles in synaptogenesis could explain why the HSN branch might not be required for HSN function. *C. elegans* muscles send processes known as muscle arms to the axons of motor neurons in both the ventral and dorsal nerve cords (White et al., 1976, 1986). These muscle arms apparently can regulate their growth to find misplaced axons. For example, a defect in circumferential axonal outgrowth in *unc-5* mutants results in the loss of the dorsal nerve cord because the axons of the motor neurons normally found in the dorsal nerve cord are displaced ventrally. Muscle arms of the dorsal body wall muscles that are normally innervated by axons in the dorsal cord regulate their growth to interact with these displaced axons in *unc-5* animals (Hedgecock et al., 1990). It is possible that although most of the synapses between the egg-laying muscles and the HSNs are normally made on the HSN branches, when the HSN axons do not branch the egg-laying muscle arms regulate their growth and nonetheless synapse with the HSN axons.

Vulval cells and neurons are both required for HSN axonal pathfinding in the VNC

As illustrated in Fig. 8B, both vulval cells and the axons of the PVP and PVQ neurons are required for the left HSN axon to fasciculate with the axonal bundle of the left VNC. The requirement for vulval cells is interesting, since in the absence of these cells the left HSN axon grows over axons that are normally chosen as substrates for fasciculation. One explanation for how vulval cells might influence the pathway choice of the left HSN axon is that the growth cone of the left HSN prefers the right VNC bundle but the vulval precursor cells, which constitute the hypodermal ridge over which the left HSN axon must pass to enter the right side of the VNC, is not a permissible substrate for HSN axonal outgrowth (Fig. 8B). In other words, the vulval cells could actively inhibit the HSN growth cones. In vertebrates, several examples of substances that are inhibitory to growth cone extension have recently been described (for reviews, see Schwab, 1990; Walter et al., 1990). An alternative hypothesis is that vulval cells play an active role in HSN axonal pathfinding. For example, vulval cells might actively cause the left HSN growth cone to interact with axons in the left VNC bundle, by altering the properties of either the HSN growth cones or the axons on which they extend.

Our observations of HSN axonal morphology in animals missing PVP and PVQ axons indicate that even in an axon bundle as simple as the left VNC, which has only four axons in the vulval region, there are subgroups of axons that selectively fasciculate with one another. Although the left HSN axon fasciculates with the PVP and PVQ axons, it does not appear to interact with the AVK axon. That the left HSN axon must pass over additional axons within the right VNC bundle before reaching the right HSN axon (White et al., 1986) further suggests that the VNC is a complex structure with subdomains created by selective fasciculation.

White and his colleagues (1985, 1986) proposed that synaptic connectivity in *C. elegans* is determined largely by the 'neighborhoods' of axons. Ultrastructural analysis of the nematode nervous system revealed that synapses are made between adjacent axons *en passant* and that axons maintain their neighbors (adjacent axons) for long distances within axonal bundles. Since within a nerve bundle *C. elegans* neurons synapse with 23% of their neighboring axons (for highly adjacent axons; Durbin, 1987), the synaptic connectivity of a neuron is dictated to a great extent by the neighborhood its axon inhabits. How are these neighborhoods established? Our results support the hypothesis (White, 1985; White et al., 1986) that neighborhoods are generated by selective fasciculation. Analogous to the 'labeled pathway hypothesis' proposed to account for selective fasciculation in grasshoppers (Raper et al., 1983b,

1984), selective fasciculation in *C. elegans* could be specified by neighborhood-specific surface molecules that act as recognition markers for individual growth cones.

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