Extracellular cues and pioneers act together to guide axons in the ventral cord of *C. elegans*

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Summary

The ventral cord is the major longitudinal axon tract in *C. elegans* containing essential components of the motor circuit. Previous studies have shown that axons grow out sequentially and that there is a single pioneer for the right axon tract which is important for the correct outgrowth of follower axons. Here, the dependencies between early and late outgrowing axons in the ventral cord were studied systematically with laser ablation experiments and a detailed analysis of mutants using multi-color GFP markers. Different classes of axon were affected to a different extent when the AVG pioneer neuron was eliminated. In the majority of the animals, axons were able to grow out normally even in the absence of the pioneer, suggesting that its presence is not absolutely essential for the correct outgrowth of follower axons. The transcription factor LIN-11 was found to be essential for the differentiation and pioneering function of the AVG neuron. UNC-30 appears to play a similar role for the PVP pioneer neurons. Later outgrowing axons typically do not simply follow earlier outgrowing ones, but subtle dependencies between certain groups of early and late outgrowing axons do exist. Different groups of axons growing in the same axon bundle apparently use different combinations of guidance cues for their navigation and can navigate largely independently.

Key words: Nervous system, Axon guidance, Laser ablation

Introduction

Establishing the correct pattern of axon outgrowth during brain development is a formidable navigational problem because of the large numbers of neurons involved. The complexity of the problem is reduced by the fact that axons from different groups of neurons grow out sequentially. Axonal pathways typically are established by one or a few early outgrowing axons known as ‘pioneers’. Later outgrowing axons frequently extend along these preformed pathways and often depend on the presence of pioneer axons. In the grasshopper limb bud, a pair of neurons has been identified, which pioneers a pathway to the central nervous system. Some of the later outgrowing axons are unable to grow out in the absence of the pioneer (Klose and Bentley, 1989), whereas others are able to do so (Keshishian and Bentley, 1983), depending on the time of outgrowth. Here, pioneers are thought to provide a pathway through the tibia-femur segment boundary, which differentiates after pioneers have grown out and apparently presents a physical barrier for later outgrowing axons originating distal to the boundary (Klose and Bentley, 1989). Several examples are known where more than one neuron can act as pioneer. The G growth cone in grasshopper depends on the presence of only one of the three P axons pioneering the AP fascicle (Raper et al., 1983a; Raper et al., 1983b; Raper et al., 1984). Similar results were also obtained in zebrafish, where follower axons can reach their targets even when one or a combination of two of the three primary motoneurons is eliminated (Eisen et al., 1989; Pike et al., 1992). Frequently, the navigation of follower axons is only partially affected in the absence of pioneers. For example, elimination of the TPOC axons in the developing zebrafish brain leads to increased errors in the pathfinding of later outgrowing nucPC axons (Chitnis and Kuwada, 1991), many of which still reach their targets. Examples, where follower axons are completely independent of all the pioneers are rare. Longitudinal axon tracts in the *Drosophila* embryo can form in the absence of the pioneers (Lin et al., 1995), although this observation has been challenged in later experiments (Hidalgo and Brand, 1997). The order of outgrowth of axons and therefore the identity of the pioneer can be different in related species. In grasshopper the intersegmental nerve (ISN) is pioneered by U axons, whereas the aCC axon follows later (Bastiani et al., 1986). In *Drosophila* the situation is reversed, as the aCC axon pioneers the ISN and the U axons follow. In grasshopper, the follower (aCC axon) cannot grow out correctly in the absence of the pioneer (du Lac et al., 1986), whereas in *Drosophila* followers (U axons) are in principle able to do so (Lin et al., 1995). Obviously, studies in different organisms with different pioneers do not provide a unified picture on the importance of pioneers for the navigation of later outgrowing axons.

Axon tracts in the *C. elegans* nervous system have been described in great detail using electron microscopic reconstructions (White et al., 1986; White et al., 1976) and a single neuron (AVG) has been identified as the pioneer for the right ventral cord axon tract (R. M. Durbin, PhD thesis, University of Cambridge, 1987). The ventral cord in *C. elegans* consists of two axon bundles flanking the ventral midline (Fig. 1). The two axon bundles are highly asymmetrically despite an

overall bilateral symmetry of the nervous system. The left ventral cord axon tract consists of only four axons, whereas the right axon tract contains about 50 axons in adult animals. The number and arrangement of neuronal processes is highly invariant from animal to animal. Elimination of the AVG pioneer by laser ablation resulted in a disorganized ventral cord with the overall asymmetry still preserved (R. M. Durbin, PhD thesis, University of Cambridge, 1987), suggesting that this pioneer is essential for the correct navigation of later outgrowing axons. The small sample size of the original experiment (the ventral cord of one operated animal was analysed in detail with EM reconstructions), however, precludes detailed conclusions on the importance of this pioneer for the outgrowth of the different classes of follower axons.

In addition to earlier outgrowing axons, extracellular signals are known to be important for directed outgrowth of axons and a number of these signals has been identified in the last decade. These are netrin/UNC-6, slit, semaphorin, ephrin and TFGβ/BMP. Mutants in *C. elegans* homologs of these signals and their receptors have been identified and in many cases shown to be involved in certain aspects of axon guidance. UNC-6/netrin, UNC-129/TGFβ and SLT-1/slit affect the dorsoventral navigation of motoneuron commissures and other neuronal processes (Colavita et al., 1998; Hao et al., 2001; Wadsworth, 2002). Fasciculation of ventral and dorsal cord axons is disturbed in *nid-1*/nidogen, *sax-3*/Robo and also *mab-20*/SemaII mutants (Kim and Wadsworth, 2000; Roy et al., 2000; Zallen et al., 1998).

With the green fluorescent protein (GFP), its derivatives and other proteins with similar properties, a range of markers with different spectral properties is now available, which allows the simultaneous inspection of axon trajectories from different classes of neurons in a single animal. For this study a number of transgenic strains was generated, which express different combinations of GFP color variants in different subsets of neurons with axons in the ventral cord. With these markers, the dependencies of different axons in the ventral cord on identified pioneers and extracellular signals were analysed systematically on a single axon level. The results show that pioneers are not absolutely essential for correct outgrowth of follower axons. Axons of different classes of neurons navigate surprisingly independently and seem to use different combinations of extracellular cues as well as adhesion to certain early outgrowing axons in their navigation. The results give fundamental insights into the logic of axon outgrowth in the ventral cord of *C. elegans*. They provide a basis for the interpretation of phenotypes and suggest strategies for genetic screens to identify the missing guidance cues used by axons navigating in the ventral cord.

Materials and methods

Generation of multicolor GFP strains

The following promoters were used to drive GFP expression in defined subsets of neurons with axons in the ventral cord. Interneurons, *glr-1* (Hart et al., 1995; Maricq et al., 1995); D-type motoneurons, *unc-47* (McIntire et al., 1997); DA/DB motoneurons, *unc-129* (Colavita et al., 1998); PVQ neurons, *sra-6* (Troemel et al., 1995); and AVG and PVP neurons, *odr-2* (Chou et al., 2001).

The GFP-coding part of the original constructs was replaced with one of the following variants of GFP (A. Fire vector kit) or DsRed (Clontech).

CFP: Y66W, N146I, M153T, V163A.

YFP: S65G, V68A, S72A, T203Y.

DsRed: insert from Clontech vector pDsRed.

DsRed2: insert from Clontech vector pDsRed2.

Using standard procedures, the following transgenic strains were made.

VH318: *hdIs10[unc-129::CFP, glr-1::YFP, unc-47::DsRed, hsp16::rol-6]* V.

VH414: *hdIs14[odr-2:CFP, unc-129:YFP, glr-1:DsRed, hsp-16::rol-6]* IV.

VH648: *hdIs26[odr-2::CFP, sra-6::DsRed2]*.

Nematode strains and mutant analysis

In addition to the multi-color GFP strains described above the following GFP strains and mutants were used: NW1229, *evIs111[F25B3.3::GFP]*; EG1306, *oxIs12[unc-47::GFP]*; PY1058, *oyIs14[sra-6::GFP]*; OH2012, otEx1082 (GFP under the control of the promoter of an innexin-like gene expressed in a subset of neurons, including AVG) (A. S. Wenick and O. Hobert, unpublished); MT633, *lin-11(n389) I; him-5(e1467)* V; NW434, *unc-6(ev400)* X; CB53, *unc-5 (e53)* IV; CB271, *unc-40(e271)* I; CX5000, *slt-1(eh15)* X; CX3198, *sax-3(ky123)* X; IM324, *nid-1(ur41)* V; NW987, *unc-129(ev554)* IV; CZ337, *vab-1(dx31*) II; VH582, *mab-20(ev574)* I; CB191, *unc-30(e191)* X. Mutants were analysed after crossing the GFP markers into the corresponding strains. Strains were grown at 20°C under standard conditions and analysed as growing population. Typically, late larval stages and adults were scored for axonal defects. DsRed matures rather slowly so that sufficient signal could only be detected in adult animals. DsRed2 in contrast already gave strong signals in newly hatched larvae. Axonal defects were scored using a Zeiss Axioplan microscope with a 40× objective and narrow band pass filters to separate the different color variants (CFP: D436/20, 455DCLP and D480/40; GFP: HQ480/20, Q495LP and HQ510/20; YFP: HQ500/20, Q515LP and HQ535/30; DsRed: HQ565/30, Q585LP and HQ620/60). Images were taken at a Leica SP2 confocal microscope equipped with Ar and He lasers for excitation at 457 nm (CFP), 488 nm (GFP), 514 nm (YFP) and 543 nm (DsRed). For clean separation of the channels it was sufficient in most cases to acquire data for the different channels sequentially. Typical emission windows used were: 463-493 nm (CFP), 520-550 nm (YFP) and 580-640 nm (DsRed).

For counting cells in the rvg in *evIs111* and *lin-11(n389); evIs111* animals stacks of confocal images of the rvg were taken from a random set of animals displaying a ventral aspect. Only samples where individual cells could be counted unambiguously were used.

Laser ablation experiments

A Photonic Instruments Micro Point Laser System in combination with a Zeiss Axioplan II microscope was used for laser ablation experiments. The laser system consists of a nitrogen-pulsed dye laser with Coumarin (440 nm, 5 mM) as dye. Ablations were carried out at the end of gastrulation at about 270 minutes of development, a time where cells can be identified easily by virtue of their position in the embryo. Cells were irradiated with 100-200 pulses at a frequency of 2 Hz. Before each session the amount of irradiation necessary for immediate killing of the cell was determined experimentally. Irradiation were carried out at about 80% of the immediate lethal dose. For AVG ablations, ABprpapppa was irradiated in VH318. This also eliminates the RIR interneuron, which sends its process into the nerve ring and has no connection to the ventral nerve cord. Ablations were validated by checking for the absence of the AVG cell body and the absence of the AVG axon in the tail spike with the *glr-1::YFP* marker, which is expressed in AVG. Axon guidance defects in the ventral cord, mainly erroneous outgrowth in the left axon tract or a switch from the right to the left axon tract were scored after operated animals were

Table 1. Ventral cord defects after laser ablation of pioneer neurons

	% animals with defects in				
	vc asymmetry*	Interneuron axons	DD/VD axons	DA/DB axons	DA/DB commissures†
lin-11(n389)‡ (*n*=123)	0	49	37	9	90
Cell ablated					
AVG (*n*=41)	0	37	32	7	77
RIFR, SABVR (*n*=7)	0	0	–	–	–
RIFR, SABVR, RIFL, SABVL (*n*=11)	0	0	–	–	–
RIFR, SABVR, RIGR (*n*=3)	0	0	–	–	–
RIFR, SABVR, RIGR, RIFL, SABVL, RIGL (*n*=5)	0	0	–	–	–

*Too many axons extending into the left axon tract after exiting the nerve ring.
†DA3-5, DB4-5 commissures scored for outgrowth on the wrong side.
‡By comparison with defects in AVG-ablated animals, differences from AVG-ablated animals are statistically not significant (χ^2-test).

allowed to grow to adulthood. For RIF ablations, the following cells were irradiated at about 270 minutes of development in VH414: RIFL and SABVL:ABplppapaaa; RIFR and SABVR:ABprppapaaa; RIGL: ABplppappa; and RIGR: ABprppappa. Ablation of ABplppappa also eliminated DD1 and ablation of ABprppappa also eliminated DD2. Animals were allowed to grow to adulthood before axon outgrowth defects were scored. Absence of RIG neurons was judged with *glr-1::DsRed*, absence of RIF neurons was confirmed using *odr-2::CFP*.

Results

Pioneer neurons and axon outgrowth in the ventral cord

The importance of the ventral cord pioneer AVG for the navigation of interneuron and motoneuron axons was tested with laser ablation experiments. Ablations were done in triple GFP labeled animals (*unc-129::CFP*, *glr-1::YFP*, *unc-47::DsRed*), allowing the simultaneous observation of interneuron axons of the motorcircuit (AVA, AVB, AVD, AVE, PVC) as well as of all embryonic and some postembryonic motoneuron axons (DA, DB, DD, VD). More than half of the AVG-ablated animals were normal with respect to outgrowth of all labeled axons. Interneuron axons and D-type motoneuron axons were affected in about a third of the animals (Table 1), showing midline crossing defects of individual axons. DA and DB motoneuron axons were affected in only 7% of the operated animals. In all cases, the overall asymmetry of the ventral cord with most axons extending in the right axon tract was preserved. Ablation of AVG also interfered with the directional outgrowth of motoneuron commissures. These grow out in a stereotypic way towards the dorsal cord, either on the right or on the left side of the animal. The directional outgrowth of DA/DB commissures was disturbed in 77% of AVG-ablated animals. Individual commissures were found to extend erroneously on the wrong side. In all cases these commissures reached the dorsal cord normally.

The RIFR and RIFL neurons pioneer the path from the beginning of the ventral cord into the nerve ring (R. M. Durbin, PhD thesis, University of Cambridge, 1987). Axons from these symmetrically positioned neurons cross the ventral midline and enter the nerve ring on the contralateral side (Fig. 1A). Shortly afterwards, two more pairs of neurons, SABVR/L and RIGR/L send their axons along the same trajectories into the nerve ring. Later outgrowing axons leaving the nerve ring on the left side cross the ventral midline at the same point where the pioneers decussated earlier (Fig. 2A,B). These pioneers could provide a pathway guiding axons into the right ventral cord fascicle. This idea was tested in another series of laser ablation experiments. Ablations were carried out in animals where RIF, RIG and interneuron axons crossing over to the right ventral cord tract were differentially labeled with GFP variants (*odr-2::CFP*, *glr-1::DsRed*). The ablation of RIFR/SABVR, the ablation of the four RIF and SABV neurons and the ablation of all six RIF/SABV/RIG neurons failed to cause any effect in the outgrowth of *glr-1::DsRed*-expressing interneuron axons (Table 1). In all operated animals, these interneuron axons were found to cross into the right ventral cord fascicle at the correct position (Fig. 2C,D).

lin-11 and *unc-30* affect axon patterning in the ventral cord

In genetic screens for mutants affecting the outgrowth of *glr-1::GFP*-expressing interneurons we identified mutants displaying a phenotype reminiscent of AVG ablated animals (H.H., unpublished). These mutants were found to be alleles of *lin-11*, a gene encoding a LIM homeobox transcription factor controlling aspects of neuronal differentiation (Hobert et al., 1998; Sarafi-Reinach et al., 2001). Ventral cord defects in *lin-11* mutants were analysed in detail with the same triple GFP marker, which was used to study the defects after AVG ablation. The overall asymmetry of the ventral cord was still present and individual navigational errors were found in *glr-1::YFP*-expressing interneurons as well as in D-type motoneurons. The defects in *lin-11(n389)* closely matched the defects observed after AVG ablation, both qualitatively and quantitatively (Table 1). In a few *lin-11* mutant animals almost half the interneuron axons extended in the left axon tract (Fig. 3A-H), a phenotype which was not observed in AVG-ablated animals. Defects in the directional outgrowth of DA/DB commissures were also very similar in AVG ablated animals and *lin-11* mutants (Table 1, Fig. 3I,J). Interestingly, individual commissures were affected with rather different frequencies. DA3 and DB4 commissures, which normally grow on the left side, were frequently affected (59% and 39% defective, *n*=110), whereas DB3 and DA2, which normally grow on the right side, were hardly affected at all (7% defective, *n*=110). Similar defects were found in D-type motoneuron commissures. Eighty-four percent of the animals had on average two D-type commissures growing on the left side rather than the right

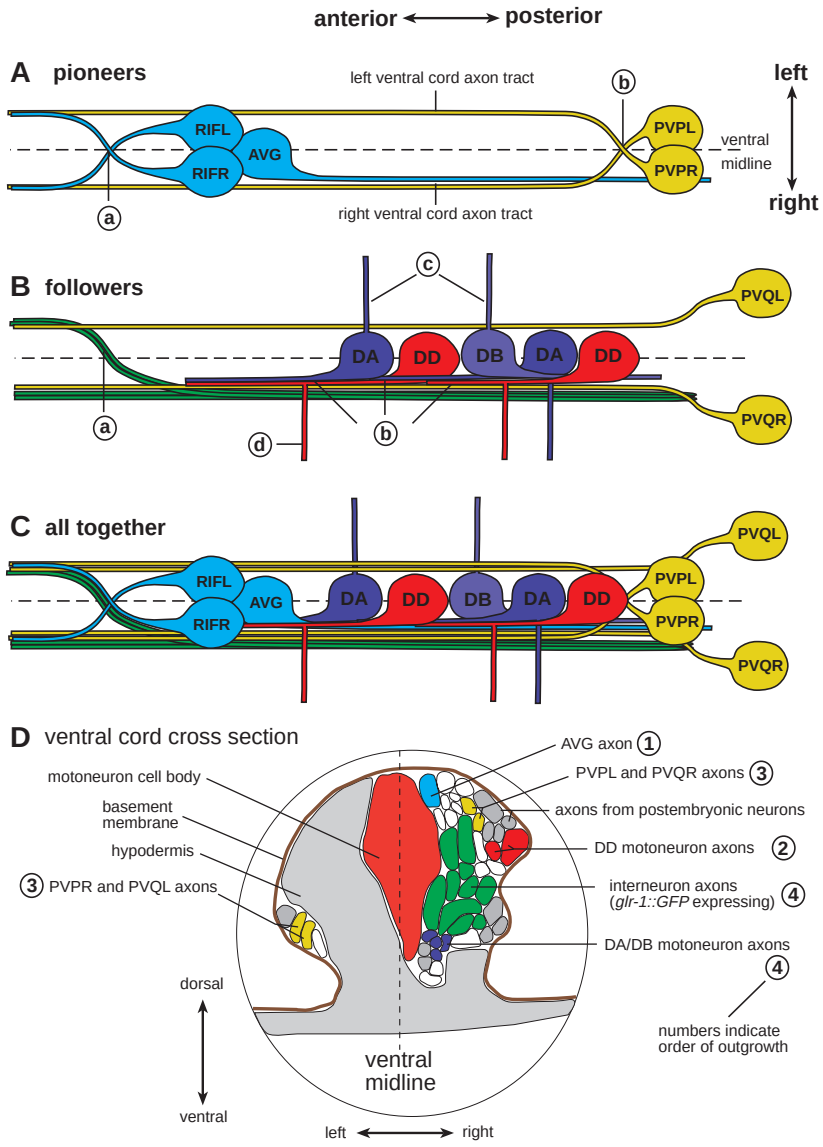

Fig. 1. Ventral cord development. (A-C) Trajectories of various axons in the ventral cord. Ventral aspect, anterior towards the left. (A) RIF axons pioneer the pathway from the anterior end of the ventral cord towards the nerve ring. RIF axons cross the ventral midline (a) and enter the nerve ring on the contralateral side (not shown). The AVG cell body is next to the RIF cell bodies in the retrovesicular ganglion (rvg). The AVG axon pioneers the right ventral cord axon tract. PVP cell bodies lie at the posterior end of the ventral cord. Their axons also cross the ventral midline (b) to grow anteriorly in the contralateral ventral cord tract, i.e. the PVPL axon grow in the right axon tract, and the PVPR axon in the left tract. The PVPR axon pioneers the left ventral cord axon tract. (B) Almost all interneuron axons growing out of the nerve ring cross over to the right side exactly where the RIF neurons crossed earlier (a). Motoneuron cell bodies lie on the ventral midline and send their axons into the right axon tract (b). DA and DB motoneuron commissure grow straight out of the cell body towards the dorsal cord (c), some on the left and others on the right side (not shown). DD motoneuron commissures branch off the ventral cord axon and always grow on the right side (d). (C) Overlay of A and B. (D) Ventral cord cross-section illustrating the position of axons within the ventral cord as well as the order of outgrowth (numbers in circles). Early outgrowing axons (AVG, DD) tend to be dorsally close to the basement membrane, whereas later outgrowing axons (DA/DB, interneurons) occupy central and ventral positions. Modified, with permission, from White et al. (White et al., 1986).

side. Differences in the penetrance of the defect for individual commissures were much less pronounced in D-type motoneurons with the DD2 commissure affected in 6% of the animals, and the DD3 commissure affected in 28% of the animals (n=50).

unc-30, another homeobox transcription factor, is expressed in D-type motoneurons and has been shown to be essential for the proper differentiation and axon guidance of the D-type motoneurons (Jin et al., 1994) (J. G. White, unpublished). Both *lin-11* and *unc-30* are also expressed in the PVP neurons, yet PVP defects have not yet been reported in mutants of these genes. The PVPR axon is essential for the correct outgrowth of the PVQL axon, which extends erroneously in the right axon tract when PVPR is eliminated (R. M. Durbin, PhD thesis, University of Cambridge, 1987). Such a PVQL defect was found in 47% of the *unc-30* mutants and in a similar percentage of *lin-11; unc-30* mutant animals, but not in *lin-11* single mutants (Table 2, column 9; see also Table 4, row 5). This suggests that *unc-30* but not *lin-11* affects the pioneer function of PVPR.

lin-11 and *unc-30* affect the differentiation of ventral cord pioneers

Interestingly, *lin-11* is expressed in the pioneer neurons AVG and PVP, but not (with one exception) in *glr-1::GFP*-expressing interneurons, suggesting that *lin-11* might control the differentiation of the AVG pioneer, which then has secondary consequences on the navigation of interneuron axons. To test this idea, we analysed the expression of AVG markers in *lin-11* mutants. *lin-11(n389)* mutants failed to express *odr-2::GFP*, a Ly-6 protein (Table 3, Fig. 4A,B) and *glr-1::GFP*, a glutamate receptor subunit (Fig. 4C,D). The same result was found with a third AVG cell fate marker gene, an innexin-like protein (Table 3). The expression of the marker in neurons other than AVG was normal, suggesting that *lin-11* specifically affects the differentiation of AVG. Unfortunately, this failure to express cell type-specific markers prevents the straightforward analysis of the axon trajectory of the AVG axon in *lin-11* mutants. The AVG neuron seems to be present and acquires at least some neuronal characteristics, as the number of cells expressing a pan-neuronal marker in the retrovesicular ganglion (rvg), where the AVG cell body is located, was not reduced in *lin-11(n389)* mutants (10.9 ± 0.4 cells in wild type (*evIs111*) versus 11.3 ± 0.7 cells in *lin-11(n389); evIs111*; n=31 for both experiments).

unc-30, another homeobox transcription factor, is expressed in D-type motoneurons as well as PVP neurons. It has been shown to be essential for the proper differentiation of the D-

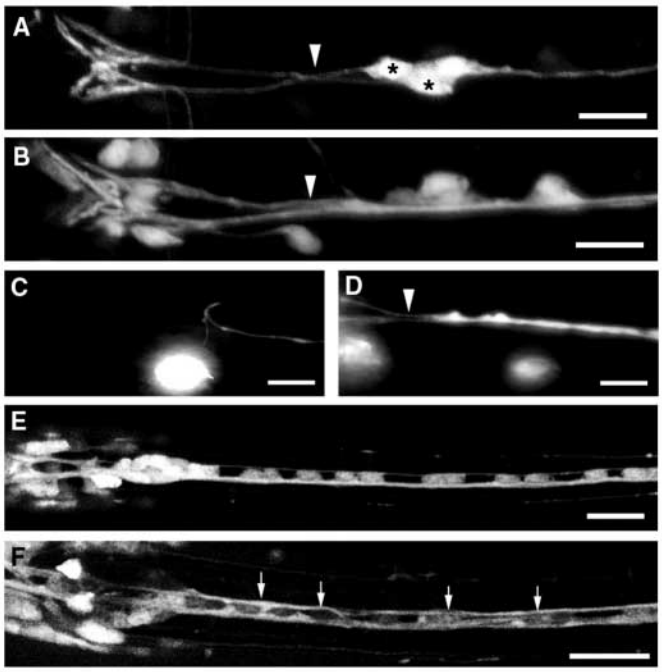

Fig. 2. Left-right asymmetry of the ventral cord. (A,B) Wild-type animal. RIF neurons and AVG are labeled with *odr-2::CFP* (A), interneurons with *glr-1::GFP*, DA/DB motoneurons with *unc-129::YFP* (B). RIF axons extend anteriorly into the nerve ring crossing the ventral midline (arrowhead, RIF cell bodies are marked with asterisks. Later outgrowing interneuron axons cross over into the right axon tract at the same position (arrowhead in B). (C,D) Animal in which RIFR/L, SABVR/L and RIGR/L have been ablated, labeled with *odr-2::CFP* (C) and *glr-1::DsRed* (D). (C) Absence of the RIF neurons (compare with A); only AVG is still present. (D) Interneuron axons still cross over into the right axon tract (arrowhead). (E,F) Wild-type (E) and *nid-1(ur41)* mutant animals (F) labeled with a pan-neuronal marker (*evIs111*). (E) The majority of the axons extends in the right axon tract. (F) Almost half of the axons extend in the left tract in the *nid-1* mutant (arrows). All animals show a ventral aspect with anterior towards the left. Scale bars: 10 μm.

type motoneurons (Jin et al., 1994). When trying to analyse the PVP axons in *lin-11* and *unc-30* mutants, I noticed that the expression of the *odr-2::GFP* marker in PVP was variable in these mutants. In about half of the *lin-11(n389)* animals, *odr-2::GFP* expression was undetectable in PVPR and PVPL (Table 3, Fig. 4H). Another quarter of the animals expressed the marker only weakly (Fig. 4G), whereas the remaining animals showed the usual strong expression of the marker. In *unc-30(e191)* mutants *odr-2::GFP* expression in PVPR was undetectable in all animals (Fig. 4F), whereas expression in PVPL was variable as in *lin-11(n389)* mutants. *lin-11; unc-30* double mutants resembled *lin-11* single mutants with respect to expression of *odr-2::GFP* in PVP, i.e. expression in PVPR was not completely abolished.

Dependencies between early and late outgrowing axons in the ventral cord

Axons grow out sequentially in the ventral cord during embryonic development. To analyse dependencies between early and late outgrowing axons, navigational errors in various classes of neurons were correlated in mutants affecting either

Table 2. Ventral cord defects in axon guidance mutants

			Ventral cord defects (% animals with defects*)								
		vc asymmetry†	Axons in right axon tract						Left axon tract		Commissure sidedness
Column number:		1	2	3	4	5	6	7	8	9	10
			Neuron								
Signal/cell affected	Genotype		AVG	DD/VD	PVPL‡	PVQR‡	DA/DB	Inter-neurons	PVPR‡	PVQL‡	DA/DB commissure§
None	Wild type	0	0	2	0	0	0	1	11 (0)	11 (0)	5
AVG	*lin-11(n389)*	11	n.s.	37¶	n.s.	4 (0)	9	49	n.s.	38 (0)	90
DD+PVP (partial)	*unc-30(e191)*	0	0	n.d.	n.s.	4 (0)	2	6	n.s.	72 (47)	11
AVG+DD+PVP	*lin-11(n389); unc-30(e191)*	19	n.s.	n.s.	n.s.	28 (22)	25	65	n.s.	62 (49)	n.d.
Netrin	*unc-6(ev400)*	8	2	42	2 (2)	3 (3)**	22	16	43 (30)	41 (30)**	22††
AVG+netrin	*lin-11(n389); unc-6(ev400)*	31	n.s.	87	n.s.	n.d.	39	46	n.s.	n.d.	n.d.
Slit receptor	*sax-3(ky123)*	16	8	30	3 (2)	7 (2)	6	10	44 (5)	43 (5)	68
Nidogen	*nid-1(ur41)*	14	7	17	10 (7)	10 (7)	11	14	69 (33)	71(33)	9
Sema II	*mab-20(ev574)*	2	0	22	0 (0)	0 (0)	2	5	12 (1)	12 (0)	15
Eph receptor	*vab-1(dx31)*	3	0	3	1 (0)	1 (0)	2	5	15 (2)	15 (2)	15
TGFβ	*unc-129(ev554)*	0	0	10	0 (0)	1 (0)	1	3	18 (0)	16 (0)	2
Slit	*slt-1(eh15)*	0	1	1	0 (0)	1 (0)	0	0	6 (0)	10 (1)	1

n.s., defects could not be scored because of lack of expression (AVG, DD/VD) or variable expression (PVP); n.d., not determined.

*n=100-178 for each data point. Markers used: evIs111 (vc asymmetry), hdIs26 (AVG, PVP, PVQ), hdIs10 (DD/VD, DA/DB, interneurons). Defects scored: midline crossing defects or axons extending inappropriately in the wrong ventral cord axon tract.

†Too many axons extending into the left axon tract after exiting the nerve ring.

‡Numbers in brackets indicate % of animals, where the axon extends over the entire distance in the contralateral axon tract.

§DA2-6, DB3-7 commissures scored for outgrowth on the wrong side.

¶DD defects scored in L1 larvae in *lin-11(n389); oxIs12*: 44% (n=110).

**Additional defects: PVQ axons fail to reach the ventral cord (27% of the PVQL axons and 14% of the PVQR axons extend laterally).

††Additional defects: individual commissures missing (frequently DB5) in 67% of the animals.

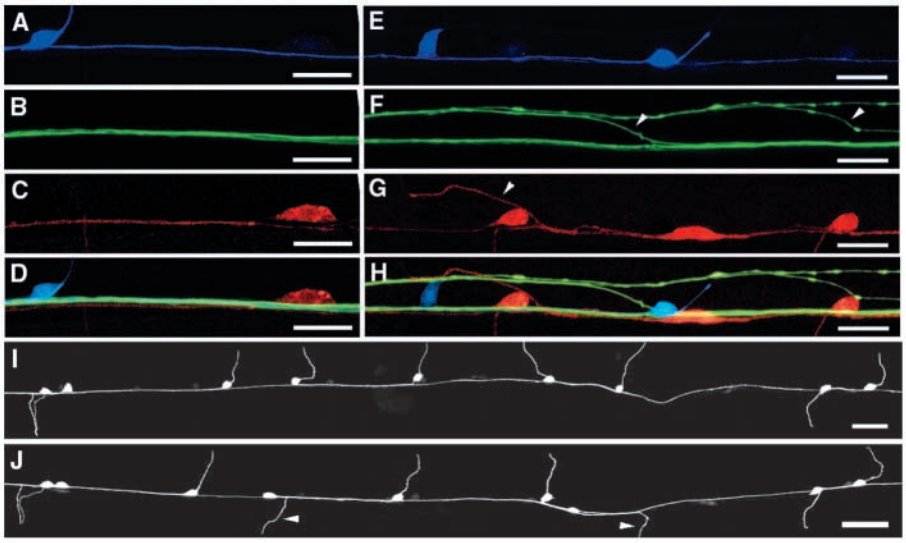

Fig. 3. Ventral cord defects in *lin-11(n389)* mutants. (A-D) Wild type, ventral cord with interneurons and motoneurons labeled with different GFP variants: (A-C) single color marker, (D) overlay. (E-H) Ventral cord defects in *lin-11(n389)*, same labels and arrangement as in A-D. Arrowheads indicate axons inappropriately crossing the ventral midline. (I,J) Wild type (I) and *lin-11(n389)* mutant (J), DA/DB motoneurons labeled. Arrowheads in J indicate commissures growing out on the wrong side. All animals are shown from a ventral aspect with anterior towards the left. Scale bars: 10 μm in A-H; 20 μm in I,J. Markers shown are *unc-129::CFP* (dark blue in A,D,E,H, gray in I,J), *glr-1::GFP* (green in B,D,F,H), *unc-47::DsRed* (red in C,D,G,H).

Table 3. Expression of AVG and PVP markers in *lin-11* and *unc-30* mutants

		% animals with expression (n=50)							
		AVG		PVPL			PVPR		
Genotype	Marker	Strong	No expression	Strong	Weak	No expression	Strong	Weak	No expression
Wild type; *hdIs10*	*glr-1*	100	0	–	–	–	–	–	–
Wild type; *otEx1082*	Innexin-like	100	0	–	–	–	–	–	–
Wild type; *hdIs26*	*odr-2*	100	0	100	0	0	100	0	0
lin-11(n389); hdIs10	*glr-1*	0	100	–	–	–	–	–	–
lin-11(n389); otEx1082	Innexin-like	0	100	–	–	–	–	–	–
lin-11(n389); hdIs26	*odr-2*	0	100	22	24	54	16	28	56
unc-30(e191); hdIs26	*odr-2*	100	0	16	28	56	0*	2	98†
lin-11(n389); unc-30(e191); hdIs26	*odr-2*	0	100	30	10	60	16*	12	72†

*Difference is statistically significant (P=0.01) (χ^2-test).
†Difference is statistically significant (P=0.0008) (χ^2-test).

the pioneer AVG or particular guidance cues using multi-color GFP strains (Table 4). Axons of two different classes of neurons only rarely crossed between axon tracts at exactly the same point. This was true for all combinations of axons analysed in all mutant backgrounds with only one exception. PVQL and PVPR axons were almost always found to switch axon tracts together (Table 4, row 1), suggesting a strict dependency of PVQL on the earlier outgrowing PVPR axon (Fig. 5D-F). However, in individual *unc-30* mutants PVQL axons were found to grow out normally despite navigational errors of PVP axons, indicating that even PVQ axons are able to navigate independently of PVP.

A number of partial dependencies among particular classes of neurons could also be detected (Table 4, rows 3-5). In *nid-1* mutants, PVPL and PVQR were occasionally found to extend in the left rather than the right axon tract. In 67% of these animals AVG was also found in the left axon tract, a defect detected in only 2% of all the *nid-1* mutants (Table 4, row 3). The AVG axons in these animals initially extended in the right axon tract and only later crossed into the left tract. PVPL and PVQR typically switched from left to right axon tract at the same point, strongly suggesting that PVPL/PVQR directly follow AVG (Fig. 5J-L). Several other examples of dependencies were revealed in *lin-11* mutants. Here, the majority of the DD axons occasionally was found to grow in the left axon tract. In 30% of these animals, the PVQR axon was also in the left axon tract, a defect that is found only in 2% of all the *lin-11* mutants scored (Table 4, row 4). However, in *lin-11* mutants the PVQL axon is sometimes found in the right rather than the left tract. In such animals, significantly less midline crossing defects of interneuron axons were found when compared with animals without PVQ defects (Table 4, row 5). No such dependencies were found between *glr-1::GFP* expressing interneuron axons and DA/DB-axons, or between interneurons and D-type motoneurons (Table 3, rows 6-8).

Extracellular cues affect ventral cord asymmetry and directional outgrowth of commissures

Pioneers as well as later outgrowing axons are known to depend on extracellular cues for their navigation. Mutants in *C. elegans* homologs of several extracellular axon guidance cues and their receptors have been identified and shown to be involved in certain aspects of axon guidance. I tested whether any of these mutants is involved in the generation of ventral cord asymmetry or is a source of information for directional outgrowth of motoneuron commissures. A small but significant

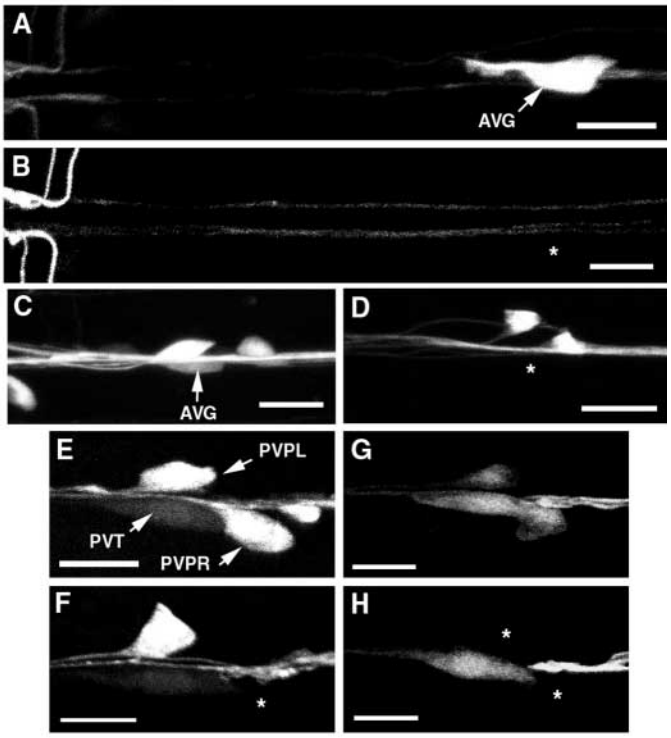

Fig. 4. AVG and PVP cell type marker expression in *lin-11* and *unc-30* mutants. (A-D) AVG marker. Ventral aspect of the anterior end of the ventral cord with the retrovesicular ganglion. (A,B) odr-2::GFP, (C,D) *glr-1::GFP* in wild type (A,C) or *lin-11(n389)* (B,D). Marker expression is absent in AVG in *lin-11(n389)* (asterisks in B and D indicate where the AVG cell body should be). (E-H) Expression of *odr-2::GFP* in the preanal ganglion. (E) wild type; expression is strong in PVPR and PVPL and weakly seen in PVT. (F) *unc-30(e191)* mutant; expression in PVPR (asterisk) is missing. (G,H) expression in *lin-11(n389)*. Expression levels in PVP neurons are either reduced (G) or expression is completely absent (H, asterisks). Scale bars: 5 μm in A,B,E-H; 10 μm in C,D.

fraction of animals with axons extending inappropriately into the left ventral cord axon tract after exiting the nerve ring was found in *unc-6*/netrin, *nid-1*/nidogen and *sax-3*/Robo mutants, as well as in *lin-11; unc-30* double mutants (Table 2, column 1). Axons found to enter the left axon tract usually stayed in this tract in *nid-1* mutants, leading to an almost symmetrical ventral cord (Fig. 2E,F). This is in contrast to *sax-3* and *unc-6* mutants, where these axons frequently crossed into the right tract.

Some of the analysed mutants had defects in the directional outgrowth of motoneuron commissures. Penetrant defects in DA/DB commissures were found in *sax-3* mutants, less penetrant defects also in *unc-6*/netrin, *mab-20*/semaII and *vab-1*/EphR mutants (Table 2, column 10).

Extracellular cues affect axon navigation within the ventral cord

I tested whether any of known guidance cues/receptors is essential for proper navigation of the ventral cord pioneer AVG. Surprisingly, mutants in *unc-6*/netrin, *slt-1*/slit, *sax-3*/Robo, *mab-20*/sema II, *vab-1* (the only Ephrin receptor), *unc-129*/TGFβ and *nid-1*/nidogen showed no significant defects in the navigation of the AVG axon (Table 2, column 2), leaving the source of information used by this pioneer obscure. In individual cases, where the AVG axon erroneously crossed into the left axon tract, this had no consequences for DD motoneuron axons, which grew out in the right axon tract even at positions where AVG was in the left axon tract (Fig. 5M-O).

Mutants in the above mentioned genes were also systematically tested for defects in the navigation of the other classes of axons present in the embryonic ventral cord (Table 2, columns 3-9). Mutations in *slt-1* and *vab-1* had no significant effects on axon outgrowth for any of the neuron classes with axons in the right axon tract. DD motoneuron axons, which grow out immediately after the AVG pioneer, were affected to a different extent in *mab-20*, *unc-129*, *sax-3*, *nid-1* and *unc-6* mutants. In a *lin-11; unc-6* double mutant these defects were additive so that almost every animal was affected. In addition to inappropriate outgrowth in the left ventral cord tract and crossing between the left and right tracts, local defasciculations within the right ventral cord axon tract could be observed in *unc-6* and *lin-11; unc-6* double mutants. PVPL and PVQR axons were essentially unaffected by mutations in any of the genes tested. By contrast, the bilateral counterparts PVPR and PVQL, which pioneer the left axon tract, are dramatically misguided in *sax-3*, *nid-1* and *unc-6* mutants, and also somewhat affected in *unc-129*, *vab-1* and *mab-20* mutants. *Glr-1::GFP* expressing interneuron axons and DA/DB motoneuron axons which grow into the ventral cord rather late were only very moderately affected in *sax-3*, *nid-1* and *unc-6* mutants, and unaffected in all other mutants tested.

Discussion

The role of pioneer neurons in guiding axons towards and within the ventral cord

The ventral cord in *C. elegans* is highly asymmetrical with the majority of the axons extending in the right axon tract. Motoneuron axons originating from cell bodies located at the ventral midline invariably grow into the right axon tract. Almost all interneuron axons exiting the nerve ring on the left side cross over to the right side at the beginning of the ventral cord and continue to grow in the right axon tract. A particular neuron, AVG, has been identified to be the first to send an axon into the right ventral cord tract (R. M. Durbin, PhD thesis, University of Cambridge, 1987). The AVG cell body is located in the retrovesicular ganglion (rvg) at the anterior end of the ventral cord. This ganglion also contains the cell bodies from neurons (RIFR/L, RIGR/L, SABVR/L), which pioneer the pathway from the ventral cord into the nerve ring. Their axons grow anteriorly from the cell bodies, cross the ventral midline and enter the nerve ring on the contralateral side (R. M. Durbin, PhD thesis, University of Cambridge, 1987).

The idea that AVG has a role in guiding later outgrowing axons in the ventral cord was first tested by Richard Durbin, who eliminated AVG by laser ablation and studied ventral cord axons in one animal in great detail using electron microscopic reconstructions (R. M. Durbin, PhD thesis, University of Cambridge, 1987). He found that the right axon tract still contained the majority of the axons. However, the axon bundle was disorganized and split into distinct sub-bundles with axons wandering between bundles and occasionally even crossing the

Table 4. Correlation of defects between early and late outgrowing axons

Row	Genotype	Primary defect scored	Total	Animals with secondary defects PVQL following: No	Animals with secondary defects PVQL following: Yes
1	*sax-3(ky123); hdIs26*	PVPR crossing the midline (*n*=72)		3%	97%
				Interneuron axons following: No	Interneuron axons following: Yes
2	*lin-11(n389); rhIs16; oyIs14*	PVQL crossing into the right tract (*n*=48)		75%	25%
Row	Genotype	Defect scored	Total	In animals with AVG axon defects: No	In animals with AVG axon defects: Yes
3	*nid-1(ur41); hdIs26*	PVQR/PVPL in left axon tract	7% (*n*=129)	2% (*n*=117)	67% (*n*=12)
				DD axons in: Right tract	DD axons in: Left tract
4	*lin-11(n389); oxIs12; hdIs26*	PVQR in left ventral cord tract	2% (*n*=257)	0% (*n*=247)	30% (*n*=10)
				PVQL axon in: Right tract	PVQL axon in: Left tract
5	*lin-11(n389); rhIs16; oyIs14*	Interneuron midline crossing	54% (*n*=164)	63% (*n*=68)	25% (*n*=24)
				DD axon defects: No	DD axon defects: Yes
6	*lin-11(n389); rhIs4; hdIs22*	Interneuron midline crossing	37% (*n*=105)	35% (*n*=77)	34% (*n*=29)
				DA/DB axon defects: No	DA/DB axon defects: Yes
7	*lin-11(n389); unc-30(e191); hdIs10*	Interneuron midline crossing	65% (*n*=155)	64% (*n*=116)	66% (*n*=39)
				Interneuron axon defects: No	Interneuron axon defects: Yes
8	*lin-11(n389); unc-30(e191); hdIs10*	DA/DB midline crossing	25% (*n*=155)	22% (*n*=55)	27% (*n*=100)

midline to run in the left tract. He also noticed that motoneuron axons were predominantly affected, whereas interneurons were much less affected. This earlier work is extended here and leads to a more refined picture concerning the importance of AVG as a pioneer. A significant number of AVG-ablated animals showed axon outgrowth defects. Affected were predominantly interneurons and D-type motoneurons and only rarely DA or DB motoneurons. This indicates that some classes of neurons depend more on the pioneer than others and implies that different classes of neurons use different combinations of cues to navigate within the same axon bundle. In more than half of the animals in which AVG was removed, no defects could be detected in the outgrowth of the command interneurons of the motorcircuit and of the major classes of motoneurons (DD, VD, DA, DB). This suggests that the pioneer AVG is not absolutely essential for the proper outgrowth of other axons in the right axon tract. Apparently, these axons can use other sources of information, most likely extracellular cues, for their navigation. The pioneer AVG is not uniquely endowed with pathfinding abilities not shared by follower axons. It might be required as additional source of information at times when a large number of axons grows out simultaneously to ensure that every single axon navigates correctly. The observation that the elimination of pioneers leads to a certain frequency of errors made by later outgrowing axons is reminiscent of the situation found in *Drosophila* or zebrafish, where similar results were obtained after elimination of particular pioneers (Chitnis and Kuwada, 1991; Lin et al., 1995). Stalling or complete failure of outgrowth as described in grasshopper after elimination of particular pioneers (du Lac et al., 1986; Klose and Bentley, 1989) was never observed in the experiments presented here. This indicates that AVG is not required for continuous growth of follower axons.

RIF axons pioneering the pathway between the ventral cord and the nerve ring are obvious candidates for guiding axons exiting the nerve ring to the right side of the ventral cord. The asymmetrical expression of the guidance cue UNC-6/netrin in one of the RIF neurons and AVG led to the proposal that RIFL and AVG generate a UNC-6/netrin labeled pathway on the right side of the ventral midline (Wadsworth et al., 1996). However,

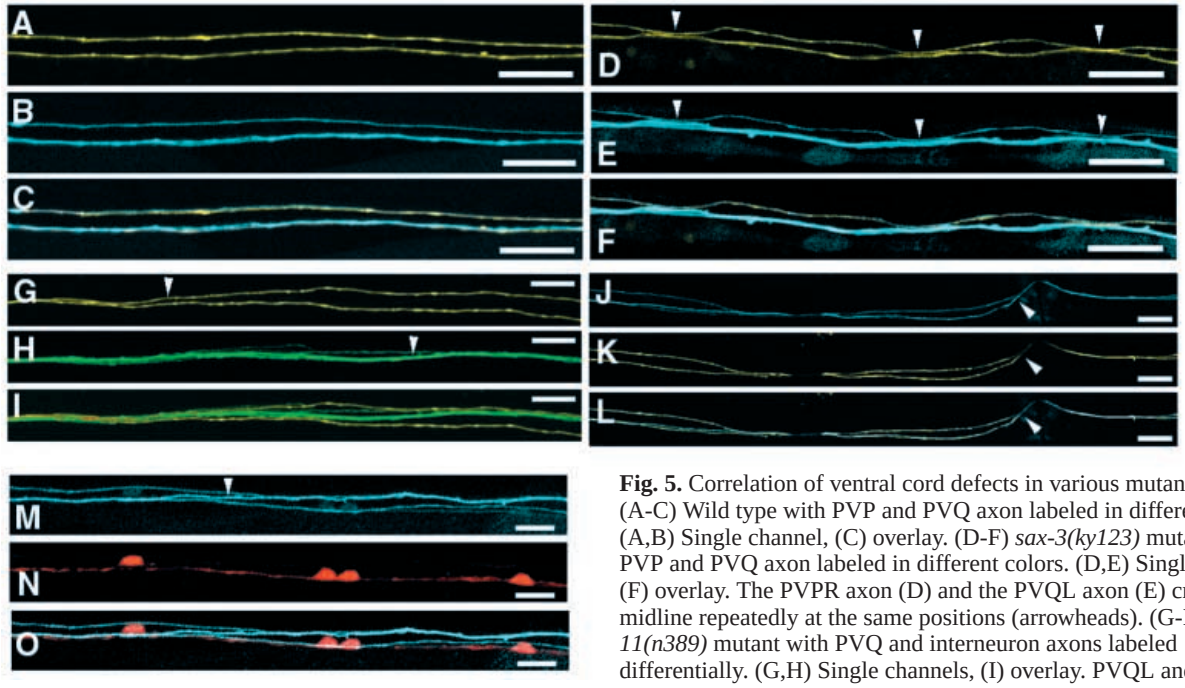

Fig. 5. Correlation of ventral cord defects in various mutants. (A-C) Wild type with PVP and PVQ axon labeled in different colors. (A,B) Single channel, (C) overlay. (D-F) *sax-3(ky123)* mutant with PVP and PVQ axon labeled in different colors. (D,E) Single channel, (F) overlay. The PVPR axon (D) and the PVQL axon (E) cross the midline repeatedly at the same positions (arrowheads). (G-I) *lin-11(n389)* mutant with PVQ and interneuron axons labeled differentially. (G,H) Single channels, (I) overlay. PVQL and interneurons cross into the contralateral axon tract at different positions (arrowheads in G and H). (J-L) *nid-1(ur41)* mutant with PVP and PVQ axon labeled in different colors. (J,K) Single channels, (L) overlay. The AVG and PVPL axons (J) and the PVQR axon (K) cross the midline at the same position (arrowheads) to run together in the left axon tract. (M-O) *unc-6(ev400)* mutant with PVP/AVG and D-type motoneuron axons labeled differentially, (M,N) Single channels, (O) overlay. The AVG axon crosses the ventral midline to grow in the left axon tract (arrowhead in M), whereas the D-type motoneuron axons all grow out correctly on the right side. All animals are shown from a ventral aspect with anterior towards the left. Scale bars: 10 μm in A-I,M-O; 20 μm in J-L. Markers shown are *glr-1::GFP* (green in H,I), *unc-47::DsRed* (red in N,O), *odr-2::CFP* (light blue in B,C,E,F,J,L,M,O), *sra-6::DsRed2* (yellow in A,C,D,F,G,I,K,L).

laser ablation of RIF neurons had no consequences for the crossing of interneuron axons into the right axon tract. Even elimination of all three pairs of neurons, which send their axons along the RIFL/R trajectories (RIF, RIG, SABV) led to the same negative result. This strongly suggests that these neurons either have no pioneering function for axons exiting the nerve ring or that redundantly acting information systems are present. More than one pioneer is also found for the longitudinal axon tracts in Grasshopper and *Drosophila*, and in zebrafish motoneuron axon tracts. In these cases, elimination of individual pioneers typically also had no consequences for follower axons (Eisen et al., 1989; Pike et al., 1992; Raper et al., 1983a; Raper et al., 1983b; Raper et al., 1984).

lin-11 and *unc-30* control the differentiation of the pioneer neurons AVG and PVP

LIN-11 and UNC-30, two homeobox transcription factors, are known to affect the differentiation of particular classes of neurons. LIN-11 has been shown to affect differentiation and function of neurons of the thermosensory circuit (Hobert et al., 1998), as well as of olfactory and chemosensory neurons (Sarafi-Reinach et al., 2001). UNC-30 is essential for the correct differentiation of D-type motoneurons (Jin et al., 1994). Both transcription factors are also expressed in pioneer neurons of the ventral cord, yet no defects have been associated with this expression so far. *lin-11* is expressed in the AVG neuron (Hobert et al., 1998) and both *lin-11* and *unc-30* are expressed in the PVP neurons (Hobert et al., 1998; Jin et al., 1994). *lin-11* mutants fail to express AVG specific markers like *glr-1::GFP* or *odr-2::GFP*, indicating that *lin-11* interferes with the differentiation of the AVG neuron. *lin-11* mutants show axon outgrowth defects in ventral cord neurons, which do not express *lin-11*, like command neurons of the motorcircuit and D-type motoneurons. These defects match the defects seen after laser ablation of AVG both qualitatively and quantitatively, suggesting that they are secondary defects caused by a failure of AVG to provide its normal pioneer function. The only difference between AVG-ablated animals and *lin-11* mutants is the severity of defects in *glr-1::GFP* expressing interneurons. The number of axons in the left axon tract is higher in some *lin-11* mutants when compared with AVG-ablated animals. One explanation for this difference could be the expression of *lin-11* in other neurons, which might directly or indirectly affect the outgrowth of these interneuron axons. *lin-11* is expressed in AVER/L, two of the *glr-1* expressing interneurons (Hobert et al., 1998), and might thus directly affect the ability of these neurons to navigate correctly. *lin-11* is also expressed in the PVP neurons, which grow out before the *glr-1* expressing interneurons. As *lin-11* appears to affect PVP differentiation (see below), this in turn could affect interneuron axon outgrowth, provided that PVP also has some pioneering function in the right axon tract.

Expression of the *odr-2::GFP* marker in PVP neurons is variable in *lin-11* as well as *unc-30* mutant animals, suggesting that these transcription factors control aspects of PVP differentiation. This idea is further supported by the nature of axon outgrowth defects found in these mutants. The PVPR neuron pioneers the left ventral cord tract and is known to be

essential for the proper navigation of the PVQL axon. When PVPR is eliminated, the PVQL axon extends in the right ventral cord axon tract (R. M. Durbin, PhD thesis, University of Cambridge, 1987). Such defects are found in *unc-30* mutants, indicating that *unc-30* affects the pioneering function of PVPR. Interestingly such PVQL defects are not found in *lin-11* mutants, suggesting that *lin-11* and *unc-30* control different aspects of PVP differentiation.

Left-right asymmetry in axon and commissure outgrowth

The overall asymmetry of the ventral cord never disappeared in any ablation experiment, suggesting that neither the ventral cord pioneer AVG, nor the neurons pioneering the path between the ventral cord and the nerve ring (RIF, RIG, SABV) are important for generating this asymmetry. However, a failure of axons to cross over into the right axon tract at the anterior end of the ventral cord was found in a small but significant fraction of animals mutant for *lin-11*. This contrasts with the observation that no such defects were observed in AVG-ablated animals. The small sample size of AVG-ablated animals might account for this difference. Alternatively, *lin-11* function in neurons other than AVG might be responsible for the crossover defects seen in *lin-11* mutants. Axons extending inappropriately into the left ventral cord tract were also observed in some animals mutant for either *unc-6*/netrin, *sax-3*/Robo or *nid-1*/nidogen. This confirms earlier observations in *nid-1* (Kim and Wadsworth, 2000) and implies that a combination of signals embedded in the basement membrane provides the essential information for the generation of an asymmetrical ventral cord.

The ventral cord consists of motoneuron axons originating from cell bodies located along the ventral midline and of interneuron axons entering the ventral cord mainly from the nerve ring in the head or from tail ganglia. Motoneuron axons invariably grow into the right ventral cord axon tract, indicating that motoneurons use positional information to distinguish left and right axon tracts. This information could either be intrinsic (polarization of the cell) or it could be extracellular information, which is read and interpreted by the neurons. The observation that the elimination of the pioneer neuron AVG leads to defects in the directional outgrowth of D-type motoneuron axons, suggests that cues provided by this pioneer are important sources of information. As a complete randomization of outgrowth was never observed in any experimental situation (laser ablation or mutant combination), other still undiscovered redundantly acting signals must exist. Navigation defects were much more prominent in D-type motoneurons when compared with DA and DB motoneurons, suggesting that different classes of motoneurons use AVG to a different extent for directed outgrowth of their axons. Directional outgrowth of DA/DB motoneuron commissures (on the left versus the right side) was affected in a large number of AVG-ablated animals, indicating that AVG provides essential left-right information for the navigation of commissures. Interestingly, the penetrance of these defects was highest for commissures normally growing on the left side, which suggests that the presence of AVG has a repulsive effect on the outgrowth of those commissures. As axons of these motoneurons were much less affected than commissures, this indicates that even different types of neuronal processes from the same neuron use different cues for their orientation.

The logic of axon navigation in the ventral cord of *C. elegans*

Axons in the ventral cord of *C. elegans* are found at very reproducible positions within the axon bundle. Axons belonging to the same class of neuron form distinct sub-bundles within the right axon tract and even sub-bundles are positioned precisely and reproducibly within the cord. Early outgrowing axons are found dorsally in close contact with the basement membrane, whereas later outgrowing ones end up in ventral positions far away from the basement membrane. This well ordered structure implies a very precise navigational system guiding axons not just towards the ventral cord but also within the ventral cord. Precise positioning of axons within the ventral cord is essential for the correct formation of synapses within the motor circuit, as synapses are made within the ventral cord between neighboring processes (White et al., 1986; White et al., 1976). Any deviation from the normal placement of neuronal processes in the ventral cord is likely to have consequences on the wiring of components of the motorcircuit and the movement behaviour of the animal. Neuronal processes grow out sequentially in the ventral cord (R. M. Durbin, PhD thesis, University of Cambridge, 1987). Navigation of later outgrowing axons could therefore depend on the presence of particular early outgrowing axons (pioneer-follower relationship) and the order of outgrowth could already be a major determinant of axon position. The data presented here argue against a strict pioneer-follower relationship between any groups of axons. The only exception are the PVQ axons, which strictly follow the earlier outgrowing PVP axons, irrespective of whether these grow out correctly or not. Apart from this example, later outgrowing axons typically do not simply follow earlier outgrowing ones. The strict dependency of the PVQ axons on PVP axons might indicate, that PVQ axons on their own are not able to navigate correctly. However, in a few *unc-30* mutants, where PVP axons were found in the wrong axon tract, PVQ axons were normal, indicating that even they are able to use other cues for their navigation.

When individual navigational errors were evaluated, no strong correlation was found between errors made by early outgrowing DD and later outgrowing DA/DB or interneuron axons. Each axon appears to commit errors individually and typically does not follow a misguided earlier outgrowing axon. Later outgrowing axons are able to ignore a misguided pioneer and to continue on their normal path, indicating that they constantly read and interpret several extracellular signals rather than simply following the trajectory of a pioneer. However, the presence or absence of early outgrowing axons is not completely irrelevant for later outgrowing ones. The percentage of animals showing *glr-1::GFP*-expressing interneuron axon defects is dramatically different depending on the presence or absence of the PVQL axon in the right axon bundle. The outgrowth of the PVQR axons is similarly affected by the presence or absence of either the AVG or the DD axons in the same axon tract. This suggests that early outgrowing axons are recognized and used as source of positional information, but not in a strict and absolute pioneer-follower relationship. This flexibility ensures that individual guidance

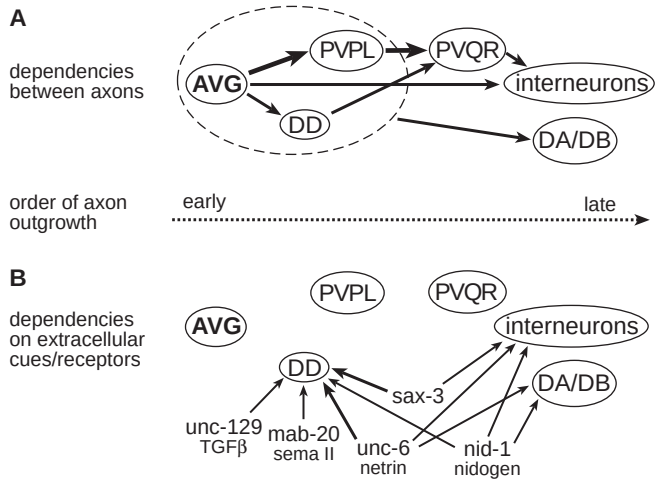

Fig. 6. The logic of axon outgrowth. The dependencies between early and late outgrowing axons (A), and the importance of extracellular cues for particular classes of axons (B). Arrows indicate the class of axons that is influenced by the extracellular cues or an earlier outgrowing axon. The thickness of the arrow corresponds to the strength of the interaction.

errors of early outgrowing axons are not inevitably amplified by affecting follower axons.

Several guidance cues are known to affect neurons with axons in the ventral cord of *C. elegans* (Colavita et al., 1998; Hao et al., 2001; Kim and Wadsworth, 2000; Roy et al., 2000; Wadsworth, 2002; Zallen et al., 1998). Early outgrowing axons should depend more heavily on extracellular cues than later outgrowing ones, which could use the presence of earlier outgrowing axons as additional source of information. However, a systematic analysis of the defects in the mutants affecting the different extracellular cues or their receptors revealed that this is not necessarily the case. Most surprisingly, the ventral cord pioneer AVG is not affected in any of the mutants tested, leaving the primordial source of navigational information for the ventral cord pioneer obscure. However, DD-motoneuron axons, which grow out immediately after the AVG axon, are affected significantly in several mutants, in particular in *unc-6*/Netrin, *sax-3*/Robo, *nid-1*/nidogen and *mab-20*/semaII mutants, suggesting that these axons depend heavily on extracellular cues and use a variety of different cues in combination to navigate within the ventral cord.

Among later outgrowing axons, the PVPL and PVQR axons are hardly affected in any of the analysed mutants. However, there is a strong correlation between AVG defects and corresponding PVQR/PVPL defects in *nid-1* mutants, arguing for a role of AVG in guiding PVPL/PVQR axons. The fact, that essentially no PVPL/PVQR defects were found in *lin-11* mutants suggests that other redundantly acting cues must be present. Most likely, these cues come from the DD axons, as there is also a strong correlation between defects in the DD axons and the PVPL/PVQR axons. DA and DB axons are only moderately affected in any of the single mutants tested. Significant defects, however, were found in *lin-11; unc-30* and even more in *lin-11; unc-6* double mutants, suggesting that *unc-6*/netrin in combination with early outgrowing axons provides guidance information for these axons. Nonetheless, even the most penetrant defects were well below 50%, indicating that major navigational cues for these axons are still unidentified. As DA/DB axons are found in ventral positions close to the epidermal cells, it might be worth considering these cells as potential sources for navigational cues. Finally, *glr-1::GFP*-expressing interneurons are moderately affected in *unc-6*/Netrin, *sax-3*/Robo and *nid-1*/Nidogen mutants. They are strongly affected in mutants interfering with the differentiation of earlier outgrowing axons, like *lin-11* and *unc-30*. These interneuron axons might predominately navigate by using interactions with other axons and to a lesser extent rely on extracellular cues for their navigation. This makes sense in the light of the fact, that these interneuron axons are found in a central position within the right axon tract and not in close contact to the basement membrane where extracellular cues are likely to be located (Fig. 1D).

Taken together, the analysis presented here provides a detailed picture of the combination of cues used by individual axons to navigate towards the ventral cord and then further within the ventral cord of *C. elegans* (Fig. 6). The main conclusions are as follows.

(1) Pioneers are not absolutely essential for the correct outgrowth of follower axons.

(2) Every class of neurons relies on several cues in combination for its navigation.

(3) Different neurons extending their axons into the same axon bundle use different sets of cues.

(4) Even different groups of early outgrowing axons, which depend strongly on extracellular cues, apparently use them in different combinations.

(5) Later outgrowing axons are influenced by the presence or absence of certain earlier outgrowing axons, but typically do not strictly follow them.

This study provides several novel insights into the logic of axon outgrowth. The dependencies discovered here provide a conceptual framework for the interpretation of axon guidance defects seen in various mutants and will help to fine tune further genetic screens aiming at the identification of the missing components of the navigational system guiding axons towards and within the ventral cord of *C. elegans*.

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