SDQR migrations in Caenorhabditis elegans are controlled by multiple guidance cues and changing responses to netrin UNC-6

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SUMMARY

SDQR migrations in C. elegans are controlled by multiple guidance cues and changing responses to netrin UNC-6. Our results show that SDQR responds to multiple guidance cues and they suggest that, besides UNC-6, other factors influence whether an UNC-6 responsive cell migrates toward or away from an UNC-6 source in vivo. We propose that multiple signals elicited by the guidance cues are integrated and interpreted by SDQR and that the response to UNC-6 can change depending on the combination of cues encountered during migration. These responses determine the final dorsoventral position of the SDQR cell and axon.

Key words: unc-6, Netrin, Caenorhabditis elegans, Cell migration, Axon guidance, SDQR, UNC-6

INTRODUCTION

The patterning of the embryonic axon scaffold positions neurons and axon tracts at defined locations. During development, axonal growth cones migrate through their environment in response to molecular guidance cues. Studies suggest that individual pioneer axons are directed to specific locations by the actions of repulsive and attractive guidance cues. (Colamarino and Tessier-Lavigne, 1995b; Keynes and Cook, 1995; Tessier-Lavigne and Goodman, 1996; Winberg et al., 1998). Axons appear to have the ability to make pathfinding and target selection decisions based on the simultaneous assessment of multiple guidance cues (Tessier-Lavigne and Goodman, 1996; Winberg et al., 1998). In this combinatorial model, the guidance cues function together as antagonists or collaborators and axons assess the equilibrium between the signals to determine a directional response.

From the genetic and molecular analyses of a guidance cue, UNC-6, and its receptors, UNC-5 and UNC-40, a model of cell and axon migrations in C. elegans has emerged. In its simplest form, the model predicts that cells or axons expressing UNC-5 together with UNC-40 are repelled from UNC-6 sources, and cells and axons expressing UNC-40 (and perhaps another coreceptor) are attracted towards UNC-6 sources. This model is supported by several pieces of evidence discussed below.

Circumferential migrations in C. elegans require the gene unc-6 (Hedgecock et al., 1990). In mutants, dorsal and ventral migrations of pioneer axons and mesodermal cells are disrupted. The UNC-6 protein is a member of the phylogenetically conserved netrin family (Serafini et al., 1994; Harris et al., 1996; Mitchell et al., 1996). The vertebrate netrins have been shown to have chemotactic and chemorepellent activities for developing axons in the embryonic nervous system (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995a; Serafini et al., 1996) and have been implicated as a guidance cue in many different regions of the nervous system (Kennedy et al., 1994; Serafini et al., 1996; de la Torre et al., 1997; Deiner et al., 1997; Lauderdale et al., 1997; Livesey and Hunt, 1997; MacLennan et al., 1997; Metin et al., 1997; Richards et al., 1997; Strahle et al., 1997; Shirasaki et al., 1998). Netrin ectopically expressed throughout the developing fly CNS and nematode nervous system causes widespread guidance defects, indicating that the location of the netrin source is critical (Harris et al., 1996; Mitchell et al., 1996; Ren et al., 1999).

We have proposed that UNC-6 is a component of an extracellular matrix cue and that it is arranged within basement membranes or along cellular surfaces in a manner that allows it to interact with cell surface receptors on migrating axons or cells (Wadsworth and Hedgecock, 1992; Wadsworth et al., 1996). The direction- and tissue-specific guidance activities are mediated by distinct domains of UNC-6, suggesting that receptors interact at unique sites within the protein (Ishii et al., 1992; Wadsworth et al., 1996). UNC-6 is expressed by
neuroglia and pioneer neurons to provide global and local netrin cues. Each cue has a characteristic role depending on the cell type in which it is expressed, its location and the developmental stage of the animal (Wadsworth et al., 1996; Wadsworth and Hedgecock, 1996). An UNC-6 cue arising from ventral epidermoblasts and their descendants creates a stable global netrin cue peaking near the ventral midline of the body wall. It is thought that each migrating cell or axon is either attracted (ventral direction) or repelled (dorsal direction) from the UNC-6 source.

One aspect of the guidance activities that is poorly understood is how mechanistically UNC-6 guides both dorsal and ventral migrations. Two receptors are known to be important for mediating responses to the UNC-6 guidance cue. UNC-5, a receptor belonging to the immunoglobulin superfamily, is expressed in motile cells and is both necessary and sufficient to direct migrations dorsally, away from ventral UNC-6 sources (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Hamelin et al., 1993). UNC-40, a C. elegans homolog of the vertebrate protein encoded by Deleted in Colorectal Cancer (DCC), is also a member of the immunoglobulin superfamily and is required in migrating cells that respond to UNC-6 (Chan et al., 1996). DCC and the vertebrate homologues of UNC-5 have been implicated as receptors for vertebrate netrin (Keino-Masu et al., 1996; Leonardo et al., 1997). The precise role that netrin receptors play in mediating migrations in vivo is only partially understood. At one extreme, different classes of axons could have distinct receptor complexes and signaling pathways to mediate either attraction or repulsion by UNC-6. However, recent data indicates that the pathways involved in netrin-mediated attraction and repulsion are interconnected (Ming et al., 1997). In culture, growth cones of embryonic Xenopus spinal neurons normally turn towards a source of netrin-1. However, with the addition of a competitive analog of cAMP or an inhibitor of protein kinase A, the axons can be switched into being repulsed from the netrin-1 source. These experiments raise the possibility that in vivo different guidance signals could be simultaneously interpreted by neurons to generate different guidance behaviors in response to netrin (Ming et al., 1997).

To study the attractive and repulsive activities of a guidance cue, we used genetic analysis in the nematode C. elegans to examine the roles that netrin UNC-6 has in the cell and axon migrations of SDQR, a neuron positioned along the right lateral side of the midbody. We altered the expression levels of UNC-6 and the position of the UNC-6-expressing cells using unc-6 null mutants and ectopic UNC-6 expression. The postembryonic migrations of the SDQR cell body and axon were then observed in larvae. We report that, while normally the migrations are away from ventral UNC-6-expressing cells, the cell body and axon will migrate towards a dorsal cell that ectopically expresses UNC-6 in the embryo and larva. This change indicates that the response of SDQR to UNC-6 is influenced by other factors. We show that the dorsal migrations require the UNC-5 receptor and are probably moderated by UNC-40. Finally, we show that the SDQR cell body and the axon migrates ventrally in the absence of UNC-6, indicating that there are ventral-directing guidance cues that can work independently of UNC-6. The response to these cues is partially mediated by UNC-40 but does not require UNC-5. To explain the cell and axon migrations of SDQR, we propose that these cues and UNC-6 are simultaneously interpreted by SDQR and that coincident guidance cues encountered by SDQR in vivo can elicit a response that is specific to the combination of cues.

**MATERIALS AND METHODS**

**General methods**

Preparation of plasmid DNA, restriction enzyme digestions, agarose gel electrophoresis of DNA and other molecular biology methods were performed by standard methods (Sambrook et al., 1989). C. elegans cultures were maintained by standard methods (Brenner, 1974). For microscopy of living animals, the animals were mounted on a slide in a small drop of M9 buffer on a 5% agar pad (Sulston and Hodgkin, 1988). The buffer was sometimes supplemented with 25% ethanol to anaesthetize the animals.

**Expression constructs**

Plasmid IM#171, an unc-6 expression vector carrying the mec-7 promoter, was constructed by subcloning the mec-7 promoter region (containing one synthetic intron) from the expression vector pPD96.41 (kindly provided by A. Fire) immediately upstream of the predicted translational start site of unc-6. The fusion was generated by using the polymerase chain reaction (PCR) to introduce unique HindIII and EcoRI sites upstream and downstream, respectively, of the mec-7 promoter sequence. A unique EcoRI site was introduced by PCR into the genomic unc-6 containing plasmid IM#58 at the predicted start site. Finally, the HindIII-EcoRI fragment of plasmid IM#58 containing the unc-6 promoter sequences was replaced with the HindIII-EcoRI mec-7 promoter fragment. Plasmid IM#175, a gfp expression vector using the unc-119 promoter, was constructed by ligating the DP#mm045 (provided by M. Maduro and D. Pilgrim) HindIII-PstI fragment of unc-119 promoter sequence into the PstI-EcoRI sites of the gfp vector pPD95.77 (provided by A. Fire).

**Transgenic animals**

Transgenic strains were obtained by microinjecting unc-6 expression and GFP reporter plasmid DNA (10 μg/ml) together with the plasmid pRF4 into the ovaries of unc-6 (+) hermaphrodites (Fire, 1986; Mello et al., 1991; Mello and Fire, 1995). Plasmid pRF4 was used as a coinjection marker to identify transgenic animals, it carries the dominant allele rol-6 (sa1006), which causes a twist in the body wall. Prenymph inheriting and expressing rol-6 (sa1006) and GFP were identified by their rolling phenotype and GFP expression. The unc-119::gfp transgene enables most neurons and some structures in the head to be visualized by fluorescence (Maduro and Pilgrim, 1995). In some lines, slight fluorescence of the body wall muscles and excretory canal was observed. At least three independent strains were established for each expression construct. From these strains, which carry the constructs as an extrachromosomal array, an integrated transgene was obtained by γ-irradiation (Mello and Fire, 1995). The resulting integrated lines were out-crossed repeatedly to N2 wild type. The strains generated and reported in this paper are: IM19; [urs1s19 IM175, pRF4]; IM30[urs23 IM171, IM175, pRF4]; IM26[urs19 IM171, IM175, pRF4]; IM29[urs22 IM171, IM175, pRF4].

By standard genetic procedure, transgenes were crossed into the unc-6(ev400) background to create: IM39[urs1s13 IM175, ev400]; IM46[urs23 IM46]; Transgenes were also crossed into different unc-5 and unc-40 backgrounds to create: IM55[unc-40(e271)]; urs1s13; IM57[unc-40(e271)]; urs1s23; IM62[unc-40(e1430)]; urs1s13; IM66[unc-40(e1430)]; urs23s12; IM68[unc-5(e53)]; urs23s12; IM106[unc-40(e1430)]; urs23s13; unc-6(ev400)]. The transgenes were also crossed into the mec-4(d) background to create:
We first examined the SDQR cell and axon migrations in direct migrations ventrally in the absence of UNC-6. UNC-6 directs migrations dorsally, but other cues control for SDQR precursor cell migration and cell lineage. Individual cell and nerve positions were recorded in fourth larval stage and young adult animals by epifluorescence microscopy. The cell body positions include ALMR, A VM, BDUR, CAN and SDQR (Fig. 1). Images were obtained using a Zeiss LSM 410 Invert Laser Scan microscope. Multiple nerve tracts were imaged using an extended depth of field. Because of the cylindrical nature of the animals, distances between individual tracts are distorted in the confocal micrographs.

In situ hybridization
To confirm the ectopic expression of the transgenes, in situ hybridizations was used to detect unc-6 RNA. A protocol developed for detection of RNA in whole-mount C. elegans embryos was used (Seydoux and Fire, 1994, 1995). AP-anti-Dig antibody (Boehringer Mannheim) was used for alkaline phosphatase (AP)-mediated detection. 1 mg/ml of DAPI was included in the staining solution to allow nuclei to be identified by epifluorescence microscopy.

RESULTS

The cell and axon migrations of the SDQR neuron
To study in detail the in vivo responses of a neuron to guidance cues, we examined the cell and axon migrations of SDQR. This neuron is part of the sublateral nerves that develop from axons of embryonic neurons in the ring ganglia that run either anteriorly or posteriorly along the epidermis beneath the four body wall muscles. The posteriorly directed sublateral nerves, in particular, comprise the axons from neurons SIA, SIB and SMB in the ventral ganglion, plus SMD in the lateral ganglia. During the first larval stage, the axons of postembryonic neurons SDQ and PLN join the dorsal anterior and ventral posterior sublateral nerves, respectively. Shortly after its birth, the SDQR neuron migrates dorsally to the lateral margin of the body wall muscle alongside the ALMR associated nerve (Figs 1, 2A). Insinuating beneath the body wall muscle and epidermis, the SDQR axon grows dorsally and then anteriorly along the dorsal epidermis toward the nerve ring. SDQR has several advantages for study. First, the cell and axon are relatively isolated from surrounding neurons. Second, the cell is born in the first larval stage, after the final expression pattern of UNC-6 has occurred (Wadsworth et al., 1996). Third, the sister neuron of SDQR, A VM, migrates ventrally to a position that is slightly posterior to SDQR (Figs 1A, 2B). This neuron serves as an easily recognizable positional reference and as a control for SDQR precursor cell migration and cell lineage.

UNC-6 directs migrations dorsally, but other cues direct migrations ventrally in the absence of UNC-6
We first examined the SDQR cell and axon migrations in unc-6 null larvae. We used an unc-119::gfp reporter which expresses green fluorescent protein (GFP) throughout the nervous system (Chalfie et al., 1994; Maduro and Pilgrim, 1995) to observe migrations. The integrated transgene itself causes no abnormalities of axonal outgrowth and guidance when homozygous. While, in wild-type larvae, the SDQR cell and axon migrate to dorsal positions, away from ventral UNC-6-expressing cells (Figs 2A, 3A), in 72% of the unc-6 null larvae, the cell body migrates ventrally (n=200, Figs 2B, 3A) and, in 78% of these cases, the cell’s axon migrates ventrally (n=143, Figs 2B, 3C). This bias towards ventral migrations contrasts with the wandering migration pattern of most axons in unc-6 null larvae (Hedgecock et al., 1990) and it suggests that SDQR responds to ventral-directing guidance cues in the absence of UNC-6.

SDQR is directed towards a dorsal ectopic UNC-6 source in unc-6 null larvae
The influence of UNC-6 expression on the SDQR cell and axon migrations was examined by observing the migrations in larvae where UNC-6 is ectopically expressed. To express ectopic netrin cues from a specific neuronal source, we fused the unc-6-6-expressing cells in the absence of UNC-6.
Neuronal ectopic expression was chosen because UNC-6 is normally expressed by some neurons, the temporal order of nervous system development is well characterized, and both the source and target are at the same interface, between the epidermis and epidermal basement membrane. For each experiment, we compared three independent chromosomal integrates, obtaining similar results. The mec-7 regulatory sequence causes sustained, high-level gene expression in the embryonic ALM/PLM and postembryonic AVM/PVM mechanosensory neurons (Savage et al., 1989, 1994; Hamelin et al., 1992, 1993; Chalfie et al., 1994; Birchall et al., 1995; Chan et al., 1996). Using an unc-6 cDNA probe for in situ hybridization, we confirmed these integrated transgenes express in the predicted patterns (data not shown). For all strains reported, the morphology of the neurons appears normal and the animals are sensitive to touch.

Although ALM and AVM are both in the vicinity of SDQR in the mature animal (Fig. 1), only ALM is born and expresses mec-7 before SDQR migrations. The ALM neuron is born in the embryo and extends a pioneering axon during early formation of the axon scaffold (Durbin, 1987). Expression of mec-7 in ALM occurs during axon outgrowth and it continues throughout development. Ectopic UNC-6 expression from ALM is effective; in mec-7; unc-6 embryos, the migrations of nearby pioneer axons are disrupted (Fig. 2D, Ren et al., 1999). On the contrary, AVM is not born until the first larval stage and mec-7 expression does not occur until axon outgrowth in subsequent developmental stages. This expression is too late to influence pioneering axons. AVM and SDQR are sister neurons, arising from an anterior-posterior division. While initially they are lateral to one another, both cells migrate so that SDQR becomes positioned anterior and dorsal to AVM (Fig. 1B). In unc-6 null animals, when SDQR migrates ventrally (Fig. 1C; discussed below), the position of AVM remains posterior to SDQR. In this case, AVM can be slightly dorsal, slightly ventral or directly lateral to SDQR because the dorsolateral position of the ventrally mispositioned SDQR cell body varies between the CANR-associated nerve and the ventral sublateral nerve (Fig. 1C). Reflecting the timing of axon outgrowth, the MEC-7 β-tubulin is first detected in AVM at the second larval stage by MEC-7 antibody, mec-7::gfp, and mec-7::lacZ (Walthall and Chalfie, 1988; Hamelin et al., 1992; Mitani et al., 1993; Chalfie et al., 1994). Compared to ALM, mec-7 expression by AVM is weaker and is in proportion to the fewer microtubules found in AVM (Chalfie and Thomson, 1979; Hamelin et al., 1992). UNC-6 expression driven by the mec-7 promoter in AVM is too late to influence the SDQR cell migration. For the initial SDQR axon migration, AVM could be a source of UNC-6 although, when axon outgrowth begins, the SDQR cell body is positioned either at the ALM source of UNC-6 or is ventral to the ALM source and lateral to the AVM cell body.

We expressed the mec-7::unc-6 transgene in unc-6 null larvae to examine how the distribution of UNC-6 influences the migrations. We expected that, if UNC-6 always acts as a repulsive guidance cue for SDQR, then the migrations should be directed ventrally, away from the dorsal UNC-6-expressing ALM neuron. Moreover, if signals from the ventral-directing cues and ectopic UNC-6 collaborate with each other then we expect that the frequency of ventral migrations should be greater than in unc-6 null larvae. We observe, however, an increase in the number of larvae with dorsal SDQR cell and axon migrations (Fig. 2C). Compared to 28% of unc-6 null larvae, the SDQR cell body migrates dorsally in 50% of unc-6 null larvae that express the mec-7::unc-6 transgene (n=200 for each; P<0.00001, one-tailed Fishers Exact Test; Fig. 3A). Even when the cell migrates to the ventral position, the axon migrates dorsally towards the UNC-6 source in 20% of the larvae with the transgene, compared to 5% in unc-6 null larvae (n=143 and 101, respectively; P<0.0006; Figs 2C, 3C). Finally, the dorsal migration of SDQR is not due to ectopic UNC-6 overexpression that obscures the pattern and directional information since, when the mec-7::unc-6 transgene is expressed in the unc-6(+)/background, the endogenous UNC-6 can be interpreted by SDQR to direct wild-type migrations (Fig. 3).

**UNC-6 expression in the embryo, not in the larva, is required to guide SDQR migrations**

How does the ectopic UNC-6 secreted by ALM establish a gradient that influences SDQR? As SDQR migrates, UNC-6 secreted by ALM into the flowing environment of the pseudocoelomic cavity would not be expected to form dorsoventral gradients. Rather, stable gradients must form by interactions with the basement membranes and cellular surfaces that SDQR will contact. In fact, in wild-type animals,
 UNC-6 expression often anticipates migrations and is not necessarily concurrent to them, suggesting that UNC-6 expression with respect to basement membrane modeling and epidermal cell development is critical (Wadsworth and Hedgecock, 1992; Wadsworth et al., 1996). Because of these considerations, we postulate that embryonic expression of UNC-6 is required to guide SDQR migrations and that concurrent larval expression has little influence. To test this, we genetically ablated ALM in the late embryo. We took advantage of the induced degeneration of the mechanosensory neurons by dominant mutations of mec-4 (mec-4(d)), a member of the degenerin gene family postulated to encode a subunit of a mechanotransducing channel (Chalfie and Au, 1989; Driscoll and Chalfie, 1991; Mitani et al., 1993; Lai et al., 1996). In mec-4(d) animals, cells develop and then degenerate. Degeneration is initiated shortly after mec-4(d) is expressed, although disrupted cells can persist for hours (Hall et al., 1997). In unc-6(–), mec-4(d) mutants that express the mec-7::unc-6 transgene, ALM is missing in embryos by hatching, while AVM often, but not always, degenerates by the young adult stage. A sensitive marker of mec-4(d)-induced cell disruption is GFP fluorescence, which disappears early in a cell that undergoes the degenerative process. We scored SDQR migrations in young adults that had either no ALM or AVM neurons or had no ALM but normal AVM cell bodies and axons as measured by GFP fluorescence.

If early embryonic expression from ALM is sufficient to direct SDQR migrations dorsally in the mec-7::unc-6 transgenic animals, then the removal of ALM late in the embryo may not have much affect on SDQR migrations. However, if concurrent expression from ALM is required to direct the SDQR migrations dorsally, then SDQR migration should be like that of unc-6 null larvae when ALM is missing. We observe that there is no significant difference in the frequency of dorsal SDQR cell migrations when ALM has degenerated in the late embryo. Compared to 50% dorsal cell migrations in unc-6 null larvae that express the mec-7::unc-6 transgene, the SDQR cell body migrates dorsally in 57% of the larvae when ALM degenerates (n=200 for each; P<0.9, one-tailed Fishers Exact Test; Fig. 4A). Also, embryonic degeneration of ALM does not significantly change the frequency of dorsal SDQR axon migrations when the SDQR cell body migrates ventrally. Compared to 20% dorsal axon migrations in unc-6 null larvae that express the mec-7::unc-6 transgene, the SDQR axon migrates dorsally in 27% of the larvae without ALM (n=143 and 86, respectively; P<0.2; Fig. 4C). Similar results were obtained when animals with ablated AVM were scored. These results support the observations made...
in wild-type animals that indicate early UNC-6 expression influences later cell and axon migrations (Wadsworth et al., 1996).

**The SDQR dorsal migrations are induced by mec-7::unc-6 expression in mec-3-expressing cells**

Current models predict that migrating axons follow concentration differences and require netrin gradients for directed outgrowth. If SDQR can only be repelled by UNC-6, then the dorsal SDQR migrations in the mec-7::unc-6 animals require an UNC-6 gradient that peaks ventrally of ALM. The possible sources that could establish such a gradient before SDQR migrations are the ventral muscle cells, the ventral epidermal cells and ventral cord neurons. However, these cells are not known to express MEC-7, and we cannot detect any mec-7::unc-6 transgene expression by these cells. Moreover, it is unlikely that undetectable levels of UNC-6 expression by these cells could direct SDQR dorsally since ALM is a major ectopic source and a competing ventral source would have to establish a steeper opposing gradient to drive SDQR towards and sometimes across ALM.

To experimentally test whether expression of the mec-7::unc-6 transgene in touch cells is responsible for the dorsal migrations of SDQR, we scored SDQR migrations in mec-3 mutant animals that have the mec-7::unc-6 transgene. The mec-3 gene encodes a LIM-type homeodomain protein required for the differentiation of the touch cells (Way and Chalfie, 1988; Duggan et al., 1998). The gene is expressed in the touch cells and the FLP and PVD cells. In mec-3 mutants, the expression of mec-7 is reduced (Hamelin et al., 1992; Mitani et al., 1993).

For example, it has been reported that, in 45% of mec-3(1338) animals, there is no detectable staining of ALM using an anti-MEC-7 antibody and, in 55%, there is only weak staining (Mitani et al., 1993).

Reducing the expression level of the mec-7::unc-6 transgene by mec-3, decreases the frequency of dorsal SDQR migrations. Compared to 50% dorsal cell migrations in unc-6(–) mutants that express the mec-7::unc-6 transgene, the SDQR cell body migrates dorsally in 40% of the mec-3(–), unc-6(–) mutants that express the mec-7::unc-6 transgene (n=200 for each; P<0.04; Fig. 3A). The cell migrates dorsally in 28% of unc-6(–) animals that do not have the mec-7::unc-6 transgene. The mec-3 mutation also reduces the frequency of dorsal SDQR axon migrations when the SDQR cell body migrates ventrally. Compared to 20% dorsal axon migrations in unc-6(–) mutants that express the mec-7::unc-6 transgene, the SDQR axon migrates dorsally in 4% of the mec-3(–), unc-6(–) mutants that express the mec-7::unc-6 transgene (n=101 and 119, respectively; P<0.0005; Fig. 3C). The axon migrates dorsally in 5% of unc-6(–) animals that do not have the mec-7::unc-6 transgene. These results indicate that expression of the mec-7::unc-6 transgene in mec-3-expressing cells induces the dorsal SDQR migrations in mec-7::unc-6 animals. We conclude that the ALM touch receptor neuron is the source of UNC-6 responsible for directing the dorsal SDQR migrations.

**UNC-5 is required for the dorsal, but not ventral migrations of SDQR**

We determined the roles that the UNC-5 netrin receptor plays in SDQR migrations. The SDQR cell and axon migrations of...
unc-5 null mutants, either with or without ectopic UNC-6 expression, are like those in unc-6 null mutants (Fig. 3). Larvae with both the unc-5 and unc-6 mutations have the same SDQR migration phenotypes as larvae with either mutation alone. These results are consistent with other evidence suggesting that the UNC-5 receptor is required for the dorsal migrations of SDQR in response to ventral UNC-6 sources (Hedgecock et al., 1990; Hamelin et al., 1993). Furthermore, we have observed that the ectopic UNC-6 expression in unc-5; unc-6 larvae causes severe abnormalities, most animals arrest during development and the rare adult hermaphrodites are sterile. In these animals, UNC-5 may be needed to prevent inappropriate responses by cells to ectopic UNC-6. Finally, ventral migrations are not affected in the unc-5 larvae, suggesting that the response to ventral-directing guidance cues are not primarily mediated by UNC-5.

**UNC-40 mediates SDQR ventral migrations**

We next determined the roles that the UNC-40 netrin receptor plays in SDQR cell and axon migrations. Compared to wild type, the SDQR migrations in unc-40 mutants are disrupted, however, the defects are less penetrant than those in unc-6 or unc-5 mutants. The cell body in 77% of the unc-40 larvae migrates to the normal dorsal position, compared to 98% in wild type, 28% in unc-6 null larvae and 35% in unc-5 null larvae (n=200 for each; Fig. 3A). This intermediate phenotype suggests that the unc-40 mutation may influence the cell migration by decreasing the response to one or more of the guidance cues. To address this, we examined the cell migrations in unc-40; unc-6 double null mutants. If the unc-40 mutation has no effect on the response to ventral-directing signals, the frequency of ventral migrations should be the same as in unc-6 null mutants. We find, however, that more cell bodies migrate dorsally in these larvae (43%) than in unc-6 null mutants (n=200 for each; P<0.002; Fig. 3A), suggesting that the unc-40 mutation impairs the SDQR cell response to the ventrally directing cues.

The pattern of axon migrations further suggests that the influence of the ventral-directing cues is stronger towards the ventral midline. Comparing unc-40; unc-6 larvae to unc-6 larvae, the axon migrations vary at cell bodies that are dorsal, but not at cell bodies that are ventral. The axons from dorsally positioned cell bodies migrate dorsally more frequently in unc-40; unc-6 larvae than in unc-6 larvae (67% for unc-40; unc-6 larvae, n=85, and 45% for unc-6 null larvae, n=57; P<0.003; Fig. 3B), whereas axons from ventrally positioned cell bodies have similar migrations that are mostly ventral (81% for unc-40; unc-6 larvae, n=115, and 78% for unc-6 null larvae, n=143; P<0.3).

UNC-40 has been implicated as a component of a netrin receptor (Hedgecock et al., 1990; Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej, 1996; Fazeli et al., 1997). Several experiments suggest that UNC-40 helps to mediate SDQR cell and axon responses to netrin UNC-6. First, the unc-40 mutation disrupts the SDQR dorsal cell migration, a migration that requires UNC-6 (98% and 77% dorsal axon migration for wild-type and unc-40 larvae, respectively; n=200 for each). Second, in contrast to ectopic UNC-6 expression in unc-6 null mutants, ectopic expression in unc-40; unc-6 mutants does not increase dorsal cell migrations (43% for unc-40; unc-6 mutants and 45% for unc-40; unc-6 larvae with ectopic UNC-6; n=200 for each; P<0.7; Fig. 3A) nor the frequency of axons from ventrally positioned cell bodies that migrate dorsally towards the ectopic UNC-6 source (12% for unc-40; unc-6 larvae, n=115, and 7% for unc-40; unc-6 larvae with ectopic UNC-6, n=110; P<0.9; Fig. 3C). These experiments, however, are not conclusive because of the possibility that the SDQR migration patterns are also influenced simultaneously by UNC-40-mediated responses to the ventral-directing cues. Currently, we cannot remove the influence of these cues. Finally, the unc-40 larvae reveal a difference between the cell body and axon responses to the guidance cues. Whereas ectopic UNC-6 expression did not influence the dorsal cell migration (77% for unc-40 larvae and 71% for unc-40 larvae with ectopic UNC-6; n=200 for each; P<0.9; Fig. 3A), it did cause more axons from ventrally positioned cell bodies to migrate dorsally (8%, n=47, for unc-40 larvae and 20%, n=58, for unc-40 larvae with ectopic UNC-6; P<0.03; Fig. 3C).

**The role of the embryonic dorsal sublateral nerve in SDQR pathfinding**

As the SDQR axon migrates dorsally, selective fasciculation along the dorsal sublateral tract could be one means by which the SDQR axon finds the correct dorsoventral position to turn anteriorly. We observed that, in a few larvae (<8%), the embryonic dorsal sublateral tract is deflected from the region where SDQR joins the tract. However, the dorsally migrating SDQR axon always finds the correct sublateral position (Fig. 2D), indicating that whether the SDQR axon adopts the sublateral tract is independent of whether embryonic axons are present in that tract. Furthermore, the SDQR axon was never observed to migrate to the dorsal nerve cord. These results suggest that cues, not associated with the nerve, are involved in dorsoventrally positioning the SDQR axon.

**The AVM cell**

The sister of SDQR, the AVM neuron, migrates ventrally and extends a process to the ventral cord (Figs 1A, 2B). In the mutant and transgenic lines, AVM migrations are distinct from that of SDQR and we conclude that the neurons retain different responses to the guidance cues. First, the anteroposterior position of AVM is always posterior to SDQR, suggesting specific responses to anteroposterior cues. Second, AVM axon migrations are only rarely abnormal. In all the larvae scored, the AVM axon migrated ventrally in 96%, laterally in 4% and never dorsally. The ectopic UNC-6 expression had no effect on AVM migrations. Finally, ventrally migrating SDQR axons often turn anteriorly at the ventral sublateral nerve whereas the AVM axons nearly always migrate to the ventral nerve cord.

**DISCUSSION**

The combined actions of attractive and repulsive guidance cues are thought to direct particular guidance decisions (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). A genetic analysis using Drosophila has shown that by manipulating the expression of genes encoding Netrin A, Netrin B, Semaphorin II and Fasciclin II, the ability of motor axons to select their muscle targets can be altered (Winberg et al., 1998). The results indicate that the signals elicited by these molecules are simultaneously assessed by the growth cones and that they
work in combination to amplify or antagonize one another. Current models of circumferential migration in *C. elegans*, put simply, predict that cells or axons expressing unc-5 together with unc-40 are repelled by UNC-6 sources, and axons expressing unc-40 (and perhaps an as yet unidentified coreceptor) are attracted towards UNC-6 sources. It is thought that during migrations axons turn and migrate along pre-existing longitudinal tracts in response to selective fasciculation cues that cause stronger responses than those to UNC-6. In our study, we used genetics to examine the ability of UNC-6-expressing cells to attract or repulse the cell and axon migrations of SDQR. We have found that, like the motor axons in *Drosophila*, the SDQR migrations are simultaneously influenced by several guidance cues. Furthermore, our results suggest that the response to UNC-6 is regulated by the combination of cues SDQR encounters. Finally, we show that UNC-5 and UNC-40 help mediate the SDQR responses and that other receptors must also be involved.

**A model for the migrations of SDQR**

From the migrational patterns of SDQR when different gene functions are altered, we can infer how some molecular guidance cues help to guide SDQR migrations. The observation that SDQR migrates dorsally, away from UNC-6 sources, in wild-type larvae, but migrates ventrally in observation that SDQR migrates dorsally, away from UNC-6 guidance cues help to guide SDQR migrations. The functions are altered, we can infer how some molecular from the migrational patterns of SDQR when different gene combinations of cues SDQR encounters. Finally, we show that UNC-5 and UNC-40 help mediate the SDQR responses and that other receptors must also be involved.

**SDQR migrations are controlled by multiple signaling pathways**

Two families of netrin receptors have been identified, DCC/UNC-40 and UNC-5. In culture, an antibody to DCC selectively blocks the outgrowth of rat commissural axons (Keino-Masu et al., 1996). Furthermore, turning of *Xenopus* growth cones in response to netrin-1 can be blocked by antibodies to DCC (de la Torre et al., 1997; Ming et al., 1997). This suggests that cell and axon responses to netrin UNC-6 in *C. elegans* are mediated by UNC-40. Since UNC-40 is involved in both dorsal and ventral migration, whereas the UNC-5 receptor is required only for dorsal migrations, and since guidance mediated by ectopic UNC-5 requires unc-40, repulsion from the ventral UNC-6 source may be controlled by UNC-5 working in association with UNC-40, perhaps as heteromeric receptor complex (Hedgecock et al., 1990; Leonardo et al., 1997; Colavita and Culotti, 1998). Our result would be consistent with the idea that UNC-40 regulates the strength of the UNC-5-mediated signal. Furthermore, our results suggest that UNC-5 may control some of the activities of UNC-40. While expression of the mec-7::unc-6 transgene in other single and double mutant backgrounds is tolerated, expression in unc-5(−); unc-6(−) larvae causes embryonic and early larval lethality. It is possible that, without UNC-5, ectopically expressed UNC-6 causes inappropriate UNC-40-mediated signals that are deleterious to the animals. These
inappropriate signals are eliminated in *unc-5* (+) and *unc-40* (−) larvae. Our results further indicate that UNC-5 is not involved in mediating the response to cues that guides SDQR ventrally in the absence of UNC-6, while UNC-40 does play a role. Together our results provide evidence that through multiple receptors different guidance cues are simultaneously interpreted by SDQR in vivo and they suggest that the signaling pathways that direct a response are interwoven so that the response to UNC-6 is regulated by the combination of guidance cues that is encountered by SDQR.

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