

Voltage-Gated Calcium Channels Direct Neuronal Migration in *Caenorhabditis elegans*

Tobey Tam,* Eleanor Mathews,† Terrence P. Snutch,†
and William R. Schafer*

*Department of Biology, University of California at San Diego, La Jolla, California 92093-0349;
and †Biotechnology Laboratory, University of British Columbia,
Vancouver, British Columbia, Canada V6T 1Z3

Calcium signaling is known to be important for regulating the guidance of migrating neurons, yet the molecular mechanisms underlying this process are not well understood. We have found that two different voltage-gated calcium channels are important for the accurate guidance of postembryonic neuronal migrations in the nematode *Caenorhabditis elegans*. In mutants carrying loss-of-function alleles of the calcium channel gene *unc-2*, the touch receptor neuron AVM and the interneuron SDQR often migrated inappropriately, leading to misplacement of their cell bodies. However, the AVM neurons in *unc-2* mutant animals extended axons in a wild-type pattern, suggesting that the UNC-2 calcium channel specifically directs migration of the neuronal cell body and is not required for axonal pathfinding. In contrast, mutations in *egl-19*, which affect a different voltage-gated calcium channel, affected the migration of the AVM and SDQR bodies, as well as the guidance of the AVM axon. Thus, cell migration and axonal pathfinding in the AVM neurons appear to involve distinct calcium channel subtypes. Mutants defective in the *unc-43/CaM* kinase gene showed a defect in SDQR and AVM positioning that resembled that of *unc-2* mutants; thus, CaM kinase may function as an effector of the UNC-2-mediated calcium influx in guiding cell migration. © 2000 Academic Press

Key Words: calcium channel; *Caenorhabditis elegans*; neuronal migration; CaM kinase.

INTRODUCTION

The proper assembly and wiring of the nervous system depends on the execution of complex and reproducible cell movements (reviewed in Tessier-Lavigne and Goodman, 1996). For example, the growth cones of extending axons are directed to their ultimate synaptic targets by localized guidance cues. In addition, many neurons arise in a part of the organism different from the location at which they function in the mature animal; thus, accurately directed cell migrations are necessary to bring these neurons to their ultimate destination. The guidance of both migrating neurons and extending growth cones depends on specifically localized guidance molecules, which serve as positional cues within the developing nervous system. A number of molecules that direct the navigation of both migrating neurons and extending growth cones have been identified, including the netrins, semaphorins, neurotrophins, and members of the slit gene family. For each of these ligand families, receptors have been identified that specifically recognize particular guidance molecules and mediate detec-

tion of these positional cues by the neurons undergoing cell migration or axon outgrowth. The binding of these specific ligands to their receptors triggers intracellular signaling cascades, which induce cytoskeletal rearrangements that lead to directed cell motility.

An important question in the study of neuronal guidance is to understand how intracellular signaling pathways regulate the pathfinding mechanism of an axon or cell body (Song and Poo, 1999). At present, the signal transduction pathways that mediate growth cone guidance are better characterized than those involved in cell migration. Several signaling pathways have been demonstrated to play key roles in axonal pathfinding, including the cyclic AMP/protein kinase A pathway. In mice, it has been shown that loss of adenylyl cyclase activity in the brain results in aberrant axonal patterning in the somatosensory cortex (Abdel-Majid *et al.*, 1998). In at least some neurons, cAMP appears to function as a switch to determine how a neuron responds to a localized source of so-called group I guidance cues (a group that includes netrin-1, BDNF, and NGF). For example, in cultured *Xenopus* spinal neurons, the growth

cones, which normally migrate toward a source of the guidance molecule netrin-1, avoid the same netrin when intracellular cyclic AMP levels are low (Song *et al.*, 1997). Thus, the activity of the cAMP pathway determines whether netrin-1 acts as an attractant or a repellent to these cells. Another cyclic nucleotide, cyclic GMP, appears to play a similar role in mediating cellular responses to the "group II" molecules such as semaphorin III and NT-3 (Ming *et al.*, 1999; Song *et al.*, 1998).

Another second messenger that has been implicated in directing growth cone guidance is calcium. It has been well established that high-frequency calcium transients slow growth cone motility, whereas a low frequency of transients enhances axonal outgrowth (Gomez and Spitzer, 1999; Gu and Spitzer, 1995). The responses of growth cones to many guidance molecules, such as netrin-1 and BDNF, are dependent on an influx of extracellular calcium, apparently through L-type voltage-gated channels (Ming *et al.*, 1997; Song *et al.*, 1998). In addition, there is evidence that IP₃-mediated calcium release from intracellular stores also regulates axonal outgrowth (Takei *et al.*, 1998). The pathways that respond to these calcium signals are not completely characterized; however, pharmacological evidence indicates that calmodulin-dependent kinase (CaM kinase) is required for at least some calcium-mediated control of axonal pathfinding (VanBerkum and Goodman, 1995; Zheng *et al.*, 1994).

Although much has been learned recently about the signal transduction pathways involved in axon guidance, much less is known concerning the mechanisms involved in directing neuronal cell migrations. It is clear that in many migrating cells, calcium signaling plays an important role (Komuro and Rakic, 1998). For example, in granule cells of the mammalian cerebellum, an influx of extracellular calcium appears to be necessary for cell motility. Pharmacological experiments have implicated N-type voltage-gated calcium channels as well as NMDA receptors in mediating the calcium influx in these cells (Komuro and Rakic, 1992, 1993). In other types of migrating neurons, including the enteric neurons of *Manduca* (Horgan and Copenhaver, 1998) and the granule cells of weaver mice (Liesi and Wright, 1996), calcium influx appears to inhibit cell motility. At present, the molecular mechanisms by which calcium signals control these migrations are not well understood.

In this study, we demonstrate a role for the voltage-gated calcium channel encoded by the *unc-2* gene in directing postembryonic neuronal migrations in the nematode *Caenorhabditis elegans*. This channel is specifically required for migrations of the neuronal cell bodies but not for the migration of their ectodermal precursors or for the navigation of their growth cones. We also show that a different voltage-gated calcium channel, encoded by the *egl-19* gene, specifically affects axon guidance in these same neurons. Finally, we present evidence that the *unc-2*-mediated calcium influx controlling cell migration may act through a CaM-kinase-dependent mechanism.

MATERIALS AND METHODS

Assay conditions and growth media. Nematodes were grown and assayed at room temperature on standard nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50 as a food source.

Isolation of *unc-2* mutants. The deletion alleles *lj1* and *lj2* were isolated in a noncomplementation screen for *unc-2* mutations. Hermaphrodites of a mutator strain containing a Tc1 transposon insertion in an intron of *unc-2* (*pk95*; obtained from R. Plasterk), along with a linked mutation in *dpy-3*, were mated with *unc-2(mu74)* males. New mutant alleles were identified among the F1 cross-progeny based on their Unc phenotype. Animals homozygous for the new mutant allele were then isolated from the self-progeny of these animals based on their Dpy phenotype; the absence of the *mu74* allele in these strains was then confirmed by PCR. A series of backcrosses with wild type (>5) eliminated the mutator and the *dpy-3* allele. The *ra605* and *ra610* alleles were isolated in a similar screen using ethylmethane sulfonate (EMS) as a mutagen. N2 males were mutagenized with EMS according to standard methods, crossed to *dpy-3(e27) unc-2(e55)* hermaphrodites, and screened for the presence of Unc non-Dpy progeny. Eleven independent Unc non-Dpy animals were isolated and picked individually to new plates. Animals homozygous for the new allele were identified from the self-progeny of these animals based on their failure to segregate Unc Dpy progeny. *md1186*, *md1064*, and *md328* were isolated by the laboratory of J. Rand in a screen for aldicarb-resistant mutants and kindly provided to the authors.

Sequencing of *unc-2* mutations. EMS mutations were localized using the RNase protection assay performed according to the procedure outlined in the Mismatch Detect II kit (Ambion). Nested primer pairs were designed such that the entire *unc-2* genomic sequence could be amplified using the PCR in segments of approximately 800 to 1000 bp. The deletion mutations *lj1* and *lj2* were isolated by DNA blot hybridizations to genomic blots of *unc-2* mutant DNA using *unc-2*-specific probes. Once a mutation had been localized to a specific region, the PCR product was sequenced directly using the BRL dsDNA Cycle Sequencing System.

A PCR-based approach was used to map the sites of Tc1 insertion in the *unc-2* alleles *md1064* and *md1186*. DNA was isolated from the RM1186 and RM1064 strains, and then the p618 primer (Williams *et al.*, 1992), which is complementary to the 3' end of Tc1 adjacent to the inverted repeat, and primers homologous to either the sense or the nonsense strand of *unc-2* were used to amplify the genomic DNA. The p618-EM56 (sequence 5'-TCATCCATCTCTCCACC-3') primer pair amplified an approximately 800-bp PCR product from RM1064 genomic DNA. This fragment was sequenced using the BRL dsDNA Cycle Sequencing System. Amplification of genomic DNA isolated from RM1186 using the p618-EM83 (sequence 5'-ATTGGCCTCTCGGAAACA-3') primer set resulted in an approximately 900-bp PCR product. This PCR fragment was subcloned into the pGEM-T vector (Promega) and the DNA sequence was determined by the dideoxy method using the Sequenase 2.0 kit (U.S. Biochemical Corp.).

Construction and characterization of double mutants. Double mutants carrying *unc-2* and an integrated GFP reporter construct were generated using standard methods. The strain CF703 (genotype *mul535[mec-7::GFP; lin-15(+)]V*), which carried a transcriptional fusion of GFP to the *mec-7* promoter, was obtained from Queelim Ch'ng in the Kenyon lab. Double mutants with *unc-2* and either *unc-43* or *unc-5* were constructed by crossing the single

mutants and first identifying animals homozygous for *unc-5* or *unc-43* in the F2 generation. These animals were picked to individual plates, and animals exhibiting the stronger Unc phenotype characteristic of *unc-2* homozygotes were identified from the F3 self-progeny of these animals. The presence of both mutations was subsequently verified by mating the putative double mutant with wild type and confirming the presence of both single-mutant phenotypes in the F2 generation.

Analysis of the QR.pa cell lineage. Animals were staged by bleaching adult hermaphrodites and picking larvae that hatched within a 30-min time window. These animals were then grown for 5–6 h on NGM at 20°C. Worms were then mounted on 2% agarose pads and observed for 4 h under a compound microscope outfitted with Nomarski optics.

Analysis of *unc-2* mosaics. Potential mosaics were identified using an *unc-2* mutant strain carrying an intact copy of the *unc-2* gene on an unstable free duplication (genotype *him-5* (*e1490*); *unc-2*(*md1186*) *osm-5*(*p503*); *yDp16*). *osm-5* was used to score for the presence of the duplication in the cells of the amphid (ASHL, ASHR, ASJL, ASJR, ASKL, ASKR, ADLL, and ADLR) and phasmid (PHAL, PHBL, PBAR, and PHBR) sensilla. The *osm-5* phenotype was scored as described (Herman, 1984); briefly, animals were stained with DiI on 2% agar plates, and staining of the amphid and phasmid cells was visualized by fluorescence.

RESULTS

Isolation of Loss-of-Function Mutations in the *unc-2* Calcium Channel Gene

The *C. elegans unc-2* gene encodes a protein with a high degree of sequence similarity to the $\alpha 1$ subunits of vertebrate voltage-activated calcium channels (Schafer and Kenyon, 1995). The UNC-2 amino acid sequence suggests that its closest vertebrate homologues are the non-L-type family of high-threshold channels, a group that includes the N-type and P/Q-type channels that promote neurotransmitter release at nerve terminals. *unc-2* also appears to function primarily in neurons, where its activity has been implicated in the control of a number of nematode behaviors, including locomotion, egg-laying, and feeding (Avery, 1993; Brenner, 1974; Miller et al., 1996; Schafer and Kenyon, 1995).

To more fully understand the functions of the UNC-2 calcium channel in the nervous system, we isolated and characterized additional mutations in *unc-2*. The UNC-2 gene product, like all calcium channel $\alpha 1$ subunits, contains four imperfectly repeated integral membrane domains, each consisting of six transmembrane α helices (S1–S6) and two shorter hydrophobic segments thought to line the channel pore (SS1–SS2). These domains are clearly essential for channel function, since they comprise the physical structure of the ion channel and contain the voltage sensor (Catterall, 1995; Stea et al., 1994). We examined the sequence alterations in eight *unc-2* mutant alleles: *md328*, *mu74*, *lj1*, and *lj2* contained small deletions; *ra605* and *ra610* contained premature stop codons; and *md1064* and *md1186* contained transposon insertions (Fig. 1). All eight disrupted the structure of at least one of these four

membrane domains, and one of these alleles, *unc-2*(*md1186*), contained a transposon insertion that interrupted the coding sequence in the middle of the first domain (Fig. 1). We therefore concluded that all eight alleles should at least result in a severe reduction in the activity of the UNC-2 calcium channel and might represent null alleles.

Effects of *unc-2* on the Postembryonic Migrations of AVM and SDQR

One phenotype observed in the *unc-2* strong loss-of-function mutants was a defect in the positioning of two migrating neurons, AVM and SDQR. AVM and SDQR are sister cells whose parent, QR.pa, is descended from the migrating neuroblast QR. In wild-type animals, the correct positioning of AVM and SDQR involves two stages of migration (Fig. 2A). First, QR and its descendants QR.p and QR.pa migrate along the anteroposterior axis from an original position in the midbody region to the anterior of the animal. Subsequently, QR.pa divides, and its descendents migrate in different directions: SDQR migrates to a dorsal and further anterior position, while AVM migrates to a ventral and slightly less anterior position. SDQR and AVM then differentiate into an interneuron and a touch receptor neuron, respectively (Sulston and Horvitz, 1977). In *unc-2* mutants, the final positioning of both AVM and SDQR were often abnormal (Fig. 2B). The most common defect was a failure to migrate an adequate distance; however, some neurons actually migrated in the wrong direction in mutant animals. For example, SDQR was sometimes found in positions that were more ventral and/or more posterior than its position in wild type. Likewise, in *unc-2* mutants AVM was found to adopt positions that were more dorsal, more posterior, or more anterior than the wild-type position. Occasionally, animals contained extraneuronal nuclei near the final positions of SDQR and/or AVM, suggesting that extra cell divisions in the QR.pa lineage occurred (see Fig. 3). Conversely, in a number of mutant animals it appeared that QR.pa had not divided, since a large undifferentiated nucleus was found in the position at which QR.pa normally divides. Overall, the penetrance of the misplacement phenotype in the deletion and transposon alleles was 20–30% for AVM and 10–20% for SDQR. With two exceptions (*md1186* and *md328*), the differences in penetrance between the different *unc-2* alleles were not statistically significant (*Z* test, *P* > 0.05); the greater (for *md1186*) or lesser (*md328*) penetrance in these alleles might be a result of strain background. All *unc-2* alleles were statistically different from wild type according to the *Z* test (*P* < 0.01). In all alleles tested, the positioning of ALM, a functional homologue of AVM that arises through a distinct developmental pathway, was unaffected by mutations in *unc-2*.

The patterns of SDQR/AVM mispositioning seen in *unc-2* animals suggested that *unc-2* did not affect the long anterior-directed migrations of QR.pa and its ancestors. Rather, *unc-2* appeared to be important for only a specific

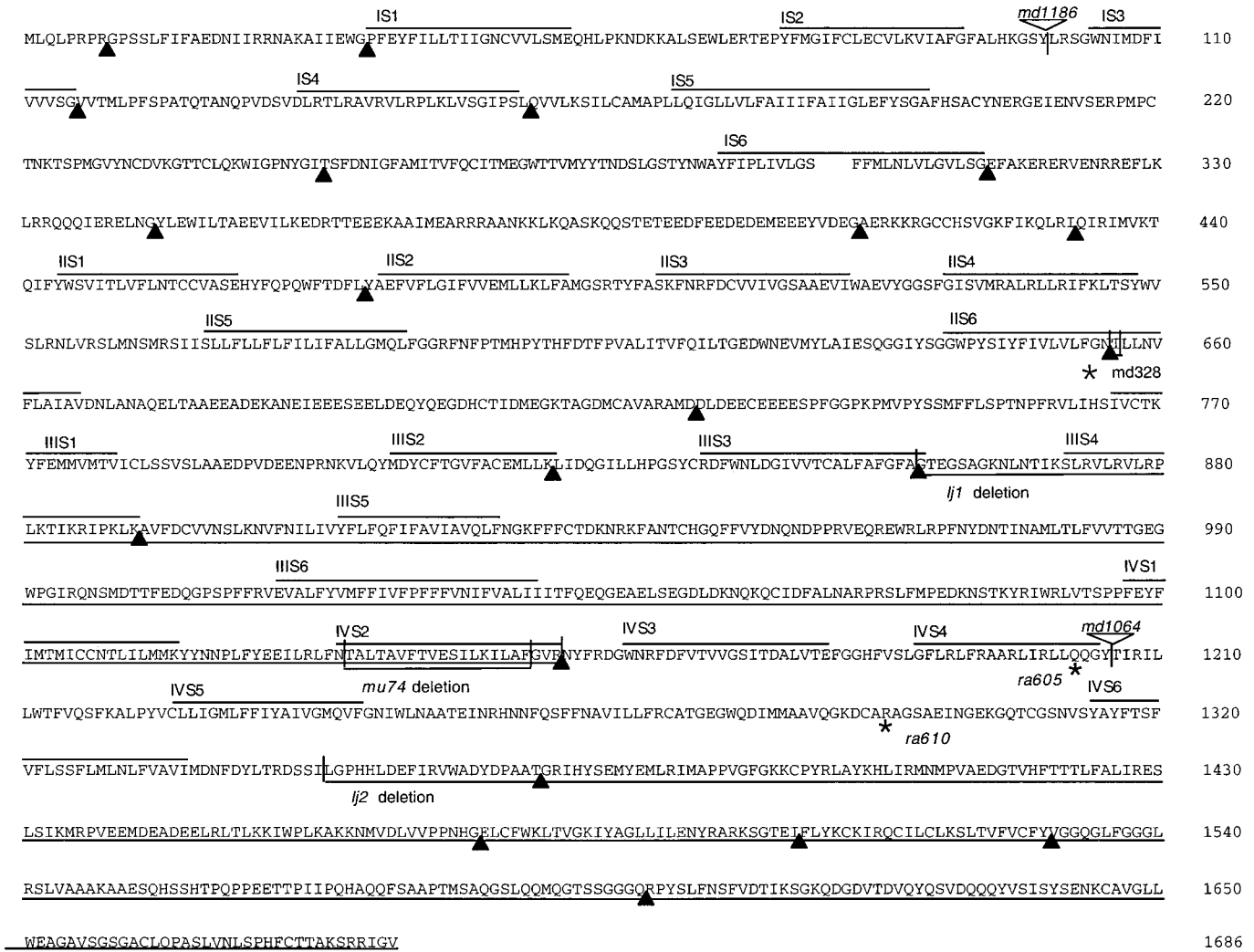
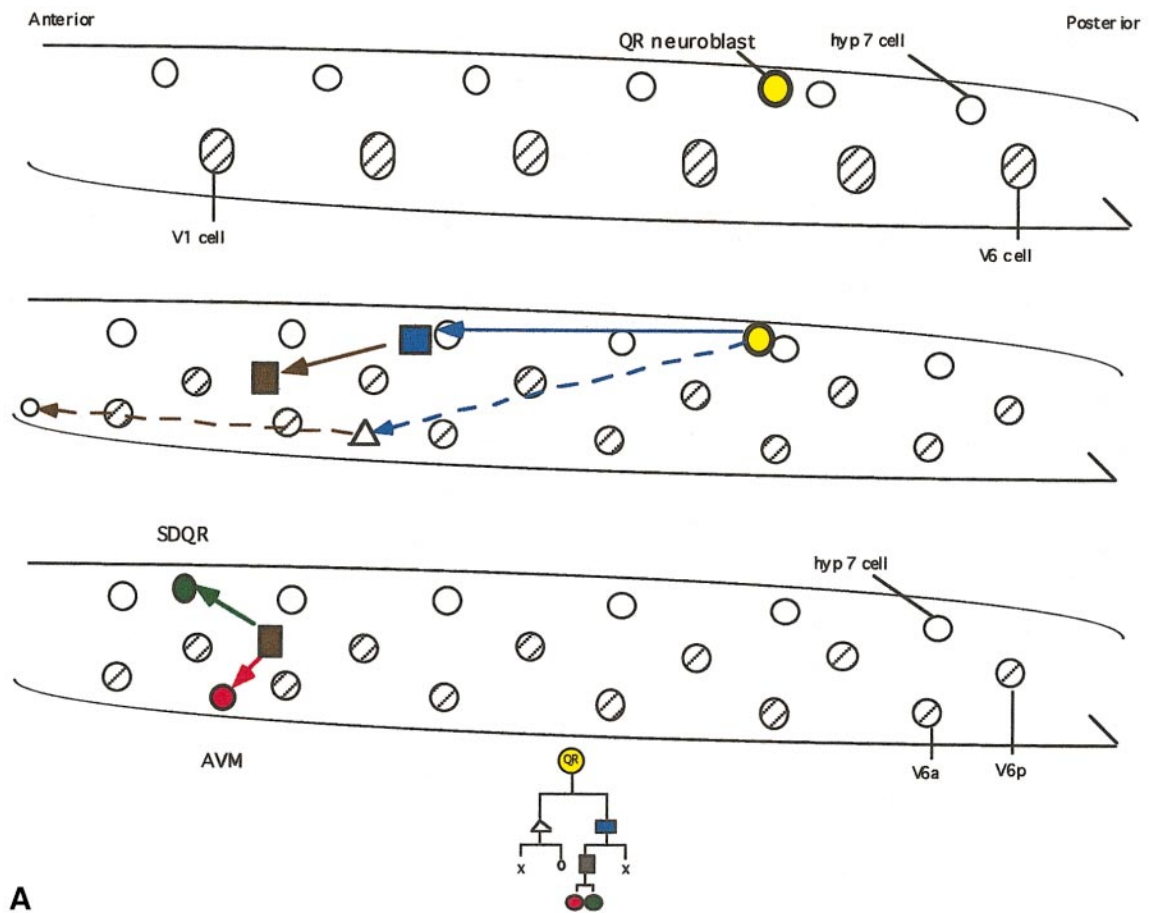


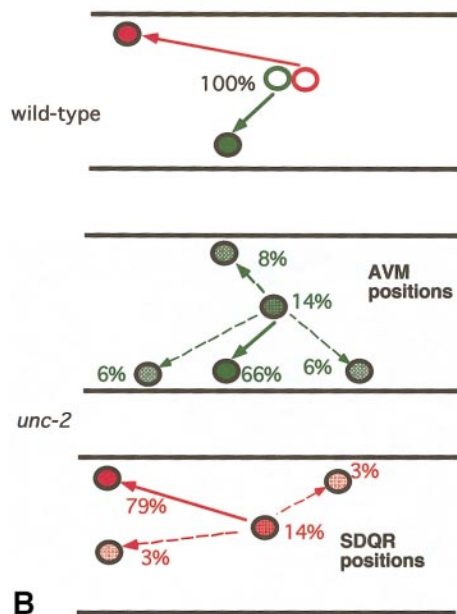
FIG. 1. Sequences of *unc-2* mutant alleles. Shown is the predicted amino acid sequence of the UNC-2 protein. Intron locations are indicated by triangles below the sequence. The cloning of the *unc-2* coding sequence will be described elsewhere (E.M. and T.P.S.). Sequence motifs corresponding to predicted structural features of the UNC-2 protein (identified through comparison with the sequences of other calcium channel $\alpha 1$ subunits) are indicated by bars above the sequence text. I-IV indicate the four repeated membrane domains, each of which contains six predicted transmembrane α helices (S1-S6). Intron positions are indicated by filled triangles. Sequence changes in *unc-2* mutant alleles are indicated above (for transposon insertions) or below (for other mutations) the sequence text; bars indicate the extent of deletions in the *mu74*, *lj1*, and *lj2* alleles; open triangles indicate the sites of transposon insertions (in *md1186* and *md1064*). The *md328* mutation deletes a splice acceptor consensus sequence and is thus presumed to truncate the protein at the point indicated by an asterisk. The point mutations *ra605* and *ra610* introduce nonsense mutations at the positions indicated by asterisks. The *lj2* deletion extends approximately 2 kb past the 3' end of the coding sequence. The sequence alteration in the *e55* allele (see Figs. 2-4) has not been determined.

stage in Q cell development, beginning roughly from the time of the division of QR.pa and lasting until completion of the dorsoventrally directed final migrations. To investigate this possibility in more detail, we traced the cell lineages of individual *unc-2* mutant animals, to determine the cause of AVM and SDQR mispositioning (Fig. 3). In all 20 mutant animals lineaged, QR.pa migrated to its correct

position before dividing. However, in 8 of these animals, either SDQR or AVM migrated to an inappropriate destination. Interestingly, some of these cells were mispositioned not because they failed to migrate, but because they migrated in the wrong direction. Thus, *unc-2* indeed appeared to be required for accurate guidance of the final migrations of SDQR and AVM.



A



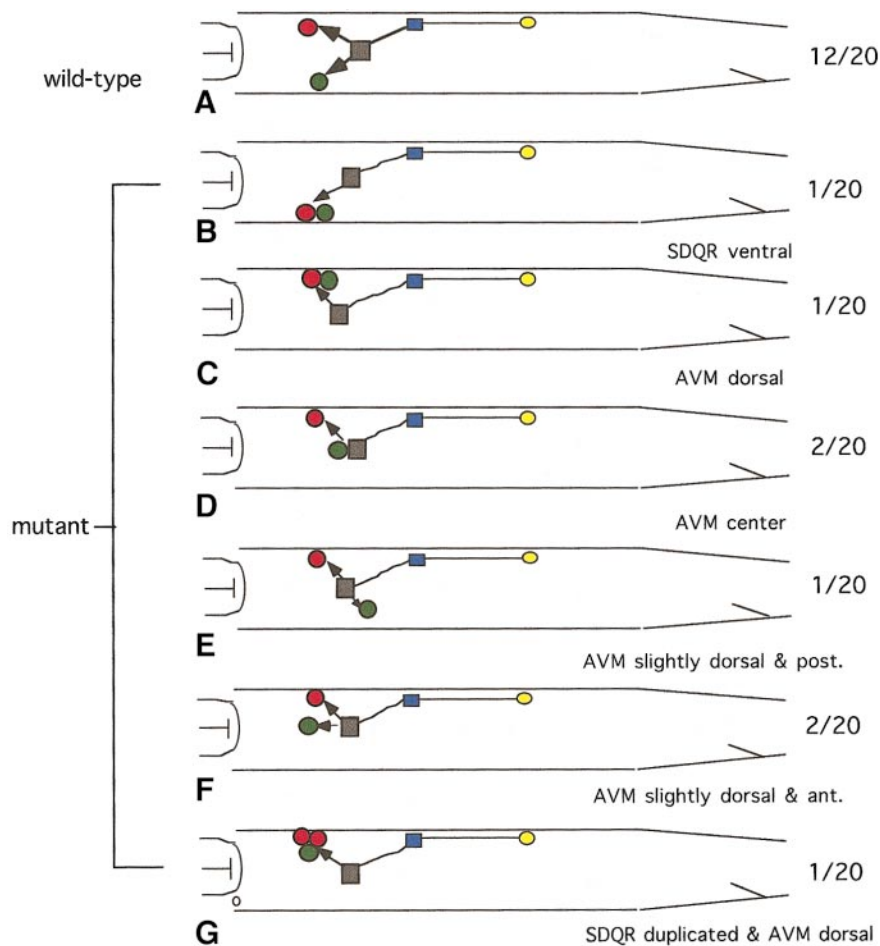


FIG. 3. Effect of *unc-2* on the migration of the SDQR and AVM precursors. Shown are representative cell lineages seen in 20 *unc-2* mutant animals. The position of QR.pa at the time it divided to produce SDQR and AVM is indicated by the gray box. The positions to which SDQR and AVM migrated are indicated by red and green circles, respectively. In the last example, QR.pa divided once, and then the anterior daughter (SDQR) divided again. All three cells subsequently migrated dorsally and anteriorly as indicated.

***EGL-19*, but Not *UNC-2*, Affects Axonal Pathfinding in AVM**

After their final migrations, AVM and SDQR differentiate and extend their axons. For example, AVM differentiates into a mechanosensory neuron and extends an axon ventrally into the ventral nerve cord, which then extends in an

anterior direction toward the nerve ring (White *et al.*, 1986). To determine whether *unc-2* affected the guidance of these processes, we used a *mec-7::GFP* transgene, which labels the cell bodies and axons of touch receptor cells, including AVM (Chalfie *et al.*, 1994; Hamelin *et al.*, 1992). Since *mec-7* encodes a touch receptor-specific tubulin (Savage *et*

FIG. 2. Migration defects of *unc-2* mutant alleles. (A) Migrations of AVM and SDQR in wild-type animals. The neuroblast QR.pa, which is born during the first larval stage in the midbody region, migrates to an anterior position and then divides. The posterior daughter cell, SDQR, migrates in a dorsal and anterior direction, differentiates into an interneuron, and projects an axon into the dorsal sublateral nerve. The anterior daughter cell of this division, AVM, migrates ventrally, differentiates into a touch receptor neuron, and projects an axon into the ventral nerve cord. The dorsal counterpart of the AVM touch receptor, ALM, arises through an unrelated lineage pathway: it is born during embryogenesis in the head region and migrates posteriorly during embryogenesis. (B) Positions of AVM and SDQR in *unc-2* mutants. The positions adopted by SDQR and AVM in wild-type and *unc-2* mutant animals are summarized. The diagram represents aggregate data from the two strong loss-of-function alleles *md1186* and *mu74*.

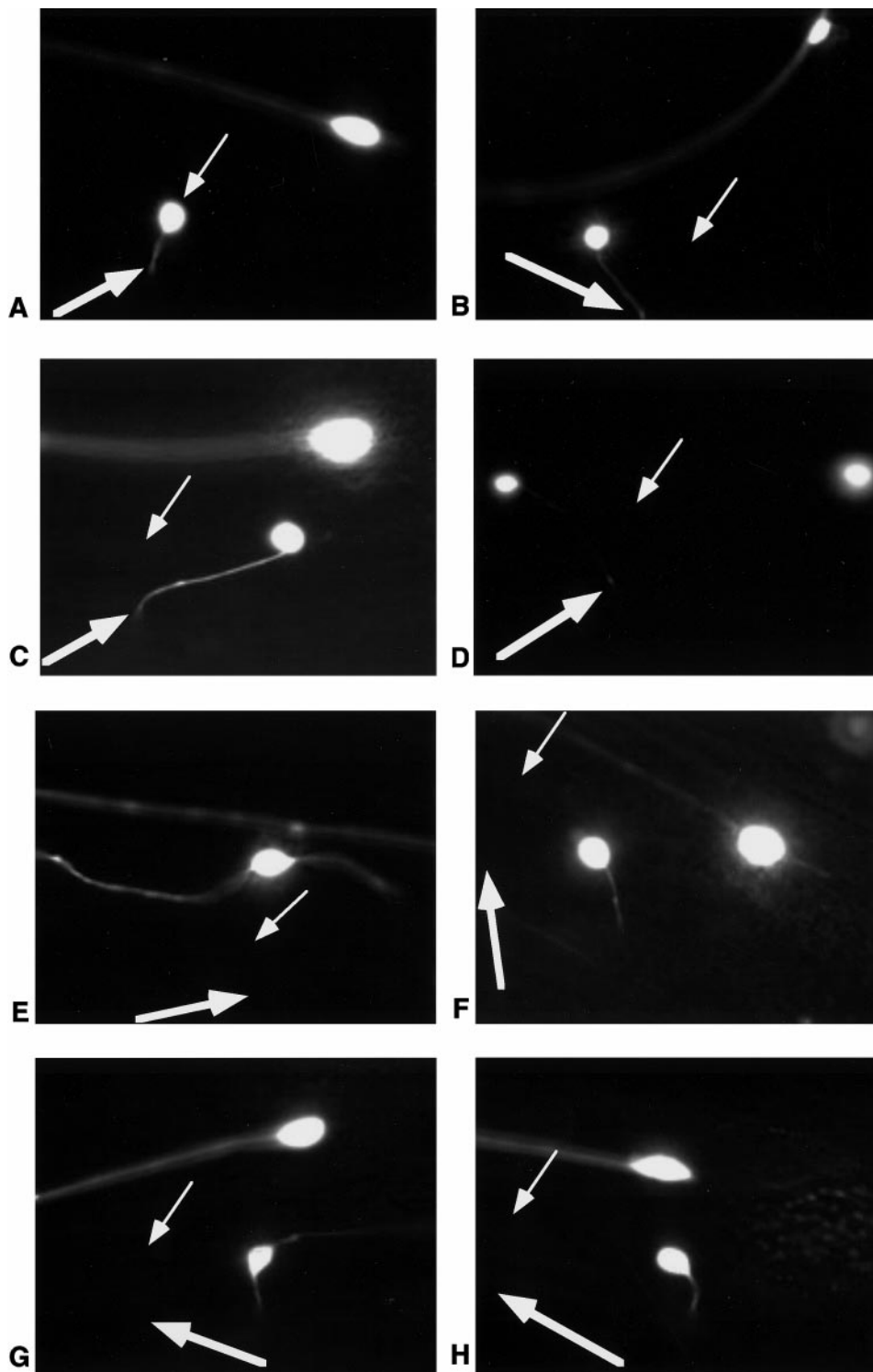


FIG. 4. Effect of *unc-2* and *egl-19* on AVM differentiation and axon guidance. Shown are fluorescence images of adult animals expressing the touch receptor-specific marker *mec-7::GFP*. Only the dorsoventrally directed process of AVM is in focus. Left is anterior and top is dorsal. Thin arrow shows the wild-type position of the AVM cell body. The thick arrow shows the position where the ventrally directed axon connects to the ventral nerve cord in wild type. The other fluorescent neuron in the pictures is ALM. Overall, the penetrances of axon guidance defects in the strains analyzed were wild-type, 0% ($n = 100$); *unc-2(mu74)*, 0% ($n = 100$); *egl-19(ad1006)*, 20% ($n = 50$);

al., 1989), its expression serves as a marker for AVM differentiation and also makes it possible to visualize the extension of the AVM axons. When we analyzed an *unc-2* mutant line containing the *mec-7::GFP* transgene (Fig. 4), we observed that the AVM neuron, like the other touch receptor neurons, was invariably fluorescent, even in animals in which the AVM cell body was misplaced. Moreover, the AVM axon still entered the ventral nerve cord in its proper location in mutant animals, despite the fact that in some cases the axon originated from a misplaced cell body (Figs. 4B–4D). Thus, *unc-2* did not appear to affect either AVM differentiation or the guidance of its axon, but rather appeared to be important only for accurate migration of the AVM cell body. Analysis of *unc-2* mutant lines expressing an *unc-119::GFP* fusion, which labels most of the nervous system, indicated no obvious abnormalities in the locations of any of the other axon tracks, including the dorsally directed SDQR process (data not shown). Thus, *unc-2* appeared to be involved in cell migration but not axon guidance.

Interestingly, the guidance of the AVM axon did appear to require the activity of a different voltage-gated calcium channel, encoded by the *egl-19* gene. *egl-19* encodes a homologue of the L-type calcium channel α -1 subunit and has shown to be expressed in both muscles and neurons (Lee *et al.*, 1997). Mutations in *egl-19*, like mutations in *unc-2*, resulted in mispositioning of the AVM and SDQR cell bodies (Table 1). Since the penetrance of AVM/SDQR mispositioning defects in *egl-19*; *unc-2* double mutants was no higher than in the *unc-2* single mutant, the EGL-19 and UNC-2 calcium channels may promote guidance of these neuronal cell bodies through a common mechanism. However, unlike *unc-2*, *egl-19* also affected the guidance of the AVM axon (Figs. 4E–4H). For example, in some *egl-19* mutant animals, the ventrally directed portion of the AVM axon was observed to wander and enter the ventral cord at an abnormal location. In other animals, extra axons that extended in essentially random directions projected from the AVM cell body. These defects were seen in both loss- and gain-of-function *egl-19* mutants, suggesting that correct axon guidance requires a properly regulated calcium influx. The positioning of the ALM touch receptor neurons was also sometimes abnormal in *egl-19* mutants, a defect not seen in *unc-2* mutant animals. In addition, extra *mec-7*-expressing cells were occasionally detected in the anterior body region of *egl-19* mutant animals, suggesting that the decision to differentiate into a touch receptor cell was defective. Thus, the UNC-2 and EGL-19 calcium channels

TABLE 1

Penetrance of Migration Defects in Calcium Channel Mutants

Strain genotype	AVM misplacement	SDQR misplacement	<i>n</i>
N2	0%	0%	100
<i>unc-2(md1186)</i>	43%	18%	60
<i>unc-2(mu74)</i>	32%	12%	50
<i>unc-2(ra610)</i>	21%	11%	50
<i>unc-2(md1064)</i>	20%	6%	50
<i>unc-2(ra605)</i>	16%	10%	50
<i>unc-2(lj2)</i>	20%	8%	50
<i>unc-2(e55)</i>	20%	10%	50
<i>unc-2(lj1)</i>	20%	6%	50
<i>unc-2(md328)</i>	10%	2%	50
<i>egl-19(ad1006)</i>	16%	0%	32
<i>egl-19(ad1006); unc-2(mu74)</i>	37%	13%	52
<i>egl-19(n582)</i>	6%	5%	85
<i>egl-19(n582); unc-2(mu74)</i>	31%	6%	36

appear to play distinct roles in regulating the development of the AVM touch receptor neurons; whereas UNC-2 appears to affect only the migration of the AVM cell body, EGL-19 appears to control both cell migration and axon guidance.

Effects of *unc-2* on Other Neuronal Migrations

AVM and SDQR are not the only neurons in *C. elegans* that undergo directed migrations during development. As noted previously, the positioning of ALMR and ALML, which migrate posteriorly during embryogenesis, was unaffected by mutations in *unc-2*. Likewise, the positions of the HSN and CAN neurons, which migrate embryonically to the midbody region, were normal in *unc-2* animals. However, *unc-2* mutants did exhibit a defect, albeit at somewhat lower penetrance, in the positioning of two neurons undergoing postembryonic migrations, VC4 and VC5 (Fig. 5). The VCs, like SDQR and AVM, arise from migratory precursors, the neuroblasts P6.p and P7.p. During the first larval stage these cells migrate from the lateral epidermis into the ventral nerve cord, where they divide to generate the VCs and other adult motor neurons. In the fourth larval stage the VCs migrate out of the ventral cord and move along the A/P axis toward the vulva; subsequently, they extend axons that

egl-19(ad695sd), 16% (*n* = 85); and *unc-43(e755)*, 0% (*n* = 200). (A) A representative wild-type animal. (B–D) Examples of *unc-2* mutant animals. The AVM cell body is displaced in a dorsal (B), posterior/dorsal (C), or anterior/dorsal (D) direction from its normal position. In each case, the ventrally directed axon enters the ventral cord in its normal wild-type position (shown by the arrow). (E–H) Examples of *egl-19* mutant animals. E and F show *egl-19(ad1006)* loss-of-function mutants; G and H show *egl-19(ad695sd)* gain-of-function mutants. In E, the cell body of AVM is dorsal of its normal position, and two misdirected axons project from the neuron. In G and H, both the cell body and the axon of AVM are posteriorly mispositioned, and in G, an extra AVM axon is also observed.

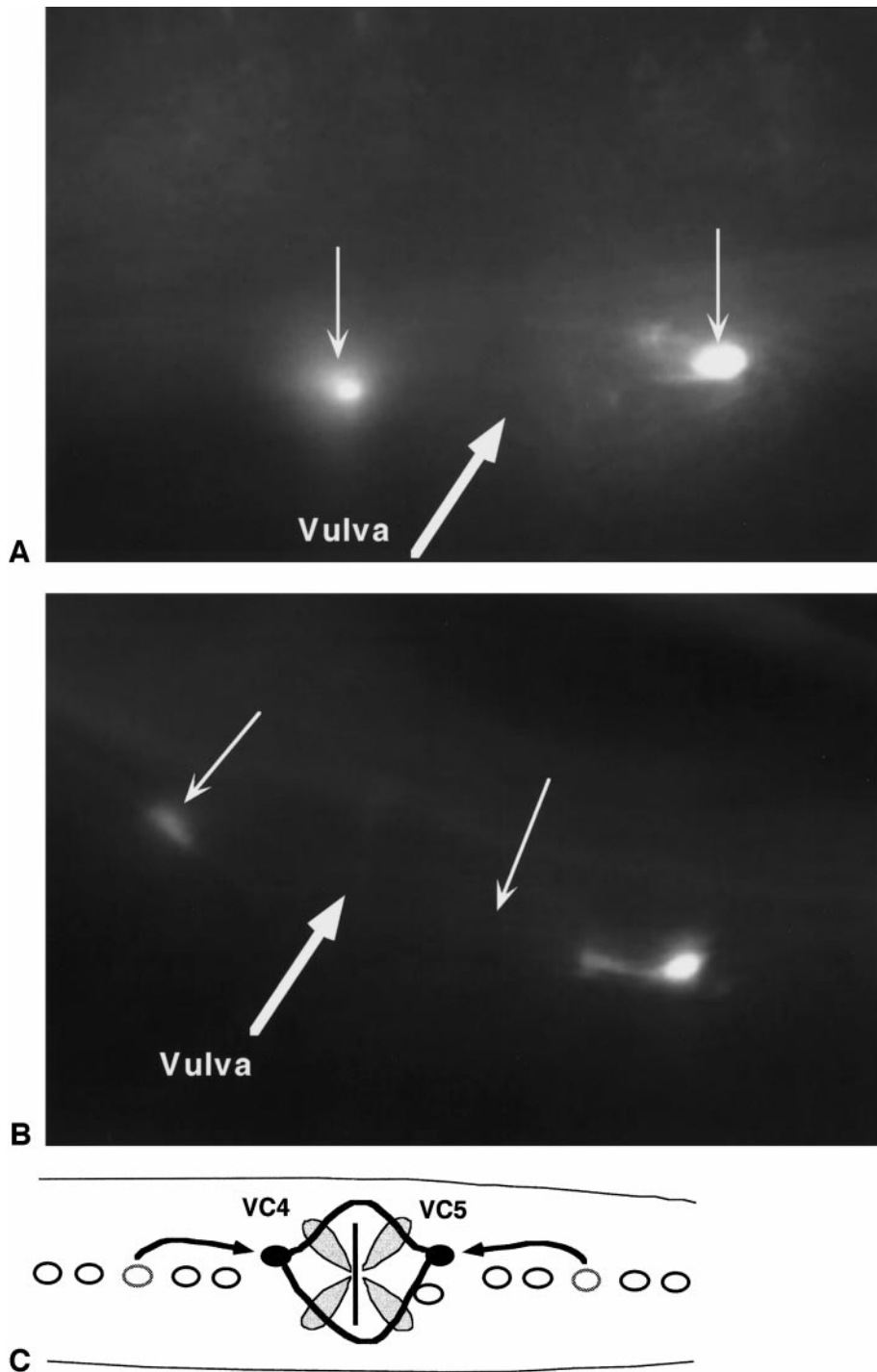


FIG. 5. Defects in positioning of VC4 and VC5 in *unc-2* mutants. Shown are fluorescence images of adult animals expressing the VC-specific marker *mab-5::GFP*. Left is anterior and top is dorsal. The thin arrows show the wild-type positions of VC4 (left) and VC5 (right); the position of the vulva is also indicated. (A) Positions in wild-type animals. VC4 and VC5 are found equidistant from the vulva on the anterior (VC4) or the posterior (VC5) side. (B) Positions in the *unc-2* mutant. In this animal, VC5 was found posterior to its normal position. The overall penetrance of VC misplacement phenotype in the mutant animals was 14% ($n = 100$). (C) Diagram of the migrations of the VC4 and VC5 neurons. Clear circles indicate the positions of the VC cell bodies following the division of the Pn.p neuroblasts; filled circles indicate their final positions.

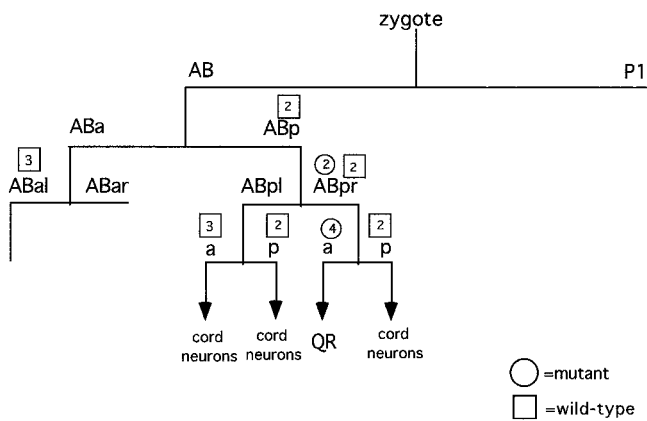


FIG. 6. Mosaic analysis of the *unc-2* cell migration defect. Shown is the cell lineage for the *C. elegans* hermaphrodite. Indicated are the precursors for QR (the progenitor of AVM and SDQR) and selected other cells in the animal. Mosaic animals were generated as described. The precursors of clones in mosaic animals are indicated by boxes or circles; circles indicate mosaics with misplaced AVM and/or SDQR cells, and boxes indicate mosaics with normally placed neurons.

innervate the vulval muscles. In about 15% of the mutant animals, the positions of the VC cell bodies were abnormal; although the cell bodies were in the general region of the ventral cord, they were displaced along either the A/P or the D/V axis. However, even in animals with misplaced VC cell bodies, the patterns of the VC axons were normal. Thus, the UNC-2 channel appeared to play a role in VC development similar to its role in AVM and SDQR: it was dispensable for precursor migrations and for axon guidance, but functioned specifically to direct the final migrations of the neurons themselves.

UNC-2 May Function within the Migrating Neurons

Where does the UNC-2 gene product function to direct these neuronal migrations? In principle, the UNC-2 calcium channel could act within the migrating neurons to direct their final migrations; alternatively, UNC-2 might function within cells that provide a guidance signal to the migrating cells. The coding regions of calcium channel genes are very large; thus, it has not been practical to address the cellular site of action of *unc-2* or *egl-19* through ectopic expression with cell-type-specific promoters. To address this question in an alternative way, we performed mosaic analysis (Herman, 1984) to determine the cellular focus of the *unc-2* cell migration phenotype. Genetic mosaics that were mixtures of wild-type and *unc-2* mutant cells were obtained using a strain with *unc-2(md1186)* mutant alleles on its chromosomes and a wild-type allele carried on a free duplication. Mitotic loss of the duplication led to clones of genetically mutant cells in an otherwise

wild-type organism, which could be scored using the cell-autonomous marker *osm-5* (Schafer and Kenyon, 1995). Mosaic animals were then analyzed for mispositioning of the AVM and SDQR cell nuclei (Fig. 6). Interestingly, these analyses suggested that the neuronal migration phenotype of *unc-2* was cell autonomous. Among 10 mosaics in which the descendants of QR were mutant for *unc-2*, 6 showed misplacement of either AVM or SDQR. In all these mosaics with misplaced AVM or SDQR cell bodies, the cells along which the neurons would be migrating (V1R and the body wall muscles) were wild-type for *unc-2*. Given the penetrance of the *unc-2* mutant phenotype, these results were consistent with the possibility that *unc-2* was required within the migrating neurons themselves. We also identified 8 mosaics in which the QR lineage was wild type for *unc-2*. All of these animals showed normal positioning of both AVM and SDQR; thus, we did not obtain any evidence supporting a site of action outside the QR lineage. Together, the results of the mosaic analysis were most simply explained by the hypothesis that UNC-2 functioned within the migrating SDQR and AVM neurons to direct their final migrations.

UNC-43/CaMII Kinase Is a Candidate Effector of the UNC-2 Calcium Channel

What molecules might function downstream of UNC-2 to facilitate guidance of AVM and SDQR? Since mutations in *C. elegans* synaptotagmin (*snt-1*; see Table 2) and RIM (*unc-10*; not shown) had no effect on AVM or SDQR migrations, we reasoned that the effects of UNC-2 on neuronal migration were probably not a consequence of its general effects on synaptic activity. One plausible candidate for an UNC-2 target was the product of the *unc-43* gene, which encodes a *C. elegans* homologue of the calcium-sensitive signaling molecule CaM kinase II (Reiner *et al.*, 1999). CaM kinases mediate the effects of calcium channels on gene expression in diverse organisms, including *C. elegans* (Troemel *et al.*, 1999). To determine if UNC-43 might function downstream of UNC-2 in the migrating

TABLE 2
Migration Defects in UNC-43/CaM Kinase Mutants

Strain genotype	AVM misplacement	SDQR misplacement	<i>n</i>
N2	0%	0%	100
<i>unc-43(e755)</i>	24%	6%	50
<i>unc-43(n498n1179)</i>	16%	0%	50
<i>unc-43(n498dm)</i>	15%	7%	30
<i>unc-2(mu74)</i>	32%	12%	50
<i>unc-43(e755); unc-2(mu74)</i>	29%	6%	45
<i>snt-1(md290)</i>	0%	0%	50
<i>snt-1(md325)</i>	0%	0%	50

AVM or SDQR cells, we analyzed the final positions of these neurons in *unc-43* mutant animals (Table 2). In three different *unc-43* mutants (two recessive and one dominant), we observed defects in SDQR and AVM positioning that were similar to those seen in *unc-2* recessive mutants: the QR.pa cell reached approximately the correct A/P position during the initial phases of migration, but the final migration of AVM and SDQR themselves were often misdirected. As with *unc-2* mutants, AVM was more often mispositioned than SDQR in *unc-43* animals; the overall penetrance of the migration defect was also comparable to that of the stronger *unc-2* alleles. Moreover, *unc-43* mutations, like *unc-2* mutations, did not affect the guidance of the AVM axon as visualized with *mec-7::GFP* (see Fig. 4 legend). When we analyzed the phenotype of an *unc-43; unc-2* double mutant, we observed no significant increase in penetrance relative to the *unc-2* single mutant (Table 2). This suggested that UNC-43 affected the same aspect of the AVM/SDQR migration process as UNC-2 and was consistent with the possibility that UNC-2 affected AVM and SDQR migration through an UNC-43/CaMII kinase pathway.

DISCUSSION

The Role of Calcium Channels in SDQR and AVM Development

We have found that loss-of-function mutations in the calcium channel gene *unc-2* cause specific defects in the development of two postembryonic neurons, AVM and SDQR. The development of these neurons involves three general stages, all of which require response to localized guidance cues. In the first stage, the progenitor of AVM and SDQR, QR.pa, arrives in the anterior body region as a result of a series of anteriorly directed migrations involving QR.pa itself and its ancestors QR.p and QR. These migrations are independent of *unc-2*, since QR.pa always divided at its normal position in *unc-2* mutants. In the second stage, QR.pa divides, giving rise to AVM and SDQR, which migrate in opposite directions (ventrally for AVM, dorsally for SDQR) to their final positions in the animal. *unc-2* is important for both of these migrations, as the cell bodies of either or both neurons were frequently mispositioned in *unc-2* mutants. Moreover, *unc-2* may play a role in regulating the cell division that generates AVM and SDQR, since *unc-2* animals can either contain extra SDQR-like or AVM-like cells or, alternatively, appear to contain an undivided QR.pa cell in the mature animal. Finally, in the third stage, AVM and SDQR extend axons; SDQR's axon extends anteriorly and posteriorly in the dorsal sublateral nerve, while AVM's axon first extends ventrally, then anteriorly in the ventral nerve cord. UNC-2 appears to be dispensable for axonal pathfinding, at least in the case of AVM; however, another *C. elegans* calcium channel, the L-type calcium channel homologue *egl-19*, appears to be involved in proper guidance of the AVM growth cone. *egl-19* has been shown

previously to affect the guidance of *C. elegans* olfactory receptor axons (Peckol et al., 1999); thus, EGL-19-mediated calcium influx may play a general role in the regulation of axon guidance throughout the *C. elegans* nervous system.

The mosaic analysis presented here argues that the UNC-2 calcium channel probably functions within the migrating neurons themselves. Thus far, we have not been able to reliably detect *unc-2* expression using either GFP reporter constructs or specific antisera, perhaps because *C. elegans* neurons may contain only a small number of calcium channels (Goodman et al., 1998). However, recent genetic studies suggest that *unc-2* probably functions in many cells of the nervous system (Berger et al., 1998; Miller et al., 1996; Rongo and Kaplan, 1999; Schafer and Kenyon, 1995; Troemel et al., 1999). Our mosaic analysis indicates that functional UNC-2 protein must be expressed within the lineage that generates AVM and SDQR for these cells to migrate properly. Since the most limited mutant clones contained almost exclusively neurons and epidermal cells that lie in other regions of the animal, the most plausible sites of action for *unc-2* within these clones are AVM and SDQR themselves. Given the penetrance of the phenotype and the number of mosaics found, we cannot rule out a requirement for *unc-2* in other cells as well, although we obtained no evidence in support of such a hypothesis. Taken together, the simplest explanation of our mosaic data is that UNC-2 functions cell autonomously within the migrating neurons themselves.

Multiple Mechanisms Control AVM and SDQR Migration

unc-2 is a member of a large group of genes that affect the positioning of AVM and/or SDQR. Some of these genes affect the migrations of the precursors of these neurons, the neuroblasts QR, QR.p, and QR.pa. Among these are Hox genes that specify Q cell identity (Clark et al., 1993; Salser and Kenyon, 1992; Wang et al., 1993), the small GTPase *mig-2* (Zipkin et al., 1997), and *mig-13*, which encodes a receptor that functions outside the migrating cells to control the stopping point of QR.pa (Sym et al., 1999). Another set of genes that control both cell migration and axonal guidance in SDQR has been identified. The products of these genes, *unc-6*, *unc-5*, and *unc-40*, function in a netrin-based process that directs both the final dorsally directed migration of SDQR and the dorsal navigation of its axon (Kim et al., 1999). *unc-5*-encoded netrin receptors in SDQR apparently mediate a repulsive response away from *unc-6*-encoded netrin ligand in the ventral nerve cord, which serves to shift the SDQR cell body dorsally from its place of birth. However, the netrin genes do not appear to be involved in the anteroposterior positioning of SDQR. Moreover, in the absence of netrin function, SDQR actually migrates ventrally, suggesting that an UNC-6-independent guidance cue may serve to ventrally bias the final position of SDQR. On the basis of these observations, it has been proposed that in addition to the netrin pathway, one or

more netrin-independent pathways also participate in the final positioning of SDQR (Kim *et al.*, 1999). Although *unc-2* also affects the SDQR migration, its phenotype is distinct from that of *unc-5* and *unc-6*: *unc-2* affects both A/P and D/V positioning of both AVM and SDQR, and it specifically affects cell migration but not axon guidance. Taken together, these results suggest that *unc-2* probably defines a new, netrin-independent pathway involved in guiding the final migrations of SDQR and AVM.

Regulators and Effectors of UNC-2 Signaling in Migrating Neurons

The molecular mechanisms through which voltage-gated calcium channels direct cell motility are not well understood. We have identified at least one putative effector of *unc-2* in directing AVM and SDQR migration—the CaM kinase encoded by *unc-43*. Both gain- and loss-of-function alleles of *unc-43* caused SDQR/AVM mispositioning phenotypes similar to those seen in *unc-2* mutants. Moreover, the phenotype of an *unc-43; unc-2* double mutant was no more severe than that of either single mutant, consistent with the hypothesis that the two genes affect a common process. CaM kinases have been shown to couple calcium influx through voltage-gated calcium channels to intracellular signaling pathways in other neurons. For example, in sensory neurons of *C. elegans*, UNC-43 CaM kinase has been shown to mediate the effects of the UNC-2 calcium channel on cell-type-specific gene expression (Troemel *et al.*, 1999). UNC-43 also appears to act downstream of UNC-2 in the regulation of glutamate receptor clustering in motor neurons (Rongo and Kaplan, 1999). Thus, UNC-43/CaM kinase is well suited to couple the UNC-2-mediated calcium influx to the activities of downstream target proteins that might promote and regulate cell motility. Interestingly, in vertebrate and *Drosophila* neurons, CaM kinase appears to function downstream of L-type voltage-gated calcium channels to direct the turning of migrating growth cones (VanBerkum and Goodman, 1995; Zheng *et al.*, 1994). However, *unc-43* mutants did not exhibit the AVM axon defects seen in worms with mutations in the *C. elegans* L-type channel homologue *egl-19*. Thus, the EGL-19-dependent calcium influx that helps guide the AVM axon probably acts through a different signaling mechanism.

Perhaps surprisingly, both loss-of-function and gain-of-function alleles of *unc-43* led to misdirection of the AVM and SDQR migrations. In fact, the nonsense allele *e755*, which should produce no functional CaM kinase, and the dominant allele *n498*, which produces a calcium-independent kinase (Reiner *et al.*, 1999), both caused random mispositioning of both SDQR and AVM with about 20% penetrance. Why might these phenotypes be so similar? One possibility is that the differential activity of UNC-43 CaM kinase in different regions of the migrating cell might be used to establish some form of cell polarity that helps orient the cell during its migration. In this case, either a lack of kinase activity or a uniformly high level of

kinase activity would make the cell blind to this polarizing signal and might lead to randomly misdirected movement. Interestingly, both gain- and loss-of-function mutations in *unc-43* were also observed to disrupt localization of glutamate receptors in motor neurons (Rongo and Kaplan, 1999), implying that regulated kinase activity may be necessary for this process as well.

Many questions remain concerning the mechanism through which the UNC-2 calcium channel directs neuronal migration. For example, the guidance cues that control the UNC-2-dependent migration pathway also remain to be identified. Voltage-gated calcium channels are controlled by both membrane potential and by G-protein-mediated signaling pathways. It is possible that UNC-2 might influence cell migration in response to an electrical signal, perhaps induced by the opening of ligand-gated ion channels. The spontaneous electrical activity of these neurons during this early stage of their development might also serve as a cue for establishing cell polarity involved in directed cell migration. Alternatively, a number of neuronal chemoattractants, such as the neurotransmitters acetylcholine and glutamate, are known to act through G-protein-coupled receptors. Thus, localized modulation of UNC-2 channels in AVM and SDQR by G-protein pathways could provide a possible mechanism for guiding the direction of these cells during their migration.

Calcium Channels and Neuronal Guidance in Other Organisms

Our observations that the UNC-2 and EGL-19 calcium channels function as regulators of neuronal migration and axonal pathfinding in *C. elegans* touch receptors have interesting parallels in other systems. The role of L-type (i.e., EGL-19-like) calcium channels in guiding neuronal growth cones has been established in both vertebrate and invertebrate neurons (Ming *et al.*, 1997; Song *et al.*, 1998; VanBerkum and Goodman, 1995; Zheng *et al.*, 1994). Though less extensively studied, there is also precedent for N-type (i.e., UNC-2-like) calcium channels controlling the motility and guidance of neuronal cell bodies. Most notably, in the mammalian cerebellum, the migration of granule cells from the premigratory zone into the granule layer is dependent on calcium influx through voltage-gated channels (Komuro and Rakic, 1996). Studies using subtype-specific inhibitors have demonstrated that these migrations depend on activation of N-type calcium channels, the closest vertebrate homologue of the UNC-2 channel (Komuro and Rakic, 1992). In cerebellar granule cells, calcium influx through N-type channels appears to promote cell motility per se, since N-type channel blockers prevent cell movement, whereas an increase in calcium influx speeds the rate of migration. Misplacement of the AVM or SDQR neurons in *unc-2* mutant animals also often resulted from a failure of the cell to migrate, although mutant animals also occasionally exhibited an improperly directed migration. Thus, it is possible that the mechanisms

through which voltage-gated calcium influx controls neuronal migration in worms and vertebrates are at least partially conserved. Further genetic analysis of SDQR/AVM migration in *C. elegans* may provide a useful approach for identifying molecules that participate generally in the control of cell movement by calcium.

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