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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Neuronal Migrations in *Caenorhabditis elegans*: Insights into the Molecular Mechanisms of Migration

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy in

Biology

By

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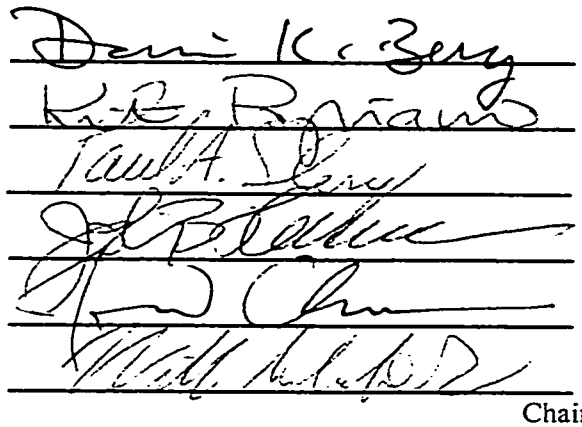

Chair

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2001

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ABSTRACT OF THE DISSERTATION

Neuronal Migrations in *Caenorhabditis elegans*:

Insights into the Molecular Mechanisms of Migration

by

Tobey M. Tam

Doctor of Philosophy in Biological Sciences

University of California, San Diego, 2001

Professor William R. Schafer, Chair

During development, neurons receive numerous external signals that act as cues for particular cellular responses. These early developmental responses aid neurons in migrating to their proper destinations. Studies have identified signaling molecules, their receptors and cytoplasmic signal transduction pathways mainly for migrating growth cones. Although calcium has been known to be important in regulating many behaviors of grown cones, a specific signaling pathway for calcium in migrating cells has not been discovered until now.

We have shown that *unc-2*, a gene that encodes the α-1 subunit of a homologue of vertebrate non-L type voltage-activated calcium channel, participates in the correct final migrations of the neuronal Q descendants AVM and SDQR. Mosaic analysis and cell lineaging seem to indicate that *unc-2* is spatially activated during the last division of QR.pa, which gives rise to AVM and SDQR. *unc-2* is necessary for the proper cell migrations of AVM and SDQR, but is not required for axonal guidance in AVM neurons. Interestingly,

egl-19, the homologue of the L-type calcium channel α-1 subunit, is required for both cell migration and axonal guidance. Downstream of *unc-2* is *unc-43*, a homologue of the calcium-sensitive signaling molecule CaM kinase, which displays the same function in cell migration and axon pathfinding as *unc-2* and may function as an effector of the calcium channel.

We have also identified serotonin as a signaling molecule that directs proper neuronal migrations in AVM and SDQR. Mutants defective in serotonin synthesis and rescue of the cell misplacement phenotype in these mutants by exogenous serotonin revealed it as a guidance molecule. Another mutant gene that displays the same misplaced Q phenotype and genetically appears to lie upstream of *unc-2* is *goa-1*, which encodes a homolog of an α subunit of the heterotrimeric guanosine triphosphate-binding protein Go. Through our studies of a *C. elegans* calcium channel, we have identified components in a signaling pathway that directs proper neuronal cell migration.

CHAPTER I INTRODUCTION

A functioning nervous system depends on the formation of proper connections between neurons and the right positioning of those neurons within an organism. Part of this organization is the result of neurons arriving in locations different from the ones in which they arise in the developing animal. Thus accurately directed cell migrations are necessary in bringing these neurons to their ultimate destinations. Many molecular processes have been found to be involved, including selection of migratory pathways by cell recognition receptors, integration of multiple cues from external and internal factors, and formation of adhesive interactions with cellular and extracellular substrates leading to neuronal motility and ultimately the proper connections in the correct location. An important issue in the study of neuronal guidance is understanding how intracellular signalling pathways regulate the guidance mechanism of cells. Learning how migrating neurons orchestrate these multiple events is critical in the study of developing higher brain functions.

Although a great deal has been learned recently about signal transduction pathways involved in axon guidance, the mechanisms involved in directing neuronal cell migration are less well characterized. In vitro studies have identified factors involved in controlling the timing and direction of neuronal migration. However, experiments that would shed light on the role of these molecules within the context of a developing nervous system have proven difficult to carry out. In vivo experiments would entail the manipulation of mobile neurons, a feat that is currently unfeasible in many interesting organisms, including humans. One solution would be to use genetically tractable organisms as tools. The invertebrate *Caenorhabditis elegans* has been demonstrated to be such an organism. This nematode has a relatively simple nervous system, precise

cell positioning and defined cell lineage. Individual neurons are accessible to ablation, generation time is short, and phenotypes are readily distinguishable. Investigations with this model may provide insight into the complex pathways bound to exist in higher order animals.

This dissertation will introduce a signalling model for neuronal migration in *C. elegans*. The following introduction will first summarize migrational studies focusing on guidance cues and their receptors and cytoplasmic signal transduction pathways in various systems, then provide an overview of neuronal migrations in *C.elegans*.

A. OVERVIEW OF NEURONAL MIGRATION

The processes underlying both migrating neurons and extending growth cones share a few features in common. Because much more has been learned about signal transduction pathways in growth cone guidance than in neuronal cell migration, some background knowledge of growth cone research will be helpful in appreciating the current understanding of cell migration.

1. Guidance Cues and their Receptors

Over a century ago Ramon y Cajal suggested the idea of chemoattraction mediating axon guidance from his studies on leucocytes (y Cajal, 1892). He imagined growth cones to be a guide, sensitive to environmental chemicals yet forceful when moving through rough terrain in order to rendezvous at the appropriate final destination at the correct time. He wondered "what mysterious forces precede the appearance of these prolongations, promote their growth and ramification, and finally

establish the protoplasmic kisses, the intercellular articulations that appear to constitute the final ecstasy of an epic love story."

We now know that pathfinding growth cones, as well as migrating neurons, are directed by specifically localized guidance molecules found in their paths, serving as positional cues within the developing nervous system. These factors include netrins, semaphorins and members of the slit gene family.

Netrins

Netrins are a small family of proteins that have been shown in vitro to act as chemotropic molecules for commisural growth cones in the developing spinal cord (Tessier-Lavigne *et al.*, 1988 #). They consist of a number of bidirectional guidance molecules, which can act either to attract or repel axons (Culotti *et al.*, 1996). Netrins are diffusible molecules whose motility may be hampered by interactions with cell surfaces or the ECM (Kennedy *et al.*, 1994 and Serafini *et al.*, 1994). In vertebrates, netrin-1 and netrin-2 attract commissural axons of the embryonic spinal cord. However, in vitro assays show netrin-1 to be capable of repelling the same axons when levels of cyclic AMP are low. There also seems to be a functional conservation of such molecules in molecular mechanisms of growth cone guidance between species (Goodman *et al.*, 1994).

Semaphorins

Semaphorins are a large family of cell-surface and secreted proteins that seem to function mainly as chemorepellents or inhibitors (Mueller *et al.*, 1999). There are at least six classes, based on sequence similarity. Depending on the family member, distinct guidance information can be given to the growth cone (Isbister *et al.*, 1999). Class I, IV, V and VI are transmembrane proteins while class II and III are secreted

proteins (Mark *et al.*, 1997 and Kikuchi *et al.*, 1997). The different classes of semaphorins are able to influence growth cone guidance in a contact-dependent manner, as long-range chemorepulsive cues, and (as in vitro studies suggest) as attractive guidance cues (Song *et al.*, 1999).

Slit-robo

In vertebrates and invertebrates, studies have shown that axons arriving at the midline receive cues so as to ensure no inappropriate crossing. In vertebrates and *Drosophila,* midline axons cross the midline before joining into two separate bundles that are aligned with the midline bilaterally. In *Drosophila*, two antagonistic genes aid as guidance cues for this crossing, roundabout (*robo*) and commissureless (*comm*) (Seeger *et al.*, 1993). Robo encodes a transmembrane protein that functions as a receptor whose activity inhibits midline crossing. Comm encodes a novel transmembrane protein that promotes midline crossing (Tear *et al.*, 1996).

In *Drosophila,* Slit encodes an extracellular matrix protein which is secreted by midline glial cells and acts as the ligand for Robo (Kidd *et al.*, 1999). Homologs for slit and robot have been discovered in vertebrate and biochemical studies demonstrate their interaction in these organisms as well (Brose *et al.*, 1999 and Li *et al.*, 1999). These vertebrate homologs have also been shown to mediate chemorepulsion of foreign axons (Ba-Charvet *et al.*, 1999) as well as promoting sensory axon elongation and branching (Wang *et al.*, 1999).

2. Cytoplasmic Signal Transduction

Investigations of intracellular signaling pathways in growth cones have delineated the roles of cyclic nucleotides, Rho GTPases, and calcium in neuronal guidance.

Cyclic Nucleotides

Two distinct categories of cyclic-nucleotide-dependent signaling pathways each involve a separate group of guidance cues for growth cone motility and axonal guidance. Cyclic AMP-dependent pathways involve guidance cues from group I, which include BDNF, NGF, netrin-1, ACh and MAG. Although these pathways involve different receptors, they share common downstream pathways, including those involving calcium, PI-3 kinase and PLC-gamma (Ming *et al.*, 1997, Song *et al.*, 1998, Song *et al.*, 1997, and Ming *et al.*, 1999). Pathways for the other cyclic nucleotide, cyclic GMP, play a similar role to cAMP in mediating the response of growth cones and use the group II molecules semaphorinIII and NT-3. These pathways are calcium independent as well as PI-3 kinase independent (Song *et al.*, 1998 and Ming *et al.*, 1999). Changing the level of cyclic nucleotide leads to either attraction or repulsion. Since these cyclic nucleotides serve as second messengers for many cell surface receptors (Cooper *et al.*, 1996, Laufer *et al.*, 1987, and Fong *et al.*, 1996), other factors influencing growth cone motility may be critical.

Rho GTPases

Rho, a member of the Ras superfamily of small guanosine triphosphatases (GTPases), has been shown to regulate cytoskeletal dynamics in fibroblasts (Ridley *et al.*, 1992) and can affect cell migration and axon outgrowth in vivo (Luo *et al.*, 1994). Three members of the Rho family, Cdc42, Rac and Rho, have important roles in the formation of filopodia, lamellipodia, focal adhesions and stress fibers of fibroblasts. In *Drosophila,* activated or dominant negative forms of Drac1 lead to axon growth truncations while activated Dcdc42 inhibits both axon and dendrite outgrowth (Luo *et*

al., 1994). Other mutations in Rho family members also cause defects in axon guidance and cell migrations in vivo (Hall, 1998). Analysis of the three members has shown that Rho activates the signaling mechanisms which link membrane receptors to the cytoskeleton, Rac is a key element in control of the reorganization of the actin cytoskeleton, and Cdc42 is required for cytoskeletal polarization (Ridley *et al.*, 1992, Ridley *et al.*, 1993, and Johnson *et al.*, 1990).

Calcium Signaling

It has been established that calcium plays an important role in growth cone migration. Although there is no clear delineated pathway through which growth cones behave, there have been important studies that have helped identify calcium as an important second messenger.

Spontaneous or induced calcium spikes slow migration in growth cones displaying the regulatory role of calcium (Gomez *et al.*, 1995). The rate of neurite expansion has also been shown to be inversely proportional to the frequency of calcium transients (Gomez *et al.*, 1999). The turning responses of growth cones to guidance molecules such as netrin-1 and BDNF are mediated through influx of extracellular calcium through L-type voltage-gated channels (Ming *et al.*, 1997 and Song *et al.*, 1998). More evidence of the importance of calcium is from studies involving IP3-mediated calcium release which plays a role in regulating nerve growth (Takei *et al.*, 1998). A few studies involving inhibition directly or through the regulation of calmodulin-dependent kinase (CaM kinase) have been shown to deviate axonal pathfinding through calcium signaling (VanBerkum *et al.*, 1995 and Zheng *et al.*, 1994).

3. Cellular Migration

Several key components in the signaling transduction pathways of extending growth cone axons have been identified. As mentioned previously, little is known about the mechanisms involved in directing neuronal cell migrations. However, from a few key studies it is clear that the regulation of intracellular calcium plays an important role in neuronal motility.

Experiments in mouse cerebellar microexplant cultures demonstrate that calcium fluctuations are correlated to the rate of cell movement (Komuro *et al.*, 1996). Real-time recordings of intracellular calcium correlated individual fluctuations of intracellular calcium concentrations to saltatory movement of migrating neurons. Influx of extracellular calcium appears to be necessary for neuronal motility. N-type voltage-gated calcium channels and NMDA receptors are also necessary in mediating calcium influx in these cells (Komuro *et al.*, 1992 and Komuro *et al.*, 1993).

Another example of calcium's role in neuronal cell migration comes from retarded migrations of acoustico-vestibular neurons from the chicken hindbrain after calcium channel blockers were added to cultures. W-conotoxin GVIA, a blocker of N-type calcium channels, slowed neuronal migration, indicating that N-type calcium channels and an influx of extracellular calcium may be involved in cellular migration (Hendriks *et al.*, 1999).

In the enteric neurons of *Maduca sexta*, increasing or buffering intracellular calcium inhibits or leads to inappropriate cell migration, respectively. Through imaging intracellular calcium concentrations, migrating cells displayed small fluctuations in

calcium concentration. Inhibition of motility in enteric neurons is induced by activation of Go-alpha, which is calcium dependent (Horgan *et al.*, 1998).

In weaver mutations, where migration of the cerebellar granular neurons are defective and ultimately die during the first two weeks of postnatal life, the L-type calcium channel blocker verapamil rescues the lethal phenotype. This study and the one preceding support a role for calcium in inhibiting cell motility (Liesi *et al.*, 1996).

4. Calcium Channels

During the early stages of neuronal cell development, calcium channels have been shown to play a prominent functional role in neuronal cell migration (Komuro *et al.*, 1992), neural crest cell migration (Moran *et al.*, 1991) and growth cone migration (Kater *et al.*, 1991) above all other channel types.

It has already been mentioned that N-type calcium channels are known to mediate cell migration (Komuro *et al.*, 1992 and Hendriks *et al.*, 1999). However, N-type channels are typically thought to play more of a role in neurotransmitter release. L-type calcium channels are typically implicated in the regulation of growth cone movement, neurite extension and neural crest cell migration (Hess *et al.*, 1990). Differences in the ratio of N-type and L-type calcium channels have been seen between in vitro and in vivo experiments and between mature and immature neurons, an observation which may explain the discrepancy. The variety and abundance of different calcium channel types may also change during translocation to the final destination of migrating neurons. Most importantly, blockade of N-type calcium channels does not inhibit cell movement but rather slows it down, hinting at the participation of other calcium

channels in the influx of extracellular calcium (Komuro *et al.*, 1992 and Hendriks *et al.*, 1999).

In the studies of Komuro *et al.*, mouse cerebellar granular neuron migrations are affected only by N-type calcium channel blockers, whereas, Liesi et al demonstrates that the same neurons are affected only by L-type blockers. It has been suggested that the differences between neuronal age, P7 verses P10, in the separate studies could explain the discrepancy (Liesi *et al.*, 1999). These results would support the idea that at distinct ages, neurons express different calcium channel subtypes with different pharmacological properties.

It may also be possible that calcium channel type, in regards to neuronal migration, is species specific since there is evidence of L-type channel participation in neural crest cell migration in salamanders (Moran *et al.*, 1991).

B. OVERVIEW OF NEURONAL MIGRATION IN NEMATODES

During *C.elegans* embryogensis, most cells move very short distances. However, twelve distinct cells travel significant lengths from their original birthplace. Among these nomadic cells are the M mesoblast, the gonad precursors Z1-Z4, the sex myoblasts (SMs), and the neuroblasts HSN, CAN, ALM and the contralateral homologs QL and QR and their daughter cells (Sulson *et al.*, 1983).

1. Q cells

Much research has been done on the neuronal Q cell migrations. The Q cells and their descendants migrate to precise positions in a selective region of the body. QL and QR are born in the same anterior/posterior axis but on opposite sides of the body: QL

on the left, QR on the right. They both undergo an identical sequence of cell divisions to give rise to a mechanosensory neuron, an interneuron, a ciliated neuron and two cells programmed for cell death. Interestingly, these two cells and their descendants migrate in opposite directions: QL posteriorly and QR anteriorly (Sulton *et al.*, 1977). This asymmetrical migration of bilateral homologues has been a focus of research performed in studying neuronal cell migration in *C. elegans*.

QL, the Hox genes, and the Wnt signaling pathway

In *C.elegans*, body pattern specification along the anterior/posterier (A/P) body axis is predominately under the control of a conserved cluster of Hox genes. Three Hox genes are expressed in broad domains along the A/P axis and function to specify cell identity in those regions. These C. elegans Hox genes are homologus to their Drosophila and vertebrate counterparts: *lin-39* specifies the mid-body region, a *Sex combs reduced* homolog; *mab-5*, specifies the posterior body region, an *Antennapedia* homolog; and *egl-5*, specifies the tail region, an *Abdominal-B* homolog.

mab-5 is the switch that controls the direction of the Q cell migrations. In QL, mab-5 is "on," hence it and its descendants migrate posteriorly. In QR, mab-5 is "off" and as a result it and its descendants migrate anteriorly. QR and its descendants can be programmed to migrate posteriorly like QL, when a heat-shock-mab-5 fusion gene is activated in QR or in gain-of-function mutants of mab-5 (Salser *et al.*, 1992). Likewise, in mab-5 loss-of-function mutants QL and its descendants adopt their new fate and migrate anteriorly (Chalfie *et al.*, 1983).

A Wnt signaling pathway is responsible for mab-5 expression in QL. This pathway includes egl-20, a Wnt homolog, lin-17, a Wnt receptor homolog, and bar-1, a B-catenin homolog. Mutations in any of the three genes result in no expression of mab-5

in QL. pry-1, a negative regulator of the Wnt pathway, limits the expression of the Hox genes to their particular domains so that in pry-1 mutants mab-5 is ectopically expressed in QR (Maloof *et al.*, 1999).

2. QR and genes that regulate its migration

As mentioned before, QR undergoes a sequence of cell divisions identical to QL. The daughters of QR are AQR, SDQR and AVM. QR.pa divides to give rise to the sister cells SDQR, an interneuron that migrates dorsally, and AVM, a touch neuron that migrates ventrally. AVM is one of three touch neurons that mediate reversal responses to touch on the anterior body. The exact role for SDQR has yet to be determined.

The existing information on the migration of QR is quite fragmentary and no definitive picture can be drawn. Predominately through genetic screens in search of genes disrupting cell migration or axonal guidance have players been identified in modulating QR and its descendants trajectories. The genes involved participate in dorsoventral guidance, extracellular matrix molecules, and regulators of actin cytoskeleton (Garriga *et al.*, 1994, Hedgecock *et al.*, 1987 and 1990, and Forrester *et al.*, 1997). Most of these genes regulate the early migration of QR and its descendants as they travel anteriorly from their birthplace.

Netrins and other migration factors

The *C. elegans* netrin guidance molecule, UNC-6, and its netrin receptors, UNC-5 and UNC-40, pattern the dorsal/ventral axis for proper migration of SDQR, daughter of QR, as it reaches its proper location. These netrin molecules govern the late stage migration of QR's descendants. Neuroglia and pioneer neurons express UNC-6, which is spatially restricted to the ventral midline. Migrating cells that express UNC-5, a

receptor belonging to the immunoglobulin superfamily, are repelled by the UNC-6 ventral source and move dorsally. Migrating cells that express UNC-40, a *C. elegans* homolog of the vertebrate protein encoded by *Deleted in Colorectal Cancer* (DCC), are attracted to the netrin source. The dorsal movement of SDQR is dependent on the UNC-5 receptor and is moderated by UNC-40 (Kim *et al.*, 1999).

Another netrin-like gene is unc-129, which encodes a member of the transforming growth factor-B (TGF-B) superfamily of secreted signaling molecules and mediates dorsoventral polarity for axon guidance. Expression of unc-129 is localized in the dorsal body wall muscles and ectopic expression causes misrouting of AVM axons, a daughter QR and sister cell to SDQR.

Genes that regulate the early migrations and/or axonal guidance of QR and its descendants include sax-3, an ortholog of the *Drosophila* Roundabout transmembrane receptor (Zallen *et al.*, 1998), epi-1, a laminin alpha chain homolog, ina-1, an alpha integrin subunit (Forrester *et al.*, 1997), pat-3, a beta integrin subunit (Gettner *et al.*, 1995), unc-73 and mig-2, genes related to a guanine nucleotide exchange factor for the Rho subfamily of Ras related G-proteins.

Although a lot of information is known about the neuroblast precursors of AVM and SDQR, not much is known about the migration of the developed neurons. In this thesis we will provide insight into the role of calcium and the neurotransmitter serotonin in the proper positioning of the neurons AVM and SDQR.

CHAPTER II VOLTAGE-GATED CALCIUM CHANNELS DIRECT NEURONAL MIGRATION IN *CAENORHABDITIS ELEGANS*

ABSTRACT

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 post-embryonic neuronal migrations in the nematode *C. 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.

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 different part of the organism 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 detection 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; Komuro and Rakic, 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 post-embryonic neuronal migrations in the nematode *C. 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.

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 (Figure 1-1). All six 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 (Figure 1-1). 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 post-embryonic 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 (Figure 1-2a). First, QR and its descendants QR.p and QR.pa migrate along the anteroposterior axis from an original position in the mid-body 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 (Figure 1-2b). 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 extra neuronal nuclei near the final positions of SDQR and/or AVM, suggesting that extra cell divisions in the QR.pa lineage occurred (see Figure 1-3). 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 strongest alleles (*md1186*, and *mu74*) was 20-40% for AVM and 10-20% for SDQR. 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 stage in Q cell development, beginning roughly from the time from 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 (Figure 1-3). 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.

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 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 (Figure 1-4), 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 (Figure 1-4b-d). Thus, *unc-2* did not appear to affect either AVM differentiation or the guidance of its axon, but rather appeared to be required 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 misposiitoning of the AVM and SDQR cell bodies (Table 1-1). 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 (Figure 1-4e-h). 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 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 mid-body 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 post-embryonic migrations, VC4 and VC5 (Figure 1-5). 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 motorneurons. 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

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 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 similar role in VC development 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, 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 (Figure 1-6). 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? One plausible candidate 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 AVM or SDQR cells, we analyzed the final positions of these neurons in *unc-43* mutant animals (Table 1-2). 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 Figure 1-4 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 1-2). 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 post-embryonic 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 contain either 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 can not 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 have been identified that control both cell migration and axonal guidance in SDQR. 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. The ability of CaM kinase to phosphorylate target proteins is regulated by calcium, since calcium activated CaM kinase both directly and indirectly through calmodulin. 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 motorneurons (Rongo and Kaplan, 1999). Thus, UNC-43/CaM kinase is well suited to couple the UNC-2-mediate 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 *e753*, 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 motorneurons (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.

MATERIALS AND METHODS

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

Isolation of *unc-2* mutants. The deletion alleles *lj1* and *lj2* were isolated in a non-complementation screen for *unc-2* mutations. Hermaphrodites of 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 ethylmethanesulfonate 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. 11 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 base pairs. 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 nonsense strand of *unc-2* were used to amplify the genomic DNA. The p618-EM56 (sequence 5'-TCATCCATCTCTTCCACC-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 Corporation).

Construction and characterization of double mutants. Double mutants carrying *unc-2* and an integrated GFP reporter construct were generated using standard methods. 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 minute time window. These animals were then grown for 5-6 hours on NGM at 20° C. Worms were then mounted on 2% agarose pads and observed for 4 hours 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,

PHBR) sensilla. The *osm-5* phenotype was scored as described (Herman, R.K. Genetics 108); briefly, animals were stained with DiI on 2% agar plates, and staining of the amphid and phasmid cells was visualized by fluorescence.

This chapter is, in full, a reprint of material in T. Tam et al. (2000) "Voltage-gated calcium channels direct neuronal migration in *Caenorhabditis elegans*" *Developmental Biology* **226**, 104-117. This dissertation author was the primary investigator and author of this paper.

Table 1-1: Penetrance of migration defects in calcium channel mutants

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

Table 1-2: Migration defects in unc-43/CaM kinase mutants

Strain genotype	AVM misplacement	SDQR misplacement	n=
N2	0%	0%	100
unc-43(e753)	24%	6%	50
unc-43(n498n1179)	16%	0%	50
unc-43(n498dm)	15%	7%	30
unc-2(mu74)	32%	12%	50
unc-43(e753); unc-2(mu74)	29%	6%	45

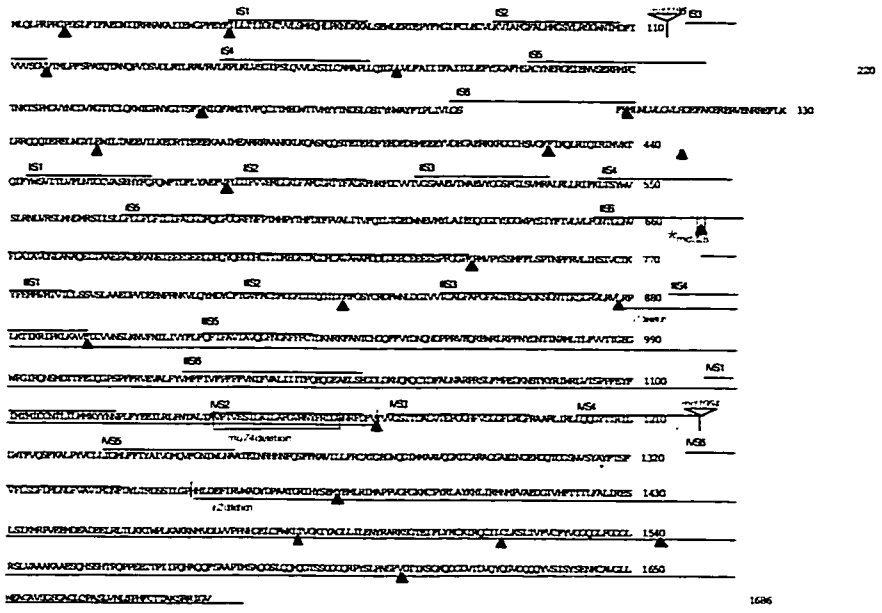
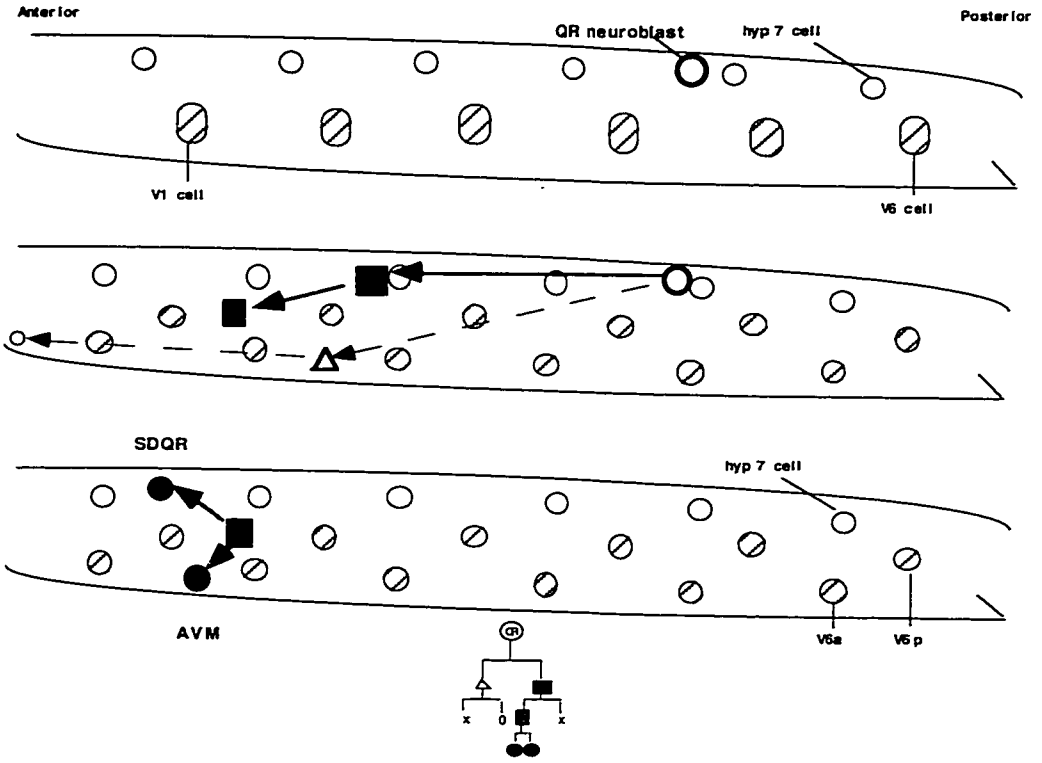
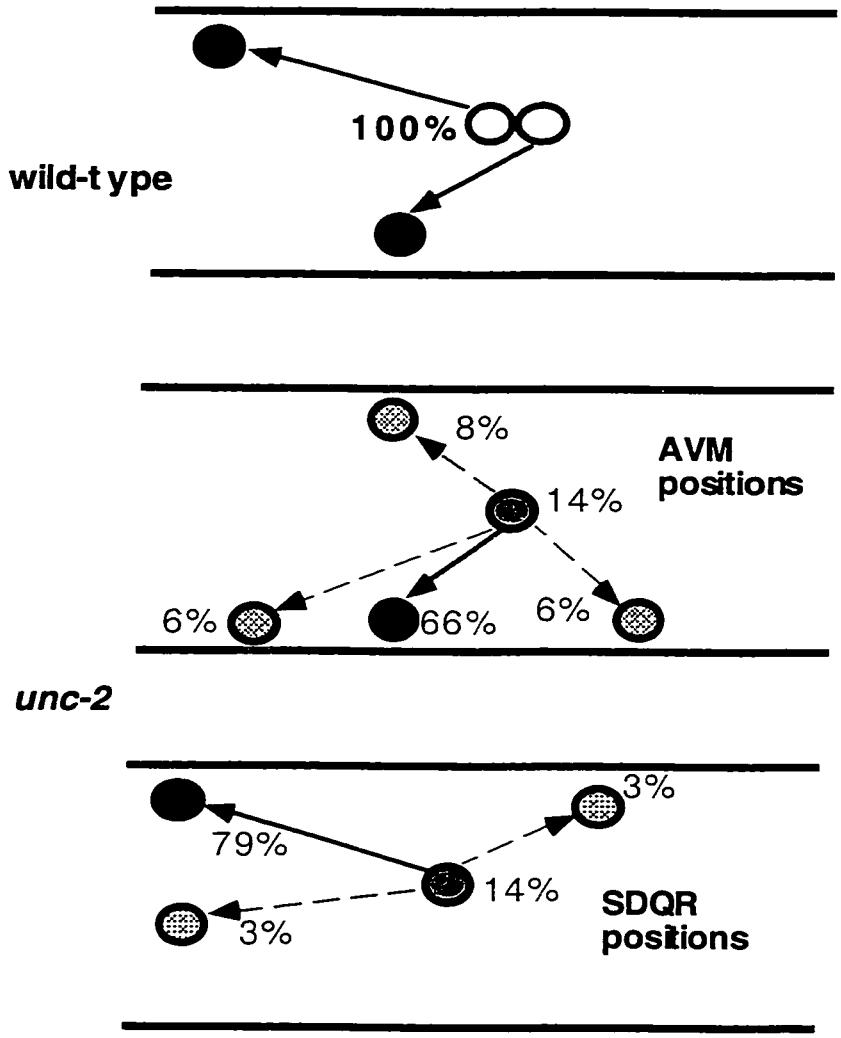


Figure 1-1: Sequences of *unc-2* mutant alleles. Shown is the predicted amino acid sequence of the UNC-2 protein. Intron locations are indicated by triangles below the sequence. The cloning of the unc-2 coding sequence will be described elsewhere (E. M. and T. P. S.). Sequence motifs corresponding to predicted structural features of the UNC-2 protein (identified through comparison with the sequences of other calcium channel α1 subunits) are indicated by blue bars above the sequence text. I-IV indicate the four repeated membrane domains, each of which contains 6 predicted transmembrane α-helices (S1-S6) and a hydrophobic segment predicted to line the channel pore (SS1-SS2). Sequence changes in *unc-2* mutant alleles are indicated above the sequence text; red 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 the asterisk. The point mutations *ra605* and *ra610* introduce nonsense mutations at the positions indicated by the arrows. The *lj2* deletion extends approximately 2 kb past the 3' end of the coding sequence. The sequence alteration in the *e55* allele (see Figures 2-4) has not been determined.



A)

Figure 1-2a. Migrations of AVM and SDQR in wild-type animals. The neuroblast QR.pa, which is born during the first larval stage in the mid-body 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 to 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)

Figure 1-2b. Migration defects of *unc-2* mutant alleles. Positions of AVM and SDQR in *unc-2* mutants. The figure summarizes the positions adopted by SDQR and AVM in wild-type and *unc-2* mutant animals. The diagram represents aggregate data from the two strong loss-of-function alleles *md1186* and *mu74*.

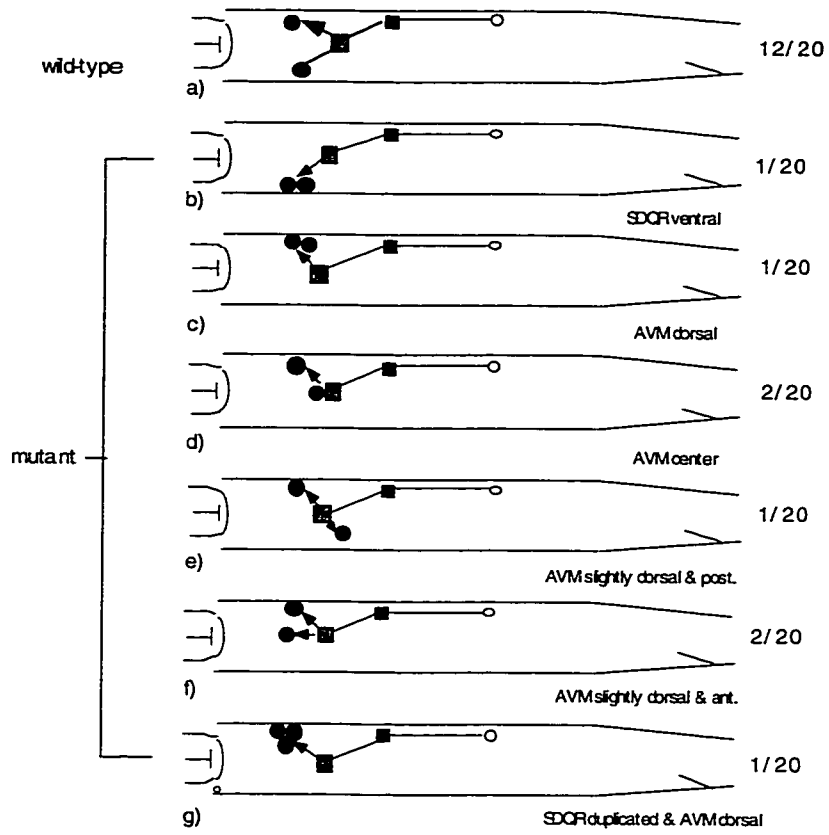
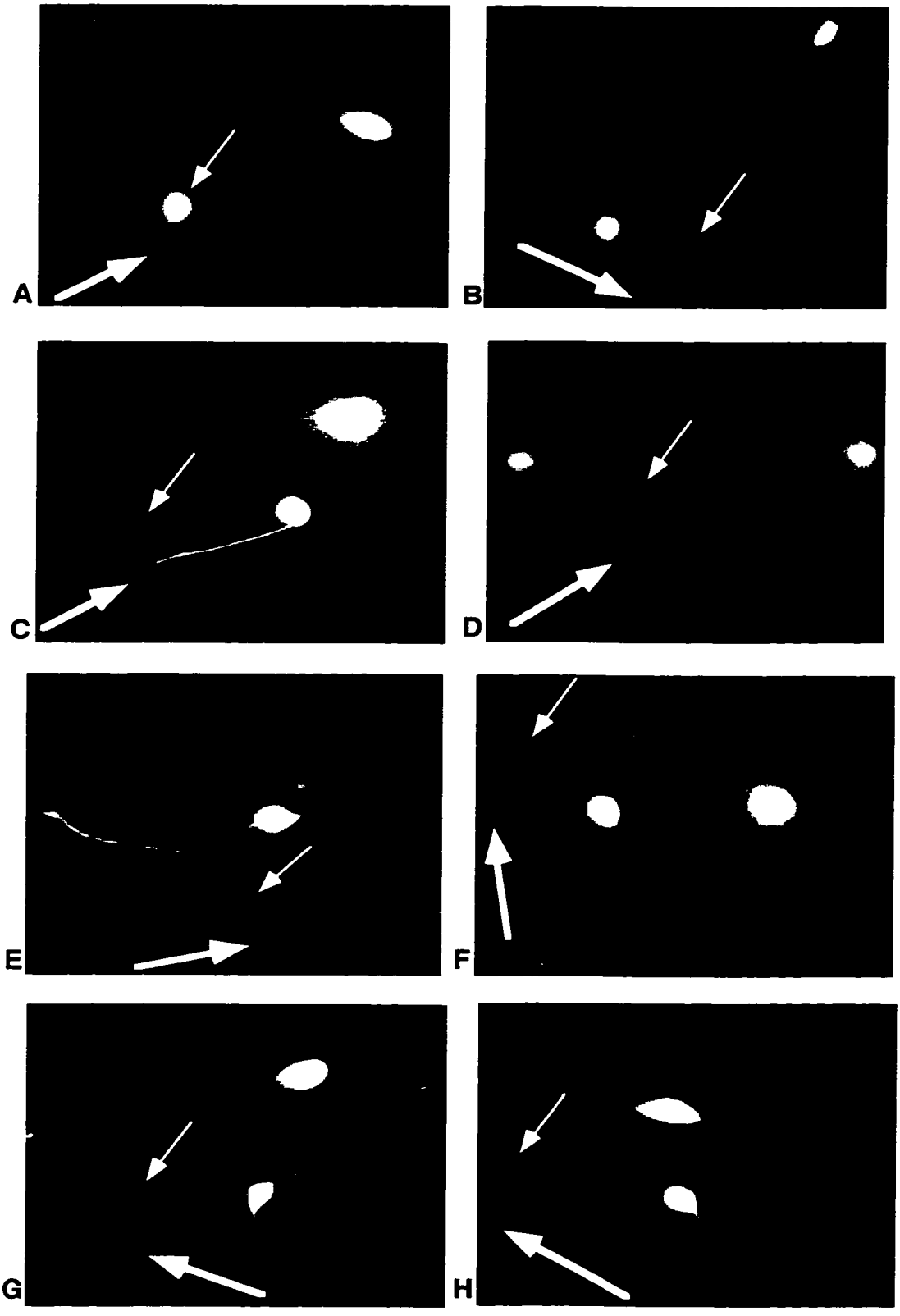


Figure 1-3: Effect of *unc-2* on the migration of the SDQR and AVM precursors. Shown are representative cell lineages seen in 20 *unc-2* mutant animals. The position of QR.pa at the time it divided to produce SDQR and AVM is indicated by the gray box. The positions to which SDQR and AVM migrated are indicated by red and green circle 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.

Figure 1-4: 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 penetrance of axon guidance defects in the strains analyzed was: wild-type: 0% (n=100) *unc-2(mu74)*: 0% (n=100), *egl-19(ad1006)*: 20% (n=50), *egl-19(ad695sd)*: 16% (n=85), *unc-43(e753)*: 0% (n=50). **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. Panels e and f show *egl-19(ad1006)* loss-of-function mutants; panels 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-h, both the cell body and the axon of AVM are posteriorly mispositioned, and in g, and extra AVM axon is also observed.


A
B
C
D
E
F
G
H

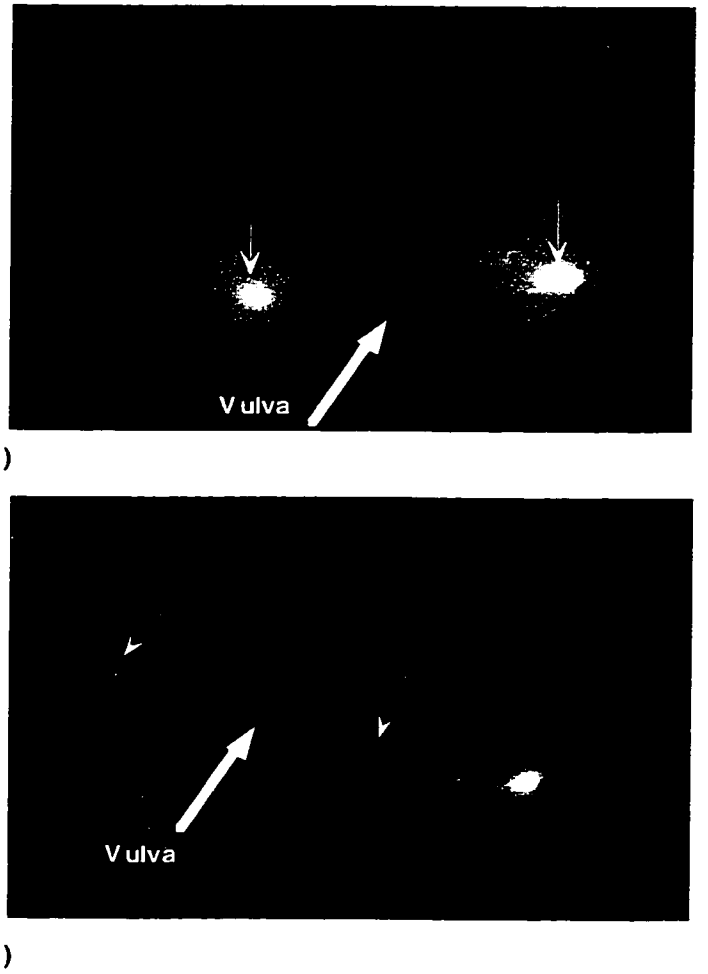


Figure 1-5: Defects in positioning of VC4 and VC5 in *unc-2* mutants. Shown are fluorescence images of adult animals expressing the VC-specific marker *mab-5*::GFP Left is anterior and top is dorsal. The thin arrows show the wild-type position of VC4 (left) and VC5 (right); the position of the vulva is also indicated. **a.** Positions in wild-type animals. VC4 and VC5 are found equidistant from the vulva on the anterior (VC4) or 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=50).

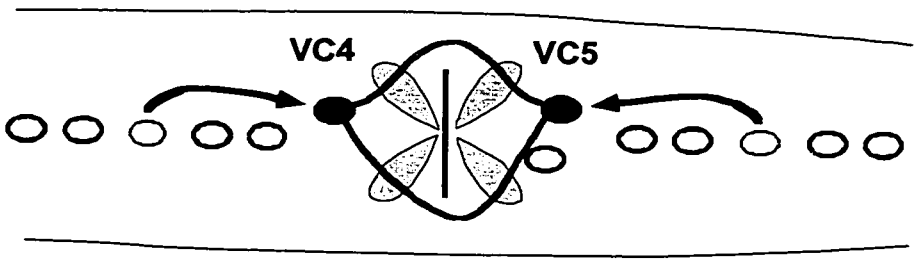


Figure 1-5: Defects in positioning of VC4 and VC5 in *unc-2* mutants. c. Diagram of the migrations of the VC4 and VC5 neurons. Clear circles indicate the positions of the VC cell bodies following the division of the Pn.p neuroblasts; filled circles indicate their final positions.

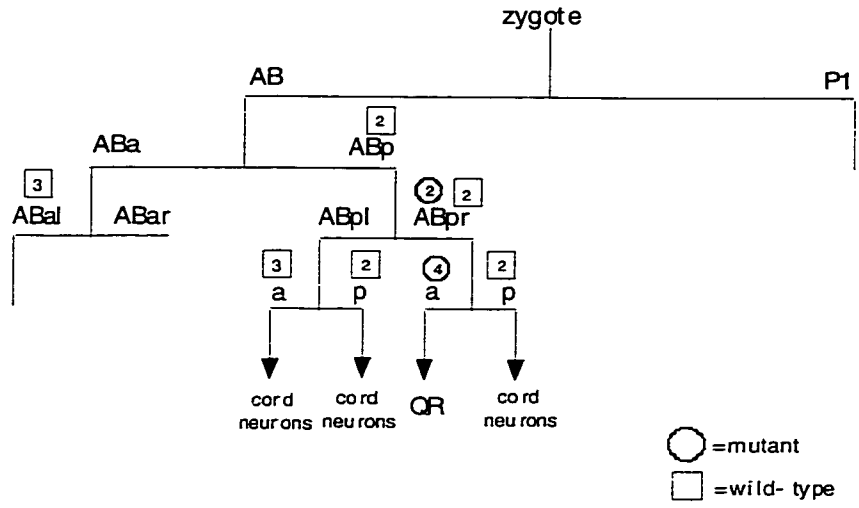


Figure 1-6: Mosaic analysis of the *unc-2* cell migration defect. Shown is the cell lineage for the *C. elegans* hermaphrodite. Indicated are the precursors for QR (the progenitor of AVM and SDQR) and selected other cells in the animal. Mosaic animals were generated as described. The precursors of clones in mosaic animals are indicated by circles; filled circles indicate mosaics with misplaced AVM and/or SDQR cells, and open circles indicate mosaics with normally placed neurons.

CHAPTER III A SIGNAL TRANSDUCTION PATHWAY MEDIATES NEURONAL MIGRATION BY SEROTONIN IN *CAENORHABDITIS ELEGANS*

ABSTRACT

Biogenic amines are known to modulate neuronal activity through activation of G-protein-mediated signaling pathways. In addition, serotonin has been shown to play a prominent role as a molecular signal in determining important aspects of neuronal growth including cell differentiation, regulation in cell shape, and cellular migration. In this study, we have identified serotonin as a signaling molecule that directs proper neuronal migrations of the *C. elegans* neurons AVM and SDQR. Mutants defective in serotonin synthesis showed a significant defect in the proper migration of the AVM and SDQR cell bodies, a defect which was rescued by the application of exogenous serotonin. Since non-localized serotonin can rescue the defect of VMAT mutant *cat-1*, it appears to be a permissive signal allowing responses to local guidance cues. The Go homologue *goa-1* is a candidate for a signaling molecule downstream of serotonin since it shows defects reminiscent of serotonin-deficient mutants and the serotonin-deficient, *goa-1* double mutant shows similar penetrance to the *goa-1* single.

INTRODUCTION

Neurotransmitters are generally thought to act as chemical mediators of intercellular communication in mature nervous systems. A growing body of evidence suggests that neurotransmitters also play important roles in the development, maintenance, plasticity and motility of individual neurons. It is speculated that neurotransmitters evolved into

the specialized role of mediating synaptic information from primitive functions in lower organisms, where they act to regulate many morphogenetic activites such as cellular development, regeneration, regulatory signals, morphogenesis, and cell motility. In particular, serotonin has been seen to play a role in the development of *Tetrahymena* (Goldman *et al*, 1981 and Blum, 1970), the regeneration of the flatworm *Planaria* (Franquinet *et al*, 1981), the motility of parasitic trematodes and cestodes within their hosts (Mansour *et al*, 1983), the gastrulation of sea urchins (Soliman *et al*, 1983 and Gustafson *et al*, 1970), and the cellular motility of chick neuroepithelial cells (Emanuelsson *et al*, 1988) and mouse cranial neural crest cells (Moiseiwitsch *et al*, 1995) .

Serotonin has also been implicated in cell motility and the control of cell migration. The role of serotonin in directing neuronal cell migration, therefore, is not a new assignment. For example, serotonin stimulates and inhibits cultured bovine pulmonary artery smooth muscle cell configuration through activation of receptors (Lee *et al*, 1991). In developing chicks, the role of serotonin in morphogenesis appears to be regulated at the cytoskeleton, particularly in the association of microfilaments and microtubules (Emanuelsson *et al*, 1988). In a variety of mammalian cells, serotonin promotes growth when it adheres to components of the extracellular matrix (Hannan *et al*, 1988).

In this study, we demonstrate a role for serotonin in directing neuronal cell migrations in *Caenorhabditis elegans*. Four different mutants that reduce serotonin expression -- *tph-1*, *cat-4, bas-1*, and *cat-1* - exhibit defects in the proper migrations of the neuronal cells AVM and SDQR. The misplacement phenotype was rescued by exogenous serotonin, a result consistent with the the misplacement being caused by a

lack of serotonin. An intracellular signaling pathway involving the calcium channel gene *unc-2* , the CaM kinase gene *unc-43* (Tam *et al*, 2000), and the G protein *goa-1* may mediate the effects of serotonin on cell migration.

RESULTS

Effects of serotonin-deficient mutants on the postembryonic migrations of AVM and SDQR.

The Q cells and their descendants migrate postembryonically to their proper positions. The migrating neuroblast QR and its descendants migrate along the anteroposterior axis from an origin in the midbody region. While traveling anteriorly, QR undergoes a few cell divisions that ultimately give rise to two sister cells, the touch receptor AVM and the interneuron SDQR (Sulston and Horvitz, 1977).

To determine whether serotonin affected AVM and SDQR migration, we assayed the positions of these neurons in serotonin-deficient mutants. We assayed four different serotonin-deficient mutants defective in the genes *tph-1, cat-4, bas-1 and cat-1*. *tph-1* encodes the only tryptophan hydroxylase gene for serotonin biosynthesis in *C. elegans* (Sze *et al*, 2000). *cat-4* also encodes a gene required for serotonin biosynthesis. (Loer *et al,* 1993). *bas-1* encodes a gene necessary for a decarboxylase step in serotonin biosynthesis (Loer *et al,* 1993). *cat-1* encodes a vesicular monoamine transporter (VMAT), which pumps serotonin into synaptic vesicles. We observed that all four mutants were defective in the proper positioning of the migrating neurons AVM and SDQR (Table 2-1), Specifically, these four serotonin-deficient mutants also seemed to be defective in the final migrations of AVM and SDQR, which occur after the cells differentiate into neurons. This phenotype is qualitatively similar to that seen in the calcium channel mutant *unc-2* and the CaM kinase mutant *unc-43*. Because of the

similar misplacement phenotype of *tph-1, cat-4, bas-1*, *cat-1* and *unc-2*, it is possible that serotonin and calcium function in the same process involved in the final positioning of AVM and SDQR (Fig.2-1).

Does serotonin signaling affect the axon placement of the touch receptor AVM? In order to answer this question, we constructed *tph-1; mec-7::GFP* double mutant animals to determine whether axon guidance was abnormal. In 42% of the animals we saw AVM's cell body misplaced but its axon made the proper connection into the ventral cord (Fig 2-2c). However, in 14% of the double mutants we saw both AVM cell body and axon misplaced posteriorly (Fig. 2-2d). Thus serotonin appeared to affect guidance of both cell body and axon of AVM. A neighboring touch receptor, ALM, was also frequently misplaced (Fig 2-2b).

Rescue of serotonin-deficient mutants by exogenous serotonin

How might serotonin promote proper cell migration? One possibility is that there is localized signaling whose local concentration determines the direction of migration. Alternatively, serotonin could be a permissive cue that allows proper directed migration. To distinguish between the two, we tested whether application of exogenous serotonin could rescue the mutant phenotype. If specific localization were required, we would expect to see no rescue. Alternatively, if serotonin is a permissive cue, exogenous serotonin should rescue the *cat-1* defect. In fact, we observed that the migration defects of *cat-1* were efficiently rescued by exogenous serotonin, supporting the second theory (Fig 2-3). Thus, serotonin appears to be a permissive signal that potentiates response to or release of localized guidance cues.

Serotonin time course

To determine when during development serotonin is necessary to direct proper migration of the neurons AVM and SDQR, we performed a time course experiment. QR undergoes migration and cell division during the first larval stage of development. Specifically, after hatching (time=0), QR divides and migrates within a time window of 4-10hrs. To determine which hours were crucial for serotonin to direct proper migration of AVM and SDQR, newly hatched *tph-1* mutants were placed on serotonin plates at varying time points. We found that when we introduced serotonin during the first two hours after hatching, we saw rescue of the migration defect (Fig 2-4). However, serotonin applied after that time point failed to rescue the defects even though division of the QR.pa precursor to generate AVM and SDQR does not occur until nine hours after hatching. This suggest that serotonin may act earlier than the division of QR.pa.

Effects of *goa-1* on migration and axonal pathfinding

What signaling molecules mediate the effect of serotonin on neuronal migration? Serotonin is usually thought to act through G-protein coupled receptors. Therefore, it seemed reasonable to search for a serotonin effector through screens of G protein mutants. Of 17 viable G protein mutants, only *goa-1* animals displayed a misplacement phenotype similar to *unc-2* (Table 2-2). *goa-1* loss-of-function allele showed migration defects similar to serotonin-deficient mutants. Interestingly, the *goa-1* gain-of-function allele, *sy1s9*, showed no misplacement phenotype, suggesting that Go, like serotonin, plays a permissive role in neuronal migration (Table 2-2). Moreover, *goa-1* loss-of-function suppressed the migration defects of *tph-1*, suggesting Go functions downstream of serotonin in this process. *goa-1* encodes the homolog of an α subunit of

the heterotrimeric guanosine triphosphate-binding protein Go is expressed in some muscles and nearly all neurons and at least in some of these cells has been implicated in the serotonin response (Segalat *et al*, 1995). Thus, Go may be a key effector of serotonin in migrating neurons.

Previously we showed that *egl-19*, the L-type voltage-gated calcium channel, was necessary for proper guidance of AVM and SDQR (Tam *et al*, 2000). We also identified *egl-19* as the calcium channel that was necessary for directing proper guidance of the AVM axon, whereas *unc-2* only contributed to neuronal cell positioning. To test whether goa-1 also contributed to axonal guidance, we constructed the double mutant *goa-1; mec-7::*GFP transgene (Fig.2-5). The double mutants were 31% penetrant for the AVM misplacement phenotype and in each case the axon made its correct connection to the proper location into the ventral cord (Fig.2-5b). By analyzing the double mutants, we concluded that *goa*-1, like *unc-2*, was not necessary for proper axonal guidance of AVM and therefore may be a regulator for this calcium channel. We also noted that *goa-1; mec7*::GFP doubles did not express fluorescence in 19% of the AVM neurons (Fig.2-5d). In 11% of AVM neurons, we also observed that the cell body itself was irregularly shaped (Fig.2-5c). This observation was not seen through DIC imaging, but could only be noted via fluorescence of the cell body. This may indicate yet another role for *goa-1,* this time in the proper differentiation of AVM neurons.

Since Go mutants showed similar migration phenotypes to *unc-2* calcium channel mutants, we wondered whether *unc-2* and *goa-1* functioned in a common pathway. To test this we made double mutants. The penetrance of the AVM/SDQR mispositioning defects in *goa-1;unc-2* double mutants was no higher than in the *unc-2* single mutants,

an indication that they may work in the same pathway (Table 2-2). Figure 2-6 displays the schematics of the misplacement phenotypes in AVM and SDQR for both *unc-2* and *goa-1* mutants. The distributions of AVM and SDQR's misplacements for *unc-2* and *goa-1* are also qualitatively similar, further supporting the idea that these two genes work together.

Serotonin does not rescue *goa-1* and *unc-2* mutants.

Another test to see whether *goa-1* and/or *unc-2* function in the same pathway as serotonin for proper cell placement was to rescue either mutant with exogenous serotonin. If serotonin were functioning downstream of *goa-1* and/or *unc-2*, we might expect to see a rescue of the misplacement phenotype in either mutant when placed on exogenous serotonin. However, when *goa-1* or *unc-2* mutants were placed on exogenous serotonin, we do not see a difference in misplacement (Fig 2-7). This result suggests that *goa-1* or *unc-2* do not regulate serotonin release for proper neuronal migration.

Analysis of double mutants

Animals mutant in the serotonin biosynthesis gene encoded by *tph-1*, the Go alpha protein encoded by *goa-1*, or the calcium channel gene *unc-2* all have similar misplacement phenotypes of the AVM and SDQR neurons. To ascertain whether all these genes work in a common pathway, we constructed double mutants (Table 2-3). Taking advantage of the fact that *goa-1* gain-of-function allele *sy1s9* displays no misplacement phenotype, we made the *tph-1*; *sy1s9* double mutant. We observed that the double mutant showed a less severe misplacement phenotype than *tph-1* by itself indicating that the *goa-1* gain-of-function mutant suppressed the *tph-1* phenotype. This result is consistent with the hypothesis that *goa-1* acts downstream of serotonin. If

serotonin and *goa-1* were acting in separate pathways or if serotonin were acting downstream of *goa-1*, the double mutant would show the same penetrance as a *tph-1* mutant. Therefore the result of the *tph-1; syIs9* double mutant suggests that serotonin not only acts in a common pathway with *goa-1*, but also acts upstream of *goa-1*.

To determine whether the calcium channel gene *unc-2* lies in a common pathway with *goa-1* and serotonin, we constructed double mutants between *tph-1* and *unc-2* and between *unc-2* and *goa-1*. We analyzed the phenotype of *tph-1;unc-2* and observed that the penetrance of the double mutant was no higher than in *tph-1* single mutants. This suggests that *unc-2* and serotonin function in a common pathway. Likewise we observed that the *unc-2; goa-1* double mutant also showed similar penetrance to the singles. This result suggests that *goa-1* may function in the *unc-2* pathway. Although *unc-2; goa-1* double mutants suggest that these two genes work in a common pathway, it is not clear which is downstream of the other. In order to determine this, we constructed a *goa-1* gain-of-function; *unc-2* double mutant. If *goa-1* were downstream of *unc-2*, we would expect to see results similar to the *tph-1; syIs9* double mutant where there was very little misplacement. However, we observed that the *unc-2; syIs9* double mutant displayed misplacement phenotypes more similar to *unc-2*. This result suggests that the calcium channel is downstream of the Go alpha protein.
From this double mutant analysis, the results not only suggest that serotonin, *goa-1* and *unc-2* work in the same pathway, but also that they function in that order.

DISCUSSION

Serotonin in cell migrations

We have found that in *C.elegans* serotonin appears to provide an important permissive cue for directed neuronal migration and axonal guidance. These results

have parallels in other organisms. For example, vertebrates, chicks and frogs synthesize norepinephrine and serotonin in the notochord where they participate in regulating proper development of the neural tube (Lauder, 1988). Relatively little is known about the signaling transduction mechanisms through which serotonin functions in these processes. Genetic studies in *C. elegans* may provide insight into these potential mechanisms by providing a useful avenue to identify molecules involved in serotonin responses in migrating cells.

The role of serotonin in controlling development may actually predate the evolution of the nervous system. From unicellular organisms to mammals, neurotransmitters such as the monoamines acetylcholine and GABA act as regulatory growth signaling molecules for particular morphogenetic activities (Lauder, 1988). In the unicellular organism *Tetrahymena*, serotonin and dopamine levels differ inversely during active and stationary phases of growth (Goldman *et al.*, 1981 and Blum, 1970). In the multicellular flatworm *Planaria*, serotonin, dopamine, and norepinephrine are involved in regeneration by inhibiting RNA synthesis and promoting DNA synthesis, restoring RNA synthesis, and promoting other regeneration mechanisms, respectively (Franquinet *et al*, 1981).

These neurotransmitters have been shown to act through receptors, second messenger pathways and association with the cytoskeleton. In *Tetrahymena*, serotonin and dopamine are released into the extracellular environment in response to elevated intracellular calcium levels. There they increase intracellular cAMP levels via cell surface receptors to promote development (Goldman *et al*, 1981 and Rodriguez *et al*, 1980). In *Planaria*, the neurotransmitters are linked to G proteins, adenylyl cyclases and receptors that mediate increases in cAMP, to aid in regeneration (Venturini, *et al*,

1989). In amphibians and avians, serotonin has been shown to bind to cytoskeletal elements in neuroepithelial cells, which may regulate changes in cell shape and morphogenetic cell movements in the neural tube (Emanuelsson *et al,* 1988).

The similarities between these mechanisms and the signaling mechanisms identified in these studies suggest that a widely conserved mechanism may mediate serotonin's developmental effects.

The role of serotonin in the development of the Q cell lineage.

Previous mutant screens for genes necessary for proper neuronal cell migrations have never isolated genes required for the biosynthesis or transportation of serotonin (Desai *et al.,* 1988, Harris *et al.,* 1996, and Forrester *et al.,* 1997). Our study is the first to identify serotonin as a necessary component for the proper migration of neurons. It is possible that previous studies concentrated on the initial long migrations of neuroblasts (Harris *et al.,* 1996), whereas our study focuses on the last migrations of a fully developed neuron. Identification of *goa-1* as another component necessary for the last migrations of the QR descendants and proper neuronal differentiation adds to the argument that there are specific genes affecting the latter stage of migration. Genes affecting the early long migrations of the Q neuroblasts mainly shorten their anterior/posterior migrations, leaving their dorsal/ventral positioning unaffected (Harris *et al.,* 1996, Zipkin *et al.,* 1997, and Sym *et al.,* 1999). To support the idea that serotonin is required for the last migrations of AVM and SDQR, lineaging serotonin mutants to ascertain exactly where the misplacement phenotype occurs would need to be performed as described in Tam *et al,* 2000.

Previous studies have also distinguished genes affecting Q migrations into two categories: those that affect the initial anterior/posterior long migrations and those that

affect the final short migrations (Harris *et al.*,1996 and Tam *et al.*, 2000). From the QR descendents' migrations in serotonin mutants and the *goa-1* mutant (Fig 2-1 and 2-6), it appears that they both fall into the second category, which affects the final migrations. The posterior counterparts of AVM and SDQR are PVM and SDQL which migrate to a location in the animal known as the *postdereid* where other neurons are found. The neurons in this area do not migrate to a highly reproducible location among themselves in wild type animals (data not shown); therefore assessing whether or not genes affecting the final migrations of AVM and SDQR also affect their counterparts PVM and SDQL cannot be absolutely determined.

Serotonin may mediate an intracellular signaling pathway involving calcium for neuronal migration

What are the signaling molecules that mediate serotonin's effect on cell migration? We have previously investigated the roles of the calcium channel genes *unc-2* and *egl-19* as well as that of the CaM kinase gene *unc-43* in the proper migrations of AVM and SDQR (Tam *et al.*, 2000). From this study, we have obtained evidence that the extracellular signaling molecule serotonin and an intracellular signaling molecule *goa-1* function in the same pathway as *unc-2* in directing the correct Q migrations.

Previous studies have linked serotonin and G proteins in the same signaling pathways. In a screen for hyperactive serotonin signaling mutants, the Gαo mutant *goa-1* was found to be resistant to exogenous serotonin by failing to rescue its hyperactive locomotion behavior. This study further implicated *goa-1* as an effector of serotonin signaling for locomotion, defecation, and egg-laying (Segalat *et al.*, 1995). To date, the studies of *goa-1* and its mutant migration phenotype, along with axonal images and double mutant analysis are consistent with the possibility that this gene

regulates the calcium channel encoded by *unc-2*. However, to argue conclusively that *goa-1* regulates *unc-2*, further genetic and possibly calcium imaging experiments need to be performed.

Calcium channels and CaM kinase: The loss of function mutants in both calcium channels encoded by *unc-2* and *egl-19* and the CaM kinase mutant *unc-43* all display similar neuronal misplacement phenotypes. However, the gain of function mutants of *egl-19* and *unc-43* also display inappropriate migration, which suggest that these molecules are not merely permissive; rather that proper regulation of both proteins appears critical for guidance. Moreover, our previous studies indicated that the *egl-19* calcium mutant had a 20% AVM axonal misplacement phenotype, one not seen in *unc-2, unc-43* and *goa-1* but seen in *tph-1* (Tam *et al.*, 2000). In fact, *tph-1; mec-7::GFP* had a simliar penetrance to that of *egl-19* with 14% AVM axonal misplacement. However, 42% percent of *tph-1; mec-7::GFP* animals had only an AVM cell body misplacement, a characteristic more similar to *unc-2* than *egl-19*. Thus, it seems plausible that serotonin may be a component in two different pathways, one directing neuronal migration and the other axon outgrowth. Further investigation into serotonin signaling will come from construction of more double mutants. This will help ascertain the possibility that serotonin directs migration through two different calcium channels.

Remaining questions

Where is the site of serotonin release? There are 11 serotonergic neurons which include two NSMs, two HSNs, VC4 and VC5, two ADF, RIH, AIM and RIC neurons (Duerr *et al*, 1999). To date, we have only a hint of the potential results from *egl-1*, a mutant lacking the serotonergic HSN neurons resulting from inappropriate

programmed cell death. *egl-1* mutants display a slight misplacement of AVM and SDQR, but the defect is not comparable to the serotonin-defective mutants (data not shown). It is possible that the HSNs, which are located in the mid-body region, are not appropriately positioned to reproduce a full Q cell misplacement effect. The slight misplacements of AVM and SDQR, however, do suggest that more proximate neurons such as the NSMs found in the head might serve as the sourse of serotonin release necessary for correct neuronal migration.

Another important question is: what is the receptor for serotonin? There are approximately ten potentional serotonin receptors known in the *C. elegans* genome. Some of these receptors are expressed in neurons, though their specific expression patterns have not be characterized.

If we could identify the serotonin receptor, we could determine whether serotonin acts directly on the migrating cell by testing whether cell-specific expression of functional receptors could rescue the neuronal migration defects. Similar experiments using the Go gain-of-function allele would allow use to test whether *goa-1* acts cell autonomously in the migrating cells.

To identify the critical source of serotonin, ablation of serotonergic neurons and a reproduction of the mutant phenotype should be performed. We would also expect a rescue of the misplacement phenotype in the neuron-ablated worms when exogenous serotonin is introduced. These further experiments would also help solidify our findings that serotonin is a signaling component necessary for the proper migrations of AVM and SDQR.

MATERIALS AND METHODS

Assay conditions and growth media

Unless otherwise noted, nematodes were grown and assayed at room temperature on standard nematode growth medium (NGM) seed with E. coli strain OP50 as a food source. For drug experiments, 5-hydroxytryptamine (creatinine sulfate complex, Sigma was added to NGM agar at 7.5 mM.

Construction and characterization of double mutants

Double mutants carrying *goa-1* or *tph-1* and an integrated GFP reporter construct were generated using standard methods. The strain CF703 (genotype *muIs35*[*mec7*::GFP;line-15(+)]V), which carried a transcriptional fustion of GFP to the *mec-7* promoter, was obtained from Queelim Ch'ng in the Kenyon lab. Double mutants with *unc-2* and *goa-1*, *unc-2* and *syIs9*, *tph-1* and *syIs9*, and *tph-1* and *unc-2* were constructed by crossing the single mutants and first identifying animals homozygous for one mutant phenotype in the F2 generation. These animals were picked to individual plates and their progeny were selected upon the revelation of the second phenotyped once homozygosed. The presence of both mutations was subsequently verified by mating the putative double mutants with wild type and confirming the presence of both single-mutant phenotypes in the F2 generation.

Table 2-1: Penetrance of migration defects in serotonin-deficient mutants.

Strain genotype	AVM misplacement	SDQR misplacement	n=
tph-1	48%	16%	100
cat-4	35%	10%	100
bas-1	30%	12%	100
cat-1	34%	6%	50

Table 2-2: Penetrance of migration defects in G protein mutants.

Strain genotype	AVM misplacement	SDQR misplacement	n=
gpa-1	0%	0%	30
gpa-2	0%	0%	30
gpa-3	0%	0%	30
gpa-4	3%	3%	31
gpa-5	0%	0%	30
gpa-6	3%	3%	31
gpa-8	0%	3%	33
gpa-9	0%	0%	30
gpa-10	0%	0%	30
gpa-11	0%	0%	30
gpa-12	3%	0%	30
gpa-13	0%	0%	30
gpa-14	6%	2%	48
gpa-15	0%	0%	30
odr-3(n2150)	0%	0%	30
egl-30 (ad805)	5%	1%	100
goa-1(n1134)	30%	8%	101
goa-1(n1134); unc-2(mu74)	24%	8%	101
svIs9 (goa-1(dm))	0%	0%	50
unc-2(mu74)	32%	12%	60

Table 2-3: Double mutants suggest genes acting in the same pathway.

Strain genotype	AVM misplacement	SDQR misplacement	n=
goa-1 (gf-*sy1s9*)	0%	0%	50
tph-1 (md280)	48%	16%	100
tph-1; goa-1 (gf)	11%	4%	100
unc-2(mu74)	32%	12%	100
unc-2; goa-1 (gf)	28%	6%	100
goa-1 (lf-*n1134*)	30%	8%	100
unc-2; goa-1(lf)	26%	9%	100
unc-2; tph-1	41%	18%	100

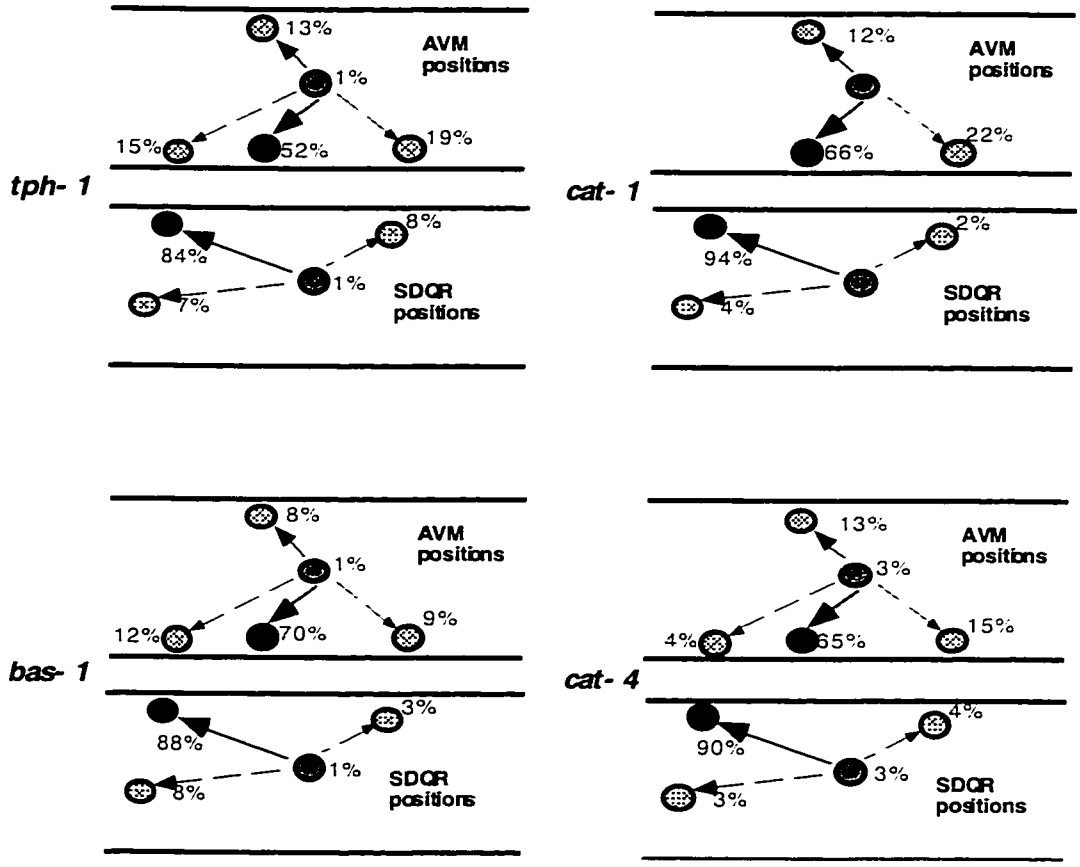


Figure 2-1: Migration defects of serotonin-deficient mutants.
Positions of AVM and SDQR in *tph-1, cat-4, bas-1* and *cat-1* mutants. The positions adopted by AVM and SDQR in wild-type, *tph-1, cat-4, bas-1* and *cat-1* mutant animals are summarized. Data from loss-of-function alleles *tph-1 (mg280), cat-4 (e1141), bas-1(ad446)*, and *cat- 1 (e1111)* were used.

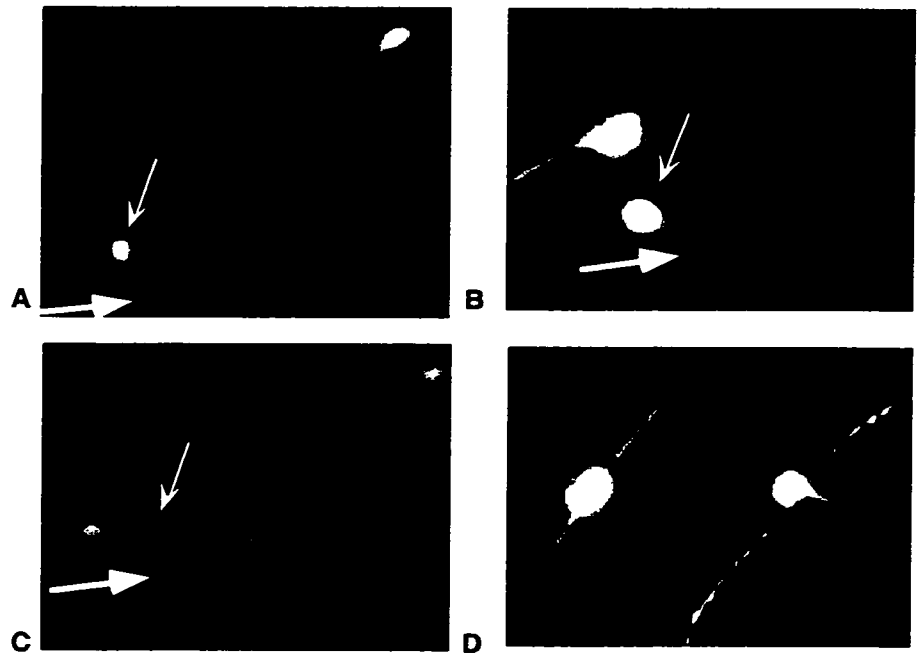


Figure 2-2: Effect of *tph-1* on AVM differentiation and axonal guidance. Shown are fluorescence images of *tph-1*; *mec7::GFP* double mutants. Only the dorsoentrally directed process of AVM is in focus. Left is anterior and top is dorsal. Thin arrow shows the wild-type position of the AVM cell body. 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) and *tph-1* (*mg280*) 14% (n=100). (A) A representative wild-type animal. (B-D) Examples of *tph-1* mutant animals. The ALM cell body is displaced in an anterior (B) direction from its normal position. C is an example of a misplaced AVM cell body with proper axon connect into the ventral cord, seen in 42% of the animals. D shows the AVM cell body much posterior to its wild type position, axon and cell body are both misplaced seen in 14% of the mutants.

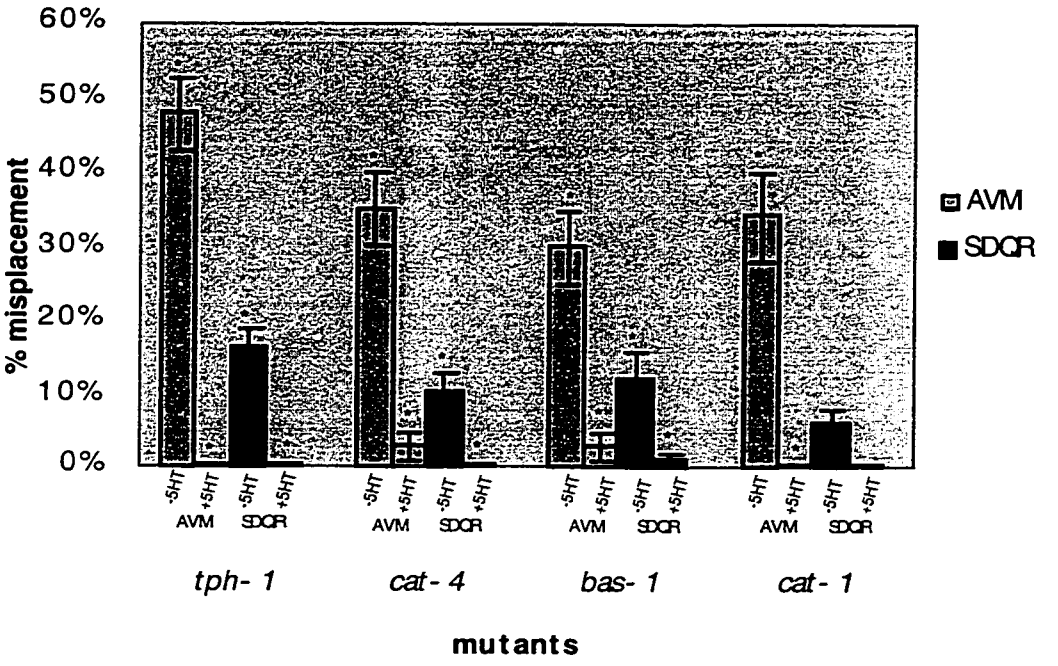


Figure 2-3: Rescue of serotonin deficient mutants. Serotonin-deficient animals animals were placed on exogenous serotonin (5HT) to test whether serotonin could rescue the migration defects. (number of animals no drug/on serotonin) *tph-1*: n=100/n=50; *cat-4*: n=100/n=52; *bas-1*: n=86/n=60; *cat-1*: n=50/n=50. * indicate statistically difference between serotonin treated and untreated animals.

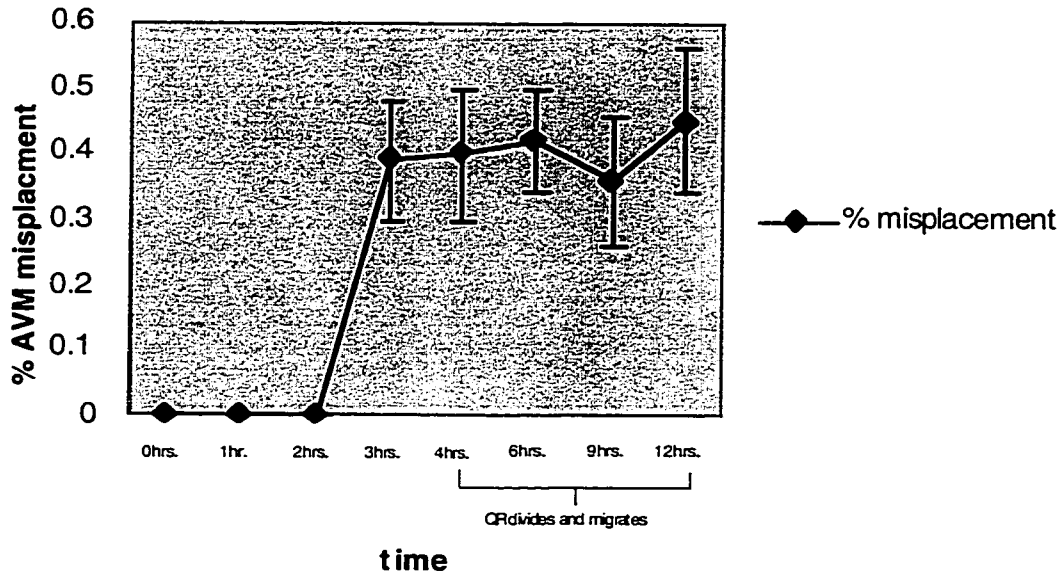


Figure 2-4: Serotonin time course: Shows percent misplacement of AVM. Serotonin-deficient *tph-1* animals placed on serotonin plates after the alloted time. n=20, 0hrs; n=20, 1hr; n=20, 2hrs; n=28, 3hrs; n=20, 4hrs; n=31, 6hrs; n=20, 9hrs; n=20, 12hrs.

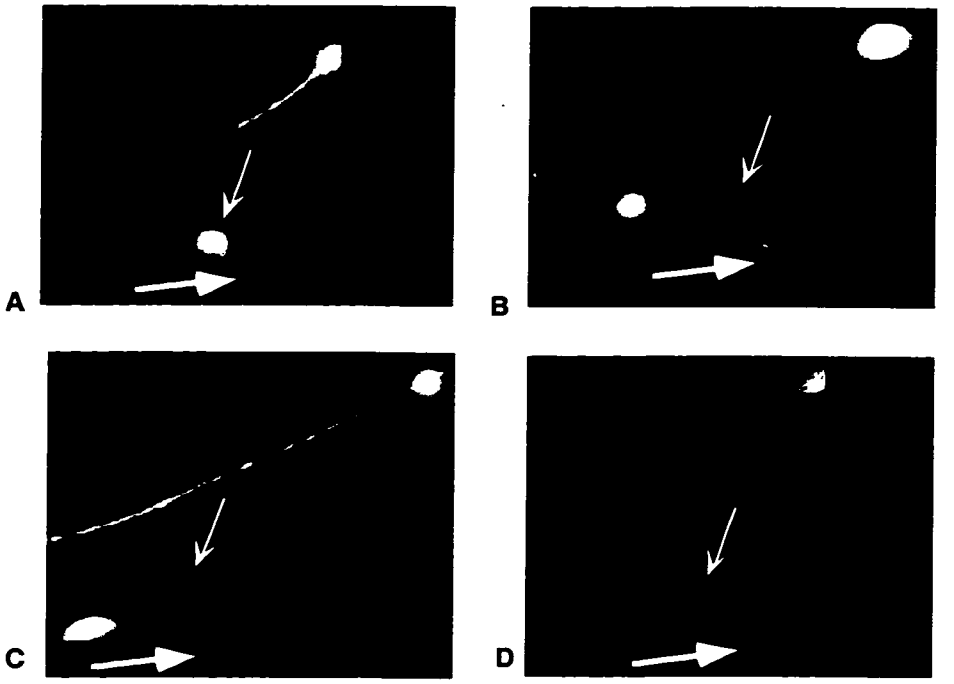


Figure 2-5: Effect of *goa-1* on AVM differentiation and axonal guidance. Shown are fluorescence images of adult animals expressing the touch receptor-specific marker *mec7::GFP*. Only the dorsoentrally directed process of AVM is in focus. Left is anterior and top is dorsal. Thin arrow shows the wild-type position of the AVM cell body. The thick arrow shows the position where the ventrally directed axon connects tot he 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) and *goa-1* (*n1134*) 0% (n=100). (A) A representative wild-type animal. (B-D) Examples of *goa-1* mutant animals. The AVM cell body is displaced in an anterior/dorsal (B & C) direction from its normal position. C also is an example of the irregular cell body shape seen in 11% of the animals. D shows no fluorescence of the AVM axon or cell body, seen in 19% of the mutants.

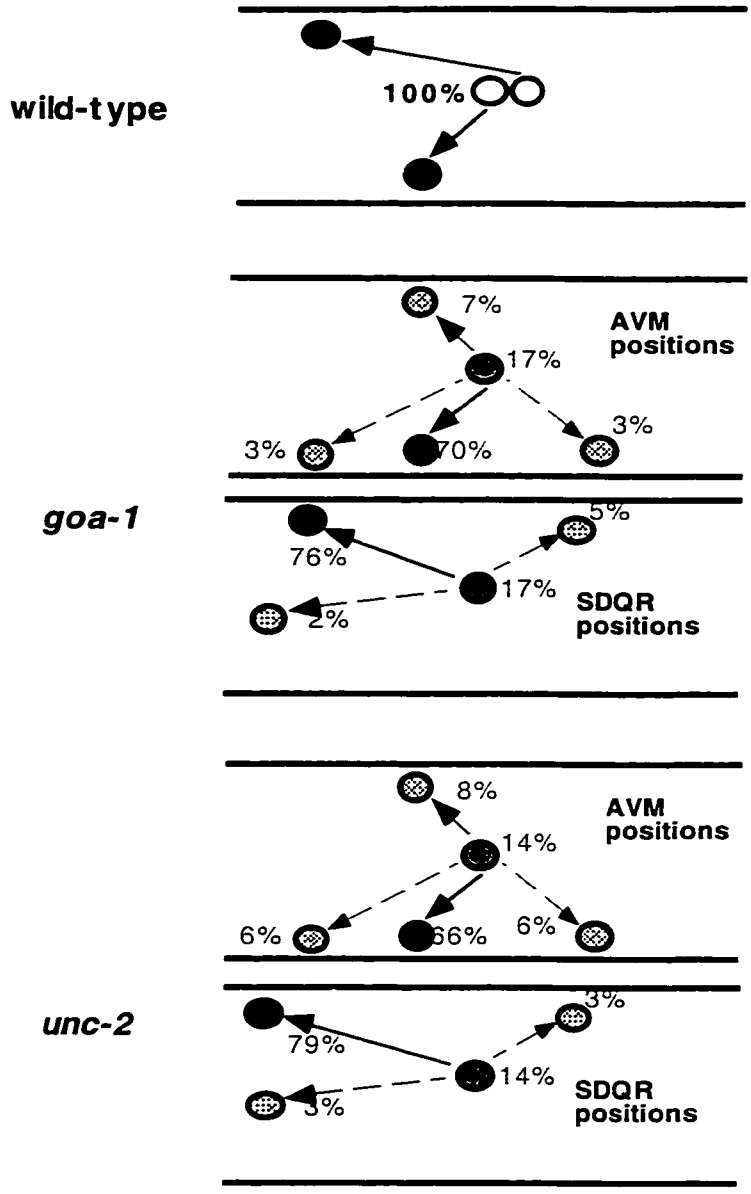


Figure 2-6: Migration defects of *goa-1* and *unc-2* mutants.
Positions of AVM and SDQR in *goa-1* and *unc-2* mutants. The positions adopted by AVM and SDQR in wild-type, *goa-1* and *unc-2* mutant animals are summarized. Data from *goa-1* loss-of-function allele *n1134* was used. For *unc-2* the diagram represents aggregate data from the two strong loss-of-function alleles *md1186* and *mu74*.

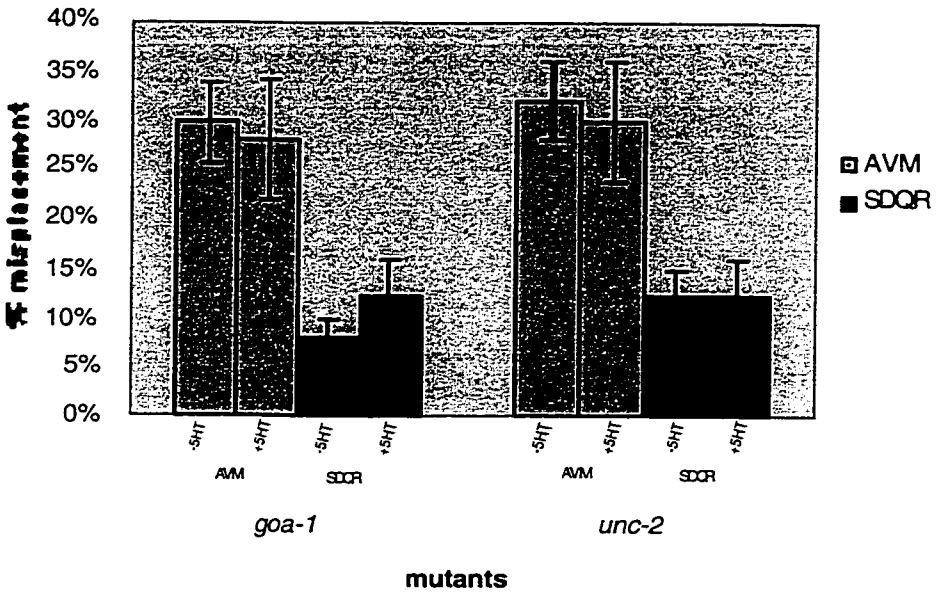


Figure 2-7: *unc-2* and *goa-1* on serotonin: *unc-2* and *goa-1* mutant animals were placed on exogenous serotonin (5HT) to test whether serotonin could rescue the migration defects. (number of animals no drug/on serotonin) *goa-1*: n=100/n=50; *unc-2*: n=100/n=50

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