Pioneer Axon Navigation Is Controlled by AEX-3, a Guanine Nucleotide Exchange Factor for RAB-3 in *Caenorhabditis elegans*

Jaffar M. Bhat and Harald Hutter¹

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

ABSTRACT Precise and accurate axon tract formation is an essential aspect of brain development. This is achieved by the migration of early outgrowing axons (pioneers) allowing later outgrowing axons (followers) to extend toward their targets in the embryo. In *Caenorhabditis elegans* the AVG neuron pioneers the right axon tract of the ventral nerve cord, the major longitudinal axon tract. AVG is essential for the guidance of follower axons and hence organization of the ventral nerve cord. In an enhancer screen for AVG axon guidance defects in a *nid-1*/Nidogen mutant background, we isolated an allele of *aex-3*. *aex-3* mutant animals show highly penetrant AVG axon navigation defects. These defects are dependent on a mutation in *nid-1*/Nidogen, a basement membrane component. Our data suggest that AEX-3 activates RAB-3 in the context of AVG axon navigation. *aex-3* genetically acts together with known players of vesicular exocytosis: *unc-64*/Syntaxin, *unc-31*/CAPS, and *ida-1*/IA-2. Furthermore our genetic interaction data suggest that AEX-3 and the UNC-6/Netrin receptor UNC-5 act in the same pathway, suggesting AEX-3 might regulate the trafficking and/or insertion of UNC-5 at the growth cone to mediate the proper guidance of the AVG axon.

KEYWORDS nervous system; axon guidance; pioneer; GEF; vesicle trafficking

PRECISE assembly of neuronal networks is a hallmark of a functional nervous system. Building these networks begins with early outgrowing axons from neurons called “pioneers.” Pioneer neurons form the initial axon scaffold used by the later outgrowing “follower” axons to extend upon. Pioneers provide guidance cues and an adhesive substrate for the follower axons to navigate properly. Sequential outgrowth of axons simplifies the problem of axonal pathfinding by allowing the majority of axons to extend along preexisting pathways rather than navigating exclusively on their own. Pioneer axons have been identified in many organisms. In grasshopper embryos, a pair of neurons (Ti) arise at the tips of the limb bud to extend axons toward the central nervous system. Follower (SGO) axons cannot extend further upon ablation of these pioneers (Klose and Bentley 1989). Similarly the *Drosophila* ventral nerve cord is pioneered by four axons forming longitudinal tracts. Ablation of these pioneers

leads to defects in the navigation of follower axons (Hidalgo and Brand 1997). The thalamus of the mouse cerebral cortex is first invaded by short-lived subplate neurons, guiding later outgrowing cortical axons to their target (McConnell *et al.* 1989). However, pioneer neurons are not always required for the guidance of follower axons (Chitnis and Kuwada 1991) and in some cases are dispensable for this purpose (Keshishian and Bentley 1983; Eisen *et al.* 1989; Cornet and Holt 1992).

In *Caenorhabditis elegans* the major longitudinal axon tract is the ventral nerve cord (VNC) (White *et al.* 1986). It consists of two axon tracts flanking the ventral midline. The right axon tract contains most of the axons (~50), whereas only around four axons form the left axon tract in the adult animal. The right side of the VNC harbors the main components of the motor circuit. This is where command interneurons connect to motor neurons, which in turn connect to nearby ventral muscles or more distant dorsal muscles. The corresponding synapses between interneurons and motor neurons can only be established between neurites in immediate contact. Even a local disorganization, *i.e.*, axons in the “wrong neighborhood” will disrupt circuitry (White *et al.* 1976). Interneuron or motoneuron axons crossing into the

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¹Corresponding author: Department of Biological Sciences, Simon Fraser University, 8888 University Dr., Burnaby, BC, Canada V5A 1S6. E-mail: hutter@sfu.ca

left axon tract will not be able to establish the correct synaptic connections, unless their synaptic partners happen to join them.

Pioneers play an important role in creating this local organization within the *C. elegans* VNC (Durbin 1987; Garriga *et al.* 1993). The AVG neuron extends the first axon and pioneers the right VNC axon tract followed by motor neuron and interneuron axons in a defined order (Durbin 1987). Removal of the AVG axon early in development does not prevent the outgrowth of follower axons. The VNC still forms but is disorganized with axons crossing between right and left tracts (Durbin 1987; Hutter 2003). The left axon tract is pioneered by the PVPR axon from the posterior side (Durbin 1987). The left axon tract fails to form in the absence of the PVPR axon (Durbin 1987; Garriga *et al.* 1993), suggesting that no other neuron can pioneer this axon tract. Under such circumstances the follower axons extend in the already established right axon tract (Durbin 1987; Garriga *et al.* 1993).

Extracellular guidance cues also mediate outgrowth and navigation of axons and pioneer axons exclusively dependent on these guidance cues to navigate. **UNC-6** (Netrin in vertebrates) is a laminin-like secreted protein that forms a gradient along the dorsoventral axis and is an essential cue for axons and cells migrating in a dorsoventral direction (Hedgecock *et al.* 1990; Ishii *et al.* 1992; Wadsworth *et al.* 1996; Wadsworth 2002). Cells and axons expressing the **UNC-6** receptor **UNC-40** (DCC in vertebrates) are attracted by **UNC-6**, whereas those expressing both **UNC-40** and **UNC-5** receptors are repelled, illustrating that response to a guidance cue can depend on receptor interactions within the neuron (Hedgecock *et al.* 1990; Ishii *et al.* 1992; Leung-Hagesteijn *et al.* 1992; Chan *et al.* 1996). **UNC-129**, a member of the TGF- β family, also affects dorsoventral migrations by promoting **UNC-40** + **UNC-5** signaling (Colavita *et al.* 1998; MacNeil *et al.* 2009). Dorsally expressed **SLT-1**/Slit repels axonal growth cones expressing the corresponding **SAX-3** receptors toward the ventral side (Zallen *et al.* 1998). Finally, **NID-1**/Nidogen, a basement membrane protein, is essential for correct positioning of axons in the sublateral nerve cord and VNC (Kim and Wadsworth 2000).

None of the known guidance cues substantially affects AVG axon navigation (Hutter 2003). Moreover, direct genetic screens for mutants affecting AVG navigation have yielded only a few new genes (Moffat *et al.* 2014; Bhat *et al.* 2015). This emphasized the need for other strategies like modifier screens to uncover AVG axon guidance genes. A good starting point for enhancer screens are *nid-1* mutants, which have weakly penetrant AVG axon guidance defects, but are healthy and viable. We isolated an allele of *aex-3* in an enhancer screen for AVG axon guidance defects in a *nid-1* mutant background. *aex-3* mutant animals display highly penetrant AVG axon guidance defects, which are dependent on *nid-1*. **AEX-3**, the homolog of the vertebrate MAP kinase-activating death domain (MADD) protein, is a guanine nucleotide exchange

Table 1 AVG cross-over (CO) defects in *aex-3* mutants with and without *nid-1* (% animals with defects)

Genotype	AVG CO	n
<i>aex-3(hd148)</i>	4*	104
<i>aex-3(hd148);nid-1</i>	56**	97
<i>aex-3(n2166)</i>	3 ns	74
<i>aex-3(n2166);nid-1</i>	46**	112
<i>aex-3(sa5)</i>	3 ns	95
<i>aex-3(sa5);nid-1</i>	43**	130
<i>aex-3(js815)</i>	4*	121
<i>aex-3(js815);nid-1</i>	39**	112
<i>nid-1(cg119)</i>	10**	118
Wild type	0	117

Marker used: *hds51[odr-2::tdTomato]*. n = number of animals. For statistical significance, single mutants were compared with wild type and double mutants with *nid-1* single mutant. * $P < 0.05$; ** $P < 0.01$; ns, not significant; χ^2 test.

factor (GEF) for the Rab3 and Rab27 GTPases (Iwasaki *et al.* 1997; Mahoney *et al.* 2006), which control various aspects of vesicle trafficking in the cell (Wada *et al.* 1997; Hutagalung and Novick 2011; Zerial and McBride 2001). Our genetic interaction data suggest that **AEX-3** activates Rab3, but not Rab27. We found *aex-3* genetically interacts with **UNC-64**/Syntaxin, a SNARE component important for exocytosis of synaptic vesicles (Saifee *et al.* 1998) and dense core vesicles (Singer-Lahat *et al.* 2008). *aex-3* also interacts with **UNC-31**/CAPS and **IDA-1**/IA-2, which are known to be involved in dense core vesicle release (Cai *et al.* 2004; Speese *et al.* 2007). In addition we found both **UNC-6**/Netrin and its receptor **UNC-5** have *nid-1*-dependent AVG axon guidance defects. Genetic interaction data suggest *aex-3* and *unc-5* are in the same genetic pathway, suggesting that **AEX-3** regulates the trafficking of the **UNC-5** receptor to the growth cone and/or its insertion into the membrane at the growth cone.

Materials and Methods

Nematode strains and alleles used

The following strains were used for phenotypic analysis: VH1775: *hds51[odr-2::tdTomato, rol-6(su1006)]* X; VH1592: *zdis13[tph-1::GFP]* IV; VH1811: *hds54[flp-1::GFP,sra-6::plum, pha-1(+)]*; VH15: *rhIs4[glr-1::GFP, dpy-20(+)]* III; VH612: *hds24[unc-129::CFP, unc-47::DsRed2]*; VH854: *hds36[rgef-1::DsRed2]*; and VH1762: *lsls1722[W05H12.1::GFP, unc-119(+)]*. **UNC-5** overexpression strain is *evIs98c [unc-5p::UNC-5::GFP]* (Levy-Strumpf and Culotti 2014).

The following alleles were used for complementation: MT5475: *aex-3(n2166)* X; JT5: *aex-3(sa5)* X; and CX4103: *sax-1(ky211)* X.

The following alleles were used for phenotypic analysis and genetic interaction studies: VH2500: *aex-3(hd148)* X; MT5475: *aex-3(n2166)* X; JT5: *aex-3(sa5)* X; NM2739: *aex-3(js815)* X; NM791: *rab-3(js49)* II; JT24: *aex-6(sa24)* I; KY46: *cab-1(tg49)* X; CB169: *unc-31(e169)* IV; CB246: *unc-64(e264)* III; CB1265: *unc-104(e1265)* II; NM1657: *unc-10(md1117)* X; VH764: *unc-6(ev400)* X; VH10: *unc-5(e53)* IV; VH28: *unc-40(e271)* I; VC226: *ida-1(ok409)* III; and

Figure 1 Molecular analysis of *aex-3* and schematics of the VNC. (A) Gene model for *aex-3* with exons shown as light blue shaded boxes and introns as dark brown lines. The gray shaded boxes in the beginning and end represent the 5' and 3' untranslated regions, respectively. The location for the *hd148* and *js815* alleles is also shown. *hd148* is a point mutation in the beginning of intron 6, which affects splicing and leads the formation of the stop codon. *js815* is a 555-bp deletion removing 159–264 aa (Mahoney *et al.* 2006), which changes the reading frame and leads the formation of the stop codon. (B) Domain organization of the AEX-3 protein. The positions of mutations are also indicated. (C) Schematic showing different types of neurons present in the VNC. The position of cell bodies and neuronal processes are also shown. Left–right and anterior–posterior sides are as indicated. For simplicity, only some of the motor neurons are shown.

CH119: *nid-1*(*cg119*) V. The strains were cultured and maintained at 20° under standard conditions (Brenner 1974).

All our experimental strains generated from VH2500: *aex-3*(*hd148*); *hdIs51*, which harbors a closely linked point mutation in *sax-1*, may also contain the *sax-1* mutation.

Isolation of the *hd148* allele of *aex-3*

The *hd148* allele of *aex-3* was isolated after EMS mutagenesis of *nid-1*; *hdIs51* [*odr-2::tdTomato, rol-6*(*su1006*)] animals in a F₂ semiclinal screen for axon guidance defects in the ventral cord pioneer AVG. Briefly, after EMS treatment, healthy P0s were allowed to self-propagate for two generations. F₂ animals were then cloned and their progeny were analyzed under the dissecting fluorescence microscope for AVG axon cross-over defects leading to the identification of several mutants, including *hd148*. The *hd148* mutant was backcrossed four times, and the whole genome was sequenced to identify changes in the coding regions of all genes. The *hd148* mutation was linked to the X chromosome by crossing it with *nid-1* males and then evaluating the phenotype of F₁ males. *aex-3* was picked as a potential candidate as the phenotypes (egg laying and body movement) of *aex-3* matched with the isolated mutant. Complementation tests confirmed that *hd148* is an allele of *aex-3*.

Fosmid rescue and expression constructs

The *aex-3* containing fosmid (WRM0629dA08) was injected (1 ng/μl) into *nid-1* mutant animals along with the coinjection marker *myo-2::GFP* (5 ng/μl) and filler DNA pBluescript KS (–) (94 ng/μl) to create transgenic lines. The *nid-1* males with the *hdEx602* [WRM0629dA08, (*aex-3*), *myo-2::GFP*] extrachromosomal array were then crossed with *aex-3*(*hd148*); *nid-1* mutant animals, and the mutant animals with *hdEx602* array were analyzed for the AVG axon cross-over defects. By injecting >600 adult animals we obtained only ~60 F₁'s and out of these, only one stable transgenic line, suggesting that the injected DNA might cause developmental problems.

Other groups who worked with *aex-3* encountered similar problems (Iwasaki *et al.* 1997; Iwasaki and Toyonaga 2000).

To express *aex-3* specifically in the AVG neuron, an enhancer fragment of *lin-11*, which drives expression specifically in the AVG neuron (B. Gupta, personal communication), and the *aex-3* cDNA were cloned into GFP vector pPD95.75 (Fire vector kit). This construct (5–15 ng/μl) was injected along with coinjection marker *unc-122::GFP* (45 ng/μl) into *nid-1* mutant animals. The *nid-1* males with an extra-chromosomal array were then crossed with *aex-3*(*hd148*); *nid-1* mutant animals and the animals from three independent transgenic lines were analyzed for AVG axon cross-over defects. Transgenic lines for fosmid as well as expression vector were generated as described (Mello *et al.* 1991).

Construction of triple mutants

Triple mutants were constructed according to Iwasaki *et al.* (1997). The following primers were used to confirm the genotypes of strains containing the *rab-3* (*js49*) and *aex-3*(*hd148*) mutants, both of which are point mutations not causing an unambiguous movement defect like *unc-31* and *unc-64*. The following forward 5' CCAGCAGACAATA CTTCGCC 3' and reverse 5' CTCCTTGGCTGATGTTTCG 3' primers were used to amplify a 610-bp PCR fragment from *rab-3* genomic sequence including the change (G to A) in the *rab-3*(*js49*) mutant. Similarly the forward 5' AGCACTTTTA TACCCACTGG 3' and reverse primer 5' CTGGACAATGA TGCTTTATTTCAG 3' primers were used to amplify a 529-bp PCR fragment from *aex-3* genomic sequence including the change (G to A) in the *aex-3*(*hd148*) mutant.

Phenotypic analysis of neuronal defects

Axonal defects were scored with a Zeiss Axiscope (40× objective) in adult animals expressing fluorescent markers in respective neurons. Animals were immobilized with 10 mM

sodium azide in M9 buffer for 1 hr and mounted on 3% agar pads before analysis.

Microscopy

Confocal images of mixed stage population of animals with respective fluorescent proteins were acquired on a Zeiss Axioplan II microscope (Carl Zeiss) connected to a Quorum WaveFX spinning disc system (Quorum Technologies). Stacks of confocal images with 0.2 to 0.5 μm distance between focal planes were recorded. Image acquisition and analysis was carried out using Volocity software (Perkin-Elmer, Waltham, MA). Images in the figures are maximum intensity projections of all focal planes. Figures were assembled with Adobe Photoshop CS8.0 (Adobe, San Jose, CA).

Statistical analysis

We used chi-square tests (χ^2) to determine the statistical significance of differences in phenotypes. Multiple comparisons were corrected by a Bonferroni correction, where the given alpha value (α) was divided by the number of comparisons (n) made so that for statistical significance in multiple comparisons individual P values are $\leq \alpha/n$.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Identification of a novel allele of *aex-3*

The molecular basis for AVG axon guidance is only partially understood. We performed enhancer screens in a *nid-1* mutant background to identify novel regulators of AVG navigation. We used the deletion allele *nid-1(cg119)*, which deletes 3133 nucleotides from 1499 to 4631 and is considered a molecular null of *NID-1* (Kang and Kramer 2000). One of the alleles we isolated, *hd148*, is a point mutation (G to A) in the splice donor at the beginning of the 6th intron of *aex-3*, preventing the removal of this intron and resulting in the early truncation of the protein after 388 of 1409 amino acids (Figure 1, A and B). Previously isolated *aex-3* mutant animals have some locomotion and egg laying defects (Thomas 1990; Iwasaki *et al.* 1997; Mahoney *et al.* 2006) similar to defects seen in *hd148*. Three known alleles of *aex-3*: *n2166*, *sa5*, and *js815* (Thomas 1990; Mahoney *et al.* 2006), showed AVG axon cross-over defects in a *nid-1* mutant background (Table 1). Two of the alleles, *n2166* and *sa5*, failed to complement *hd148* for the AVG navigation defects. Complementation tests were done so that half of the progeny were *hd148/n2166; nid-1/nid-1* and the remaining half *hd148/n2166; nid-1/+*. This mixture showed 31% AVG cross-over defects ($n = 70$) and similarly the mixture of *hd148/sa5; nid-1/nid-1* and *hd148/sa5; nid-1/+* F_1 animals showed 32% AVG cross-over defects ($n = 84$). Since the AVG cross-over defects of

Table 2 AVG cross-over defects are rescued by an *aex-3*-containing fosmid, expression of the cDNA in AVG and *unc-5* overexpression (% animals with defects)

Genotype	AVG CO	<i>n</i>
<i>aex-3</i> fosmid (WRM0629Da08)		
<i>aex-3(hd148); nid-1; hdEx602(+)</i>	24**	102
<i>aex-3(hd148); nid-1; hdEx602(-)</i>	50 ns	104
<i>aex-3</i> cDNA		
<i>aex-3(hd148); nid-1; hdEx610(+)</i>	18**	128
<i>aex-3(hd148); nid-1; hdEx610(-)</i>	45 ns	95
<i>aex-3(hd148); nid-1; hdEx611(+)</i>	16**	130
<i>aex-3(hd148); nid-1; hdEx611(-)</i>	48 ns	100
<i>aex-3(hd148);nid-1; hdEx612(+)</i>	13**	116
<i>aex-3(hd148); nid-1; hdEx612(-)</i>	47 ns	94
<i>unc-5</i> overexpression		
<i>aex-3(hd148); nid-1; evls98c</i>	25**	132

Marker used: *hds51[odr-2::tdTomato]*. *n* = number of animals. For rescue experiments, animals with (+) and without (−) extrachromosomal arrays were counted. For the *aex-3* rescue the following independent transgenic lines were analyzed: *hdEx602* [WRM0629dA08; *myo-2::GFP*]; *hdEx610*, 611, and 612 [*plin-11::aex-3* cDNA::GFP; *unc-122*]; and *evls98c* [*unc-5p::UNC-5::GFP*]. For statistical significance, we compared transgenic lines with *aex-3(hd148); nid-1* double mutant. ** $P < 0.01$; ns, not significant; χ^2 test.

hd148 mutants are strictly dependent on the *nid-1* mutation, these defects are not different from 56% AVG cross-over defects shown by our *hd148* alone, if the 50% *nid-1* heterozygote progeny are ignored, indicating that *hd148* does not complement known alleles of *aex-3*. As revealed by whole genome sequencing the *hd148*-containing strain also carries a missense mutation in *sax-1*, a gene closely linked to *aex-3*. *sax-1* is required for neurite initiation and outgrowth in sensory neurons (Zallen *et al.* 2000). However, *sax-1(ky211)* complements *hd148* for AVG navigation defects as the mixture of *hd148/ky211; nid-1/nid-1* and *hd148/ky211;nid-1/+* F_1 animals showed only 10% AVG cross-over defects ($n = 62$), suggesting that the mutation in *sax-1* does not contribute to the navigation defects seen in our isolate. In addition, a fosmid containing the *aex-3* gene rescued the AVG axon cross-over defects of *aex-3(hd148); nid-1(cg119)* mutant animals. The rescuing line shows only 24% AVG axon cross-over defects, significantly fewer than the 56% defects seen in *aex-3; nid-1* mutant animals (Table 2). Taken together, these data suggest that *hd148* is an allele of *aex-3*.

aex-3 mutant animals show *nid-1*-dependent AVG axon navigation defects

In wild-type animals the AVG axon pioneers the right axon tract of the VNC extending from the anterior end and terminating in the tail (Figure 1C and Figure 2, A, C, and E). In 56% of *aex-3(hd148); nid-1* mutant animals the AVG axon crosses the ventral midline from right to left, referred to as AVG cross-over (AVG CO) defects. These cross-overs can occur anywhere along the anterior–posterior axis (Figure 2, B, D, and F), but occur more frequently in the anterior half of the animal. After crossing, AVG axons remain in the left axon tract in the majority of animals. However, in 19% of the mutant animals, AVG axons cross back to the right axon tract. AVG cross-over defects in *aex-3* are almost completely dependent

Figure 2 AVG cross-over (CO) defects in *aex-3(hd148); nid-1* mutant animals. (A, C, and E) wild type; (B, D, and F) *aex-3(hd148); nid-1*. (A, C, and E) In wild-type animals, AVG extends an axon from its cell body (arrow) on the right side of the VNC that never crosses the ventral midline. In the majority of the *aex-3(hd148); nid-1* mutant animals the AVG axon crosses the ventral midline somewhere in the anterior half (B and D) and in some animals in the posterior half of the animal (F) and joins the left axon tract of the VNC. The AVG axon stays mostly on the left side of the VNC after crossing the midline but in some animals it switches back to the right side. Arrows indicate the position of the AVG cell body, arrowheads indicate the position of AVG axon cross-over, and the stars mark the vulva position. Marker used: *odr-2::tdTomato*. Bar: 10 μ m.

on *nid-1*, as *aex-3(hd148)* on its own has only 4% defects (Table 1), whereas *nid-1* single mutant animals have ~10% AVG cross-over defects (Table 1). Three other alleles (*n2166*, *sa5*, and *js815*) of *aex-3* show *nid-1*-dependent AVG cross-over defects with a penetrance similar to *hd148* (Table 1). Defects are somewhat higher in *hd148*, but not significantly different from the other alleles except for *js815*, which has the least penetrant defects (Table 1). The molecular nature of the *hd148* allele—expected to lead to an early truncation of the protein—matches well with the strong defects observed and suggests it is a strong loss-of-function allele.

Follower axon navigation is disrupted in *aex-3; nid-1* mutant animals

Since the AVG pioneer is important for correct organization of the VNC, we wanted to test whether follower axons are also affected, and we evaluated *aex-3(hd148); nid-1(cg119)* mutant animals with neuron-specific markers for follower defects. Command interneurons initially exit the nerve ring in two fascicles, one each on the right and left sides. Immediately after leaving the nerve ring, the left side fascicle crosses and extends into the right axon tract and both fascicles follow the AVG axon (Figure 1C and Figure 3E). In *aex-3; nid-1* double mutant animals, 49% of animals show cross-over defects where interneuron axons cross from the right to the left side of the VNC (Figure 3F; Table 3). Among these, 35% are AVG dependent, *i.e.*, interneurons cross the midline at the same position where the AVG axon crosses (Figure 3F; Table 3). In the remaining 14% of animals, interneuron axons cross independently of the AVG axon (Table 3). This suggests that while the majority of the interneuron cross-over defects are likely secondary consequences of AVG defects, some of the defects are likely primary defects in interneurons themselves.

The two AVK neurons also send axons into the VNC from the nerve ring. However in this case one of them extends in the left tract, whereas the other one extends in the right tract (Figure 1C and Figure 3A). In *aex-3; nid-1* double mutant animals we observed that both AVKR/L axons frequently cross the ventral midline with equal penetrance (Figure 3B; Table 3). However we did not observe any correlation between the AVG and AVK axon cross-overs, suggesting that

these defects are likely independent. In some mutant animals AVK axons terminate prematurely, but this is not significantly different from *nid-1* single mutants (Table 3).

The hermaphrodite-specific neurons (HSN) neurons are a symmetrical pair of neurons born in the tail during embryogenesis. They migrate toward the midbody, where each neuron extends an axon on either side of the ventral midline along the VNC toward the nerve ring (Figure 1C and Figure 3C). *nid-1* single mutants have highly penetrant HSN cross-over defects, which are not enhanced in *aex-3; nid-1* double mutant animals (Figure 3D; Table 3). However in *aex-3; nid-1* double mutant animals some HSN neurons fail to migrate properly, defects not seen in *nid-1* single mutants (Table 3).

***aex-3; nid-1* mutant animals show motor neuron axon navigation defects**

The VNC in *C. elegans* contains essential components of the motor circuit. DD/VD GABAergic motor neuron cell bodies are located along the ventral midline and send out processes in the right axon tract that branch and extend commissures dorsally (Figure 1C and Figure 4, A, C, and E). In *aex-3; nid-1* double mutants, 28% of animals show DD/VD axon guidance defects, where motor neuron axons cross from the right to the left tract of the VNC (Figure 4B; Table 3). These defects are mostly dependent on AVG, since DD/VD axons cross together with the AVG axon. Additionally in 36% of *aex-3; nid-1* mutant animals, some DD/VD axons grow in the left rather than the right axon tract, which makes the VNC symmetrical in appearance (symmetric VNC) (Figure 4, B and D; Table 3). DD/VD commissures individually (as pioneers) navigate toward the dorsal side, where they extend to form the dorsal nerve cord (DNC) (Figure 4, E and G). A total of 65% of *nid-1* single mutants have commissural defects, where on average, 5 of 19 commissures per animal fail to reach the dorsal cord leading to gaps (DNC gaps, Table 3). In *aex-3* single mutants, 14% of animals have commissural defects (1 of 19 commissures fails to reach the dorsal side, *i.e.*, <1% of commissures show defects) with fewer gaps in the DNC (Table 3). We observed that in *aex-3; nid-1* double mutant animals these defects are enhanced to 100%, with every animal showing commissural defects (7 of 19 commissures per animal fail to

Figure 3 Interneuron, AVK, and HSN axon defects in *aex-3(hd148); nid-1* mutant animals. (A, C, and E) Wild type; (B, D, F, F', and F'') *aex-3(hd148); nid-1*. (A) In wild-type animals, AVK axons extend in both VNC axon tracts and never cross the midline. (B) In *aex-3(hd148); nid-1* mutant animals, AVK axons frequently cross the ventral midline (arrowhead). (C) In wild-type animals, HSN axons are ipsilateral and extend axons in both VNC axon tracts on their respective sides. (D) In the majority of the *aex-3(hd148); nid-1* mutant animals, the left HSN axon crosses the ventral midline and joins the right axon tract (arrowhead). (E) In wild-type animals, command interneurons extend into the right axon tract of the VNC. (F and F') In *aex-3(hd148); nid-1* mutant animals, the command interneurons cross the midline frequently together with the AVG axon and extend into the left axon tract of the VNC. F'' is the merged image of F and F'. Stars mark the vulva position. The dashed lines (white) mark the normal axon trajectories. Markers used: *glr-1::GFP* (interneurons), *flp-1::GFP* (AVK), and *tph-1::GFP* (HSN). Bar: 10 μ m.

reach the DNC) and gaps in the DNC (Figure 4, F and H; Table 3). This suggests that *aex-3* is required independently of *nid-1* for the guidance of commissures and the proper formation of the DNC.

DA/DB motor neuron cell bodies are also located along the ventral midline. They send their dendrites into the right axon

Table 3 Other neuronal defects in *nid-1* single and *aex-3(hd148); nid-1* double mutants (% animals with defects)

Phenotype	<i>aex-3(hd148)</i>	<i>nid-1(cg119)</i>	<i>aex-3(hd148); nid-1</i>
Interneuron cross-over defects			
AVG dependent ^a	6 (101)	6 (114)	35** (104)
AVG independent ^b	5 (101)	0 (114)	14** (104)
AVK defects			
VNC cross-over	6 (107)	18 (115)	49** (109)
Premature stop	0 (107)	12 (115)	22 ns (109)
Leaving VNC	0 (107)	1 (115)	3 ns (109)
HSN neurons			
VNC cross-over	9 (132)	58 (103)	68 ns (109)
Undermigration and VNC cross-over	6 (132)	3 (103)	16** (109)
Motor neuron defects			
DD/VD			
VNC cross-over ^c	6 (102)	8 (133)	28** (123)
Symmetrical VNC	0 (102)	0 (133)	36** (123)
Commissural defects	14 (102)	65 (133) ^d	100** (123) ^e
DNC gaps	31 (95)	85 (53)	100* (26)
DA/DB			
VNC cross-over ^f	6 (101)	8 (110)	29** (103)
Symmetrical VNC	0 (101)	0 (110)	20** (103)
Commissural defects	0 (101)	0 (110)	18** (103)

Values in parentheses indicate *n*. Markers used: Interneuron (*glr-1::GFP*), AVK (*flp-1::GFP*), HSN (*tph-1::GFP*), DD/VD/Commissures (*unc-47::DsRed-2*), and DA/DB (*unc-129::CFP*). For statistical significance, *aex-3; nid-1* double mutants are compared with *nid-1* single mutant. * $P < 0.05$; ** $P < 0.01$; ns, not significant; χ^2 test.

^a Interneurons cross the midline together with AVG.

^b Interneurons cross the midline without AVG.

^c DD/VD axons cross the midline at the same position where the AVG axon crosses.

^d On average, 5 of 19 commissures fail to reach the DNC per animal.

^e On average, 7 of 19 commissures fail to reach the DNC per animal.

^f DA/DB axons cross the midline at the same position where the AVG axon crosses.

tract of the VNC and axons toward the DNC (Figure 1C and Figure 4, I, K, and M). We found in 29% of *aex-3; nid-1* double mutant animals, DA/DB motor axons cross from the right to the left axon tract of the VNC (Figure 4J; Table 3). These defects are mostly dependent on AVG, as cross-overs occur together with the AVG axon, again suggesting these are secondary defects. In addition, some DA/DB motor neurons extend dendrites into the left axon tract (Figure 4L; Table 3). We observed very few DA/DB commissural defects (Figure 4N) and no DNC gaps in *aex-3; nid-1* double mutant animals, suggesting commissural defects are neuron specific.

We used a panneuronal marker to assess the overall state of the nervous system as well as selected neurons with axons outside the VNC. We did not observe guidance defects in touch receptor axons, other longitudinal axons, or neuronal processes in the head region (data not shown). Defects in *aex-3; nid-1* mutant animals are largely confined to the VNC and some commissural axons.

AEX-3 activation of RAB-3 GTPase is required for AVG navigation

AEX-3 is a GEF and regulates the activity of the RAB-3 and AEX-6/Rab27 GTPases (Mahoney *et al.* 2006). Both bind to synaptic vesicle precursors in their GTP form and regulate trafficking of these vesicles (Nonet *et al.* 1997; Mahoney *et al.* 2006). In wild type, RAB-3 and AEX-6/Rab27 are localized to axons and enriched in synaptic regions, whereas in *aex-3* mutant animals, RAB-3 as well as AEX-6/Rab27 are mislocalized to the cell body (Iwasaki and Toyonaga 2000; Mahoney *et al.* 2006). We found that mutations in *nid-1* do not affect RAB-3 localization. Even though both RAB-3 and AEX-6/Rab27 are activated by AEX-3, they act through different downstream effectors (Mahoney *et al.* 2006). To test whether AEX-3 regulates RAB-3 or AEX-6/Rab27 (or both) in the context of AVG axon navigation, we constructed *rab-3; nid-1* and *aex-6; nid-1* double mutants and evaluated them for AVG axon cross-over defects. *rab-3; nid-1* mutant animals show AVG axon cross-over defects with a penetrance similar

Figure 4 DD/VD and DA/DB motor neuron defects in *aex-3(hd148); nid-1* mutant animals. (A, C, E, G, I, K, and M) Wild type; (B, D, F, H, J, L, and N) *aex-3(hd148); nid-1*. (A, C, E, and G) In wild-type animals, DD/VD motor neurons extend axons anteriorly in the right axon tract that branch and extend commissures toward the dorsal side to form the DNC. In *aex-3(hd148); nid-1* mutant animals motor neuron axons either cross the ventral midline (arrowhead) and extend into the left axon tract (B) or grow directly into the left axon tract (arrow) (B and D). Some DD/VD commissures (F) fail to reach the dorsal cord, resulting in gaps (H, arrow with two heads). (I, K, and M) In wild-type animals, DA/DB motor neurons extend dendrites into the right axon tract and commissures that grow from the cell body toward the dorsal cord. (J, L, and N) In *aex-3(hd148); nid-1* mutant animals, DA/DB dendrites cross the ventral midline (arrowhead) and extend in the left axon tract (J) or grow directly in the left axon tract (L, arrow). Some commissures show navigation defects (N). Stars mark the vulva position. Markers used: *unc-47::DsRed2* (DD/VD), *unc-129::CFP* (DA/DB). Bar: 10 μ m.

to *aex-3; nid-1*, whereas *aex-6; nid-1* double mutants do not (Figure 5). *rab-3* single mutants have weakly penetrant AVG axon cross-over defects and *aex-6* single mutants do not have any such defects (Figure 5). The penetrance of AVG axon cross-over defects in *aex-3; rab-3; nid-1* triple mutant animals is not significantly different from *aex-3; nid-1* double mutants (Figure 5), suggesting that *aex-3* and *rab-3* are in the same genetic pathway. This indicates that activation of *RAB-3* but not *AEX-6/Rab27* is required for AVG navigation.

AEX-3 physically interacts with *CAB-1* to regulate defecation, a pathway distinct from the *rab-3* pathway (Iwasaki and Toyonaga 2000). We tested *cab-1; nid-1* double mutant animals for AVG axon cross-over defects and observed only a mild enhancement of the defects (Figure 5), suggesting *CAB-1* does not have a major role in the context of AVG navigation.

Since *RAB-3*-associated vesicles are transported by the *UNC-104/Kinesin-3* motor in *C. elegans* (Hall and Hedgecock 1991; Nonet *et al.* 1997), we wanted to test whether *UNC-104* plays any role here. AVG axon cross-over defects are enhanced in *unc-104; nid-1* double mutant animals compared to either single mutant, but these defects are less penetrant compared to *aex-3; nid-1* or *rab-3; nid-1* double mutants (Figure 5). However, the penetrance of AVG cross-over

defects in *aex-3; unc-104; nid-1* triple mutants is not significantly different from the *aex-3; nid-1* double mutants (Figure 5). This suggests that *aex-3* and *unc-104* are in the same genetic pathway and that *AEX-3* could be involved in transport of vesicles in outgrowing axons in part mediated through *UNC-104*.

UNC-10/Rim is a *RAB-3* effector molecule, which interacts with *RAB-3* during priming of synaptic vesicles prior to their release at the synapse (Koushika *et al.* 2001). *unc-10* single mutants have no AVG axon cross-over defects, and *nid-1* defects are not enhanced in *unc-10; nid-1* double mutants (Figure 5), indicating that *UNC-10/Rim* is not involved in the navigation of the AVG axon. This is not unexpected as there are no synapses at that stage of pioneer axon outgrowth. In summary, the above data suggest that *AEX-3* activates *RAB-3* but not *AEX-6/Rab27* in the context of AVG navigation and is potentially involved in *UNC-104*-dependent vesicle transport.

Given that *AEX-3* regulates *RAB-3* in the context of AVG navigation, one would expect *AEX-3* to act in a cell-autonomous manner. To determine whether *AEX-3* is required within the AVG neuron for its axon navigation, we expressed an *AEX-3* complementary DNA (cDNA) specifically in the AVG neuron in *aex-3; nid-1* double mutants. We evaluated the AVG axon

Figure 5 *aex-3* and *rab-3* act in the same genetic pathway in AVG axon navigation. Columns are percentages of animals with AVG midline crossing defects ($\pm$ SE) with genotypes as indicated. In the bar diagram, the single mutants are represented by black bars, the double mutants by blue bars, and the triple mutants by green bars. For each strain, $n > 100$ animals except for *aex-3*; *nid-1* ($n = 97$). χ^2 tests were used to establish statistical significance between the mutants. Single mutants were compared with wild type, double mutants with *nid-1* single mutant, and the triple mutants with *aex-3*; *nid-1* double mutant (* $P < 0.05$; ** $P < 0.01$; ns, not significant). Mutant alleles used: *aex-3*(*hd148*), *nid-1*(*cg119*), *rab-3*(*js49*), *aex-6*(*sa24*), *cab-1*(*tg49*), *unc-104*(*e1265*), and *unc-10*(*md1117*). Marker used: *odr-2::tdTomato*.

cross-over defects in these transgenic lines and observed that *AEX-3* was able to rescue the AVG axon cross-over defects in all three independent transgenic lines (Table 2). For all three lines, both animals with and without extrachromosomal arrays were counted. Defects were only rescued in animals containing the extrachromosomal array (Table 2). Thus *AEX-3* is required in the AVG neuron for its axon navigation and acts in a cell-autonomous manner.

***AEX-3*, *UNC-31/CAPS*, *IDA-1/IA-2*, and *UNC-64/Syntaxin* act in the same genetic pathway**

aex-3 genetically interacts with *unc-31* and *unc-64* in dauer formation constitutive (*Daf-c*) phenotype (Iwasaki *et al.* 1997). *UNC-31* is the nematode homolog of CAPS (Ca^{2+} -dependent secretion activator) and is required for secretion of dense core vesicles (Livingstone 1991; Walent *et al.* 1992; Speese *et al.* 2007). *unc-64* encodes the nematode homolog of Syntaxin, a SNARE component involved in the fusion of synaptic vesicles (Saifee *et al.* 1998). Recently it has been shown that Syntaxin1 binds to the guidance receptor DCC in the growth cone, where it is required for chemoattraction of migrating axons to the guidance cue Netrin (Cotrufo *et al.* 2012). We wanted to test whether *unc-31* and *unc-64* mutants have AVG axon guidance defects similar to those seen in *aex-3*. Neither *unc-31* nor *unc-64* single mutants have AVG axon cross-over defects; however, both mutants enhance AVG defects of *nid-1* mutants but do not enhance *aex-3* mutant defects (Figure 6). Both *unc-31*; *nid-1* and *unc-64*; *nid-1* double mutants have defects that are significantly less penetrant than defects in *aex-3*; *nid-1* double mutants (Figure 6). However, both *unc-31*; *aex-3*; *nid-1* and *unc-64*; *aex-3*; *nid-1* triple mutants are not significantly different from defects in *aex-3*; *nid-1* double mutants (Figure 6), suggesting that all three genes act in the same pathway.

unc-31 has been shown to interact with *ida-1* for dense core vesicle exocytosis (Cai *et al.* 2004). *ida-1*; *nid-1* double

mutant animals have AVG axon cross-over defects with a penetrance close to *aex-3*; *nid-1* double mutants, whereas *aex-3*; *ida-1* double mutants do not show these defects (Figure 6). Defects in *ida-1*; *aex-3*; *nid-1* triple mutant are not enhanced compared to *aex-3*; *nid-1* double mutants (Figure 6), again suggesting that these genes act in the same pathway.

AEX-3* might act through the Netrin receptor *UNC-5

Since Syntaxin1 (*UNC-64*) has recently been directly implicated in Netrin-mediated axonal navigation (Cotrufo *et al.* 2012), we examined whether *UNC-6*/Netrin is involved in AVG axon navigation. *unc-6* mutant animals display only weakly penetrant AVG axon cross-over defects similar to *nid-1* mutant animals (Figure 7). AVG defects are significantly enhanced in *unc-6*; *nid-1* double mutants, however not as much as in *aex-3*; *nid-1* double mutants (Figure 7). This suggests *unc-6* indeed plays a role in the navigation of the AVG axon. Two receptors, *UNC-40/DCC* and *UNC-5*, mediate responses to *UNC-6*/Netrin in *C. elegans*. *unc-5* mutants have mild AVG cross-over defects, but *unc-40* mutants do not and *unc-5* defects are not enhanced in *aex-3*; *unc-5* double mutants (Figure 7). *unc-5*; *nid-1* double mutant animals have penetrant AVG cross-over defects comparable to *aex-3*; *nid-1* (Figure 7). These defects are not further enhanced in *unc-5*; *aex-3*; *nid-1* triple mutants. In contrast, *unc-40* does not enhance *nid-1* defects (Figure 7). This suggests *UNC-5*, but not *UNC-40*, is required for the navigation of the AVG axon.

The above data suggest that *AEX-3* might regulate trafficking of the *UNC-5* receptor to the AVG growth cone or insertion of the receptor into the cell membrane of the growth cone. Since *aex-3* mutants do not show an *unc-5* loss-of-function phenotype, *aex-3* cannot be absolutely required for transport of *UNC-5* but it could reduce the effectiveness of the transport such that navigation defects become apparent only in a sensitized (*i.e.*, *nid-1* mutant) background. We

Figure 6 *aex-3*, *ida-1*, *unc-31*, and *unc-64* act through the same genetic pathway in AVG axon navigation. Columns are percentages of animals with AVG midline crossing defects ($\pm$ SE) with genotypes as indicated. In the bar diagram, the single mutants are represented by black bars, the double mutants by blue bars, and the triple mutants by green bars. For each strain, $n > 100$ except *aex-3; nid-1* ($n = 97$) animals. χ^2 tests were used to establish statistical significance between the mutants. Single mutants were compared with wild type, double mutants with the strongest single mutant, and the triple mutants with *aex-3; nid-1* double mutant (* $P < 0.05$; ** $P < 0.01$; ns, not significant). Mutant alleles used: *aex-3(hd148)*, *nid-1(cg119)*, *ida-1(ok409)*, *unc-31(e169)*, and *unc-64(e264)*. Marker used: *odr-2::tdTomato*.

found that a functional UNC-5::GFP overexpression construct (Levy-Strumpf and Culotti 2014) partially rescues the AVG cross-over defects of *aex-3; nid-1* mutant animals (Table 2). We then used the same UNC-5::GFP reporter to determine whether there are obvious changes in UNC-5::GFP levels in growth cones in *aex-3* single and *aex-3; nid-1* double mutant animals. While it is not technically possible to visualize the UNC-5::GFP reporter in the AVG growth cone, it can be observed in postembryonic VD growth cones as they navigate toward the dorsal cord. UNC-5::GFP expression in VD growth cones of both *aex-3* single and *aex-3; nid-1* double mutant animals is comparable to expression in wild type (Figure 8), suggesting that as expected, *aex-3* is not absolutely essential

for transport of UNC-5 to the growth cone. Since GFP expression levels and GFP distribution in individual growth cones are quite variable, we found it difficult to determine whether there are more subtle changes in UNC-5::GFP expression levels. Furthermore the UNC-5::GFP reporter is mainly localized in puncta (likely vesicles) within the growth cone and not localized to the plasma membrane as expected and as observed with an UNC-40::GFP reporter (Norris *et al.* 2014). It is therefore impossible to judge with this reporter whether insertion of UNC-5::GFP into the plasma membrane of growth cones is affected in *aex-3* mutant animals. Taken together the observations support a role for *aex-3* in trafficking of UNC-5.

Figure 7 *aex-3* and *unc-5* act through the same genetic pathway in AVG axon navigation. Columns are percentages of animals with AVG midline crossing defects ($\pm$ SE) with genotypes as indicated. In the bar diagram, the single mutants are represented by black bars, the double mutants by blue bars, and the triple mutant by a green bar. For each strain, $n > 100$ except *aex-3; nid-1* ($n = 97$) animals. χ^2 tests were used to establish statistical significance between the mutants. Single mutants were compared with wild type, double mutants with the strongest single mutant, and the triple mutant with *aex-3; nid-1* double mutant (* $P < 0.05$; ** $P < 0.01$; ns, not significant). Mutant alleles used: *aex-3(hd148)*, *nid-1(cg119)*, *unc-6(ev400)*, *unc-40(e271)*, and *unc-5(e53)*. Marker used: *odr-2::tdTomato*.

Figure 8 Localization of UNC-5::GFP in growth cones in *aex-3* mutants. Fluorescent micrographs of postembryonic VD growth cones of L2 stage larvae are shown. In wild type, *aex-3* single and *aex-3; nid-1* double mutant animals, UNC-5::GFP is localized to the growth cones as indicated by white arrows. White star highlights fluorescent granules in the gut. Bar: 5 μ m. Marker strain used: *evls98c [unc-5p::UNC-5::GFP]*.

Discussion

Ventral cord axon guidance requires aex-3 in a Nidogen-dependent manner

The AVG axon pioneers the right axon tract of the VNC in *C. elegans* and is critical for the navigation of follower axons (Durbin 1987; Hutter 2003). We isolated an allele of *aex-3* in an enhancer screen for AVG axon guidance defects in a *nid-1* mutant background. In *C. elegans*, *nid-1* encodes the sole homolog of nidogen (entactin), a basement membrane component. *nid-1* is required for the correct positioning of longitudinal axons and proper organization of presynaptic zones (Kim and Wadsworth 2000; Ackley *et al.* 2003), but not for basement membrane assembly in general. *nid-1* mutant animals thus are viable and healthy. *nid-1* mutant animals have weakly penetrant AVG axon cross-over defects, which are substantially enhanced in *aex-3; nid-1* double mutants, suggesting that *aex-3* is required for AVG axon navigation. AEX-3 is the nematode homolog of the GDP/GTP exchange factor (GEF) for Rab3/Rab27, and is expressed in most neurons (Iwasaki *et al.* 1997). AEX-3 activates the RAB-3 and AEX-6/Rab27 GTPases through distinct pathways regulating synaptic vesicle transport and exocytosis (Mahoney *et al.* 2006). Since *aex-3* and *nid-1* are not obviously in a common pathway, synergistic effects on axon pathfinding are likely due to the disruption of functionally redundant pathways in *nid-1* mutants. How *nid-1* controls axon navigation is currently unknown.

AVG pioneer navigation defects were expected to have secondary consequences on follower axon navigation (Hutter 2003). We found navigation defects in several classes of VNC follower neurons in *aex-3; nid-1* mutant animals. In many cases these axons followed misguided pioneers, suggesting these defects are indeed secondary. However, in some cases follower defects were not correlated with pioneer defects, raising the possibility that primary defects are not limited to AVG. For example, we found no correlation between AVK and AVG defects, indicating that AVK defects are not secondary consequences of AVG defects and that AVK navigation is independent of AVG. This is consistent with earlier studies, where defects in AVK axon navigation in the left axon tract were also independent of pioneer defects (Steimel *et al.* 2010; Unsoeld *et al.* 2012). The idea that *aex-3* defects are

not limited to the VNC pioneer is strengthened by the observation of synergistic effects in *aex-3; nid-1* double mutants in commissural navigation, which is completely independent of AVG navigation. On the other hand, visualization of the entire nervous system suggests that the overall structure of the nervous system is intact in *aex-3; nid-1* mutant animals and that the defects are largely limited to the VNC and commissures.

We did not observe any misplacement of neuronal cell bodies (with one exception, see below), indicating that neuronal cell migration is not affected in *aex-3; nid-1* double mutants. However, in a small fraction (16%) of animals HSN neurons fail to reach their normal position at the vulva. HSN migration is controlled by Wnt signaling and both *egl-20*/Wnt and *mig-1*/Frizzled mutants display similar but more penetrant HSN defects (Pan *et al.* 2006). Mutations in other Wnts or Frizzled receptors do not cause these defects, but they enhance HSN migration defects of *egl-20* and *mig-1*, suggesting a partially redundant function for Wnts and their receptors in this process (Pan *et al.* 2006). *aex-3* so far has not been linked to Wnt signaling. However, LIN-44/Wnt has been proposed to control sorting of presynaptic RAB-3 to axons and precludes its entry into the dendrite (Poon *et al.* 2008). This raises the possibility that HSN migration defects in *aex-3(hd148); nid-1* mutant animals could be a secondary consequence of the observed mislocalization of RAB-3.

AEX-3 and its interacting partners in AVG axon navigation

AEX-3 activates RAB-3 and AEX-6/Rab27 to regulate synaptic transmission (Mahoney *et al.* 2006) and physically interacts with CAB-1 to control the defecation motor program (Iwasaki and Toyonaga 2000). Moreover, *aex-3* genetically interacts with *unc-31* and *unc-64* in the context of dauer formation (Iwasaki *et al.* 1997). Taken together this indicates that AEX-3 regulates multiple processes through different downstream effectors. We found that *rab-3* but not *aex-6*/Rab27 mutant animals have AVG axon cross-over defects in a *nid-1* mutant background. Our genetic interaction data suggest *aex-3* and *rab-3* act in the same genetic pathway to affect AVG navigation and that *aex-6*/Rab27 is not required for this process. Activated RAB-3 binds to synaptic precursor vesicles (Mahoney *et al.* 2006), which are transported from the cell

Figure 9 The *aex-3* pathway in AVG axon navigation. The figure describes the observed genetic interactions in the context of the known molecular functions of the proteins involved (e.g., *aex-3* is upstream of *rab-3* because it is known to activate *rab-3*).

body to synapses by the Kinesin-3 motor *UNC-104* (Hall and Hedgecock 1991), which is also involved in the anterograde transport of dense core vesicles (DCVs) (Zahn *et al.* 2004). We observed some AVG axon cross-over defects in *unc-104*; *nid-1* mutant animals, but significantly fewer compared to either *aex-3*; *nid-1* or *rab-3*; *nid-1* mutants, which are not further enhanced in *aex-3*; *unc-104*; *nid-1* triple mutant animals. This suggests that while *aex-3* might be involved in *UNC-104*-mediated vesicle transport in the context of axonal navigation, these vesicles are likely not exclusively transported by *UNC-104*. The *C. elegans* genome encodes 21 kinesins involved in transport, spindle movement, and chromosome segregation (Siddiqui 2002). *UNC-116/KIF5* is the kinesin heavy chain anterograde motor protein involved in the transport of synaptic vesicle components and glutamate receptors (Patel *et al.* 1993; Sakamoto *et al.* 2005; Hoerndli *et al.* 2013). *VAB-8*, an atypical kinase, which controls the posteriorly directed cell migrations and axon outgrowth, also regulates the levels of axon guidance receptors *UNC-40/DCC* and *SAX-3/Robo* in neurons (Wightman *et al.* 1996; Levy-Strumpf and Culotti 2007; Watari-Goshima *et al.* 2007). Moreover, the actin based minus-end-directed motor Myosin V1 transports both dendritic and axonal surface proteins (Lewis *et al.* 2011). It is likely that some of these other motor proteins contribute to the vesicular transport required for proper AVG axon navigation. Synaptic vesicles are made competent for fusion by the Rab3 effector molecule *UNC-10/Rim* (Koushika *et al.* 2001). We did not observe AVG axon cross-over defects in the *unc-10*; *nid-1* mutant animals, suggesting that *unc-10/Rim* and by inference synaptic vesicle release is not involved in AVG axon navigation. Since the AVG axon extends before synapses are formed, this is not unexpected and indicates that the role of *aex-3* and *rab-3* here is independent of synaptic vesicle release.

Both *unc-31* and *ida-1*, which are required for dense core vesicle release (Cai *et al.* 2004; Speese *et al.* 2007), have AVG axon cross-over defects in a *nid-1* mutant background. Both genes as well as *UNC-64/Syntaxin*, a component of the SNARE complex (Saifee *et al.* 1998) required for synaptic and DCV release (Singer-Lahat *et al.* 2008), act in the same pathway as *aex-3*. Since neither mature synaptic vesicles nor mature DCVs are expected to be found in neurons at the beginning of axonal outgrowth, it seems more likely that these proteins are involved in the release of some precursor vesicles in the growth cone during AVG axon outgrowth. Since both *unc-31*; *nid-1* and *unc-64*; *nid-1* double mutants have less penetrant AVG defects than *aex-3*; *nid-1* double

mutants, it is possible two different populations of vesicles are involved. Alternatively *unc-31* and *unc-64* might have a partially redundant role in the release of a single type of precursor vesicle. It has been shown that mature synaptic vesicles from vertebrate cultured neurons are different both in function and composition than the vesicles found in growth cones. The SNARE complex proteins are present in both, but are regulated differently (Igarashi *et al.* 1997). The SNARE complex proteins and Rab3a appear early in the growth cone (Igarashi *et al.* 1997).

There are at least two possible functions for vesicles in the growth cone in the context of axon navigation. First they can deliver molecules essential for navigation, such as receptors for guidance molecules, to the membrane of the growth cone. We found that *UNC-5*, one of the receptors for the guidance cue *UNC-6/Netrin*, acts in the same pathway as *AEX-3* in AVG axon navigation. This raises the possibility that *AEX-3* is involved in the transport of vesicles carrying *UNC-5* to the growth cone. An *UNC-5::GFP* reporter appears in punctate form in growth cones and axons, suggesting localization to vesicles (Ogura and Goshima 2006; Norris *et al.* 2014). *UNC-51*, a serine/threonine kinase and *UNC-14*, a RUN (RPIP8, *UNC-14*, and NESCA) domain-containing protein have been proposed to cooperate with an unknown motor protein to regulate the formation, processing, and transport of *UNC-5*-containing vesicles (Ogura and Goshima 2006).

A second possible role of vesicles in axon guidance arises from the observation that Syntaxin1 associates with DCC (the other Netrin receptor) at the growth cone. This interaction is required for *UNC-6/Netrin*-dependent migration of axons (Cotrufo *et al.* 2012). Localized insertion of membrane at sites of activation of guidance receptors is thought to be the key function of this interaction (Tojima *et al.* 2011; Tojima and Kamiguchi 2015). This raises the possibility that *aex-3* is involved in the localized insertion of membrane into the growth cone in response to activation of *UNC-5*. Distinguishing between these two models would require *in vivo* observations of growth cones, which unfortunately is not possible for AVG.

In summary, we found that *AEX-3* is required for axon guidance in pioneer neurons during nervous system development in *C. elegans*. *aex-3* genetically interacts with *rab-3*, several genes controlling vesicle release in neurons, and the axon guidance receptor *unc-5* (Figure 9). *aex-3* is likely involved in transport of vesicles to the growth cone and/or release of vesicles at the growth cone. It could control delivery and/or insertion of *UNC-5* protein into the membrane of the growth cone or be involved in targeted insertion of membrane after receptor activation. Although the conventional role for *UNC-5* is to mediate repulsion away from the ventral *UNC-6/Netrin* source, recent studies have also demonstrated a redundant role in attracting some growth cones toward the ventral side (Kulkarni *et al.* 2013; Levy-Strumpf and Culotti 2014). Moreover, *UNC-5* acts redundantly with Wnt signaling to regulate anterior–posterior guidance of neurons and distal tip cells of gonad (Levy-Strumpf and Culotti

2014). It is therefore conceivable that *unc-5* plays a partially redundant role in posterior navigation of the AVG axon. Given the evolutionary conservation of all genes we found to be involved in this process, it seems likely that *AEX-3* and *RAB-3* homologs have a similar role in mammalian nervous systems.

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