Regulation of Axonal Midline Guidance by Prolyl 4-Hydroxylation in Caenorhabditis elegans

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Neuronal wiring during development requires that the growth cones of axons and dendrites are correctly guided to their appropriate targets. As in other animals, axon growth cones in Caenorhabditis elegans integrate information in their extracellular environment via interactions among transiently expressed cell surface receptors, their ligands, and the extracellular matrix (ECM). Components of the ECM undergo a wide variety of post-translational modifications that may affect efficacy of binding to neuronal guidance molecules. The most common modification of the ECM is prolyl 4-hydroxylation. However, little is known of its importance in the control of axon guidance. In a screen of prolyl 4-hydroxylase (P4H) mutants, we found that genetic removal of a specific P4H subunit, DPY-18, causes dramatic defects in C. elegans neuroanatomy. In dpv-18 mutant animals, the axons of specific ventral nerve cord neurons do not respect the ventral midline boundary and cross over to the contralateral axon fascicle. We found that these defects are independent of the known role of dpv-18 in regulating body size and that dpv-18 acts from multiple tissues to regulate axon guidance. Finally, we found that the neuronal defects in dpv-18 mutant animals are dependent on the expression of muscle-derived basement membrane collagens and motor neuron-derived ephrin ligands. Loss of dpv-18 causes dysregulated ephrin expression and this is at least partially responsible for the neurodevelopmental defects observed. Together, our data suggest that DPV-18 regulates ephrin expression to direct axon guidance, a role for P4Hs that may be conserved in higher organisms.

Key words: axon guidance; collagen; ephrin

Introduction

Faithful development of the nervous system requires that neurons assimilate guidance information supplied from other cells and the extracellular environment (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001). Such guidance molecules are sensed and processed by neurons to facilitate accurate targeting of axonal and dendritic projections. The ventral nerve cord (VNC) of Caenorhabditis elegans contains many neurons that project along the length of the animal (White et al., 1976). The guidance of these neurons is regulated by multiple conserved molecular pathways and is also sensitive to environmental perturbations (Zallen et al., 1998; Hobert and Bülow, 2003; Bülow et al., 2004; Rhiner et al., 2005; Boulin et al., 2006; Pocock and Hobert, 2008; Steimel et al., 2010).

Basement membranes (BMs) are specialized areas of extracellular matrix (ECM) that act as integration centers for cell-surface and extracellular-released guidance molecules (Varela-Echavarria and Guthrie, 1997). This environment provides a complex arena for axonal growth cones to precisely map their position in time and space to enable correct axon guidance. A major structural and signaling constituent of the BM is collagen (Paulsson, 1992) and functions of collagen in the control of axon guidance has been reported in a number of systems. Collagen XVIII is required for motor neuron axon guidance in both C. elegans and zebrafish (Ackley et al., 2001; Schneider and Granato, 2006), and collagen XIXα1 regulates motor axon guidance in zebrafish (Hilario et al., 2010). Central in the control of collagen biosynthesis are the collagen prolyl 4-hydroxylases (P4Hs), which hydroxylate multiple proline residues in collagen (Myllyharju, 2003). This modification is required for collagen triple helix formation and stability. To date, however, the importance of prolyl 4-hydroxylation in the control of neuronal development is not well understood.

In C. elegans, four genes encode collagen P4Hs: dpv-18 (also known as phy-1), phy-2, phy-3, and phy-4 (Friedman et al., 2000; Hill et al., 2000; Keskiaho et al., 2008). These genes control embryonic development, body size, and morphogenesis (Friedman et al., 2000; Hill et al., 2000). Previous work described a role for the HIF-1–prolyl hydroxylase EGL-9 in axon guidance regulation (Pocock and Hobert, 2008); however, there is no known role for the collagen P4H enzymes in neuronal development. In this study, we found that loss of dpv-18 causes defects in hermaphrodite-specific neuron (HSN) development, in addition to a subset of other VNC neurons in C. elegans. Genetic analysis re-
Significance was assessed by ANOVA followed by Dunnett’s multiple-comparison test. The following strains were used:

- C. elegans organisms.

Guidance regulation a likely conserved mechanism in higher organisms. Together, we have identified a novel function for prolyl 4-hydroxylation in axon guidance defects. Mutants HSN development scored using:

- Figure 1.

A. Quantification of HSN axonal cross-over defects in collagen P4H mutants. HSN development was scored using a transgene (zdIs13). Data are expressed as mean ± SD from 3–7 independent experiments. The asterisk indicates significant difference compared to wild type by multiple-comparison test. 

B. C. C. HSN anatomy of wild-type and dpy-18(ok162) mutant animals. HSN cell bodies migrate to their correct position just anterior to the HSNR axon (red arrowhead). This left–right bias in axon defects occurs in 94% of defective animals. Vulval position is marked with a red asterisk. Ventral view, anterior to the left. Scale bar, 20 μm.

Figures: RJP133 (is[tph-1prom::gfp]); RJP1325 dpy-18(ok162); is[tph-1prom::gfp]); RJP1472 (phy-2(ok802)); is[tph-1prom::gfp]); RJP1547 (phy-3(ok199)); is[tph-1prom::gfp]); RJP1473 (phy-4(tm3539)); is[tph-1prom::gfp]); RJP1272 (dpy-18(ok162)); is[sra-6prom::gfp]); RJP1231 (dpy-18(ok162)); is[odr-2prom::CFP::sra-6prom::DsRed2]); RJP1476 (dpy-18(ok162)); is[tph-1prom::gfp]); RJP882 (rpe4442; dpy-18prom::gfp')); RJP6 (rpe4442; dpy-18prom::gfp')); RJP7 (rpe4442; dpy-18prom::gfp')); RJP8 (rpe4442; dpy-18prom::gfp')); RJP127 (rpe4442; dpy-18prom::gfp')); RJP1476 (dpy-18(ok162)); is[tph-1prom::gfp])); RJP1477 (dpy-18(ok162)); is[tph-1prom::gfp])); RJP882 (rpe4442; dpy-18prom::gfp')).

Materials and Methods

Strains. C. elegans strains are listed in the order in which they appear in the figures first and then in tables. The following strains were used:

- dpy-18 is required for HSN axon guidance. A. Quantification of HSN axonal cross-over defects in collagen P4H mutants. HSN development was scored using a tph-1prom::gfp transgene (zdIs13). Data are expressed as mean ± SD and statistical significance was assessed by ANOVA followed by Dunnett’s multiple-comparison test. n > 50, ****p < 0.0001. n.s. Not significant.

B. HSN anatomy of wild-type and dpy-18(ok162) mutant animals. HSN cell bodies migrate to their correct position just anterior to the vulva. However, the HSNL axon crosses over to the right VNC fascicle (white arrowhead) and the HSNL axon extends anteriorly with the HSNR axon (red arrowhead). This left–right bias in axon defects occurs in 94% of defective animals. Vulval position is marked with a red asterisk. Ventral view, anterior to the left. Scale bar, 20 μm.

- Figures: RJP133 (is[tph-1prom::gfp]); RJP1325 dpy-18(ok162); is[tph-1prom::gfp]); RJP1472 (phy-2(ok802)); is[tph-1prom::gfp]); RJP1547 (phy-3(ok199)); is[tph-1prom::gfp]); RJP1473 (phy-4(tm3539)); is[tph-1prom::gfp])); RJP1272 (dpy-18(ok162)); is[sra-6prom::gfp]); RJP1231 (dpy-18(ok162)); is[odr-2prom::CFP::sra-6prom::DsRed2]); RJP1476 (dpy-18(ok162)); is[tph-1prom::gfp])); RJP882 (rpe4442; dpy-18prom::gfp')).
18(ok162); vab-2(e96); Is[tph-1prom::gfp]); RJP1860 (vab-1(e2); Is[tph-1prom::gfp]); RJP1861 (dpv-18(ok162); vab-1(e2); Is[tph-1prom::gfp]); RJP1849 (vab-2(ju1); efn-2(ev658); Is[tph-1prom::gfp]); RJP1850 (vab-2(ju1); efn-3(ev696); Is[tph-1prom::gfp]); RJP1859 (efn-2(ev658); efn-3(ev696); Is[tph-1prom::gfp]); RJP1851 (vab-2(ju1); efn-2(ev658); efn-3(ev696); Is[tph-1prom::gfp]); RJP1852 (rpEx771 Es[unc-47prom::vab-2 cDNA]; dpy-18(ok162); vab-2(ju1); Is[tph-1prom::gfp]); RJP1853 (rpEx772 Es[unc-47prom::vab-2 cDNA]; dpy-18(ok162); vab-2(ju1); Is[tph-1prom::gfp]); RJP1854 (rpEx773 Es[unc-47prom::vab-2 cDNA]; dpy-18(ok162); vab-2(ju1); Is[tph-1prom::gfp]); RJP1855 (rpEx774 Es[unc-129prom::efn-3 cDNA]; dpy-18(ok162); Is[tph-1prom::gfp]); RJP1856 (rpEx775 Es[unc-129prom::efn-3 cDNA]; dpy-18(ok162); Is[tph-1prom::gfp]); RJP1857 (rpEx776 Es[unc-129prom::efn-3 cDNA]; dpy-18(ok162); Is[tph-1prom::gfp]).

DNA constructs and transgenic lines. Rescue constructs were injected into the dpy-18(ok162) mutant background at 5–15 ng/µl with myo-2prom::mcherry (5 ng/µl) as injection marker. Expression constructs were injected into N2 background at 50 ng/µl with myo-2prom::mcherry (5 ng/µl) as injection marker.

Fluorescence microscopy. Animals were grown at 20°C and neuroanatomy was scored in L4 and young adult hermaphrodites by mounting on 5% agarose on glass slides. Images were taken using an automated fluorescence microscope (AXIO Imager M2, Zeiss) and Zen software (version 3.1, Zen Software).

qRT-PCR assays. RNA was isolated from mixed worm population using standard Trizol-based methods (Chomczynski and Sacchi, 1987). Total cDNA was obtained using TaqMan Reverse Transcription Reagents (Invitrogen, catalog #N8080234). qRT-PCR reactions were performed in triplicates on a LightCycler 480 System (Roche) using the Maxima SYBR/ROX qRT-PCR Master Mix (Fermentas, catalog #N8080234). qRT-PCR reactions were performed in triplicates on a LightCycler 480 System (Roche) using the Maxima SYBR/ROX qRT-PCR Master Mix (Fermentas, catalog #N8080234). qRT-PCR reactions were performed in triplicates on a LightCycler 480 System (Roche) using the Maxima SYBR/ROX qRT-PCR Master Mix (Fermentas, catalog #N8080234). qRT-PCR reactions were performed in triplicates on a LightCycler 480 System (Roche) using the Maxima SYBR/ROX qRT-PCR Master Mix (Fermentas, catalog #N8080234). qRT-PCR reactions were performed in triplicates on a LightCycler 480 System (Roche) using the Maxima SYBR/ROX qRT-PCR Master Mix (Fermentas, catalog #N8080234).

Electron microscopy. Animals were fixed by high-pressure freezing in 2% osmium tetroxide, 4% distilled water in acetone, warmed to 0°C and rinsed several times in cold 10% acetone before embedment into Embed812 plastic resin (Hall et al., 2012). Single animals were trimmed by hand and serial thin sections collected onto slot grids, followed by poststaining with uranylacetate for transmission electron microscopy on a Philips CM10 microscope. To detect potential axon crossings between the major and minor fascicles of the VNC, high-power images were collected using a digital camera for every third transverse section proceeding from the anterior preanal ganglion forward along the VNC in several animals.
Table 1. Neuronal development in dpy-18(ok162) mutant animals

<table>
<thead>
<tr>
<th>Neurons examined (marker used)</th>
<th>Wild type</th>
<th>dpy-18(ok162)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inteneurons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKV interneurons (bwIs2)</td>
<td>0</td>
<td>3</td>
<td>n.s.</td>
</tr>
<tr>
<td>PVP interneurons (hdsIs26)</td>
<td>6</td>
<td>96</td>
<td>****</td>
</tr>
<tr>
<td>PVQ interneurons (oyiIs14)</td>
<td>6</td>
<td>94</td>
<td>****</td>
</tr>
<tr>
<td>Motor neurons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSN motor neurons (zdsIs13)</td>
<td>2</td>
<td>100</td>
<td>****</td>
</tr>
<tr>
<td>DA/DB motor neuron (evIs82b)</td>
<td>0</td>
<td>13</td>
<td>**</td>
</tr>
<tr>
<td>Defasciculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D motor neuron (axIs12)</td>
<td>7</td>
<td>15</td>
<td>n.s.</td>
</tr>
<tr>
<td>DA/DB motor neuron (evIs82b)</td>
<td>2</td>
<td>34</td>
<td>****</td>
</tr>
<tr>
<td>Ventral nerve cord</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D motor neuron (axIs12)</td>
<td>4</td>
<td>80</td>
<td>****</td>
</tr>
<tr>
<td>DA/DB motor neuron (evIs82b)</td>
<td>3</td>
<td>14</td>
<td>**</td>
</tr>
<tr>
<td>Mechanosensory neurons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Touch cells (zdsIs4)</td>
<td>4</td>
<td>10</td>
<td>n.s.</td>
</tr>
<tr>
<td>ALM synapse formation</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
| Neuronal score and cell migration in wild-type and dpy-18(ok162) mutant animals. Cell position and axon guidance were assessed using neuronal type-specific gfp reporter strains. The position of the neuronal cell bodies and the extension of the axons were compared to control gfp reporter animals. Animals were scored 1 d after L4 on ≥2 consecutive days. n > 50. ***p < 0.0001, **p < 0.002, n.s., Not significant.

Results

**dpy-18 mutants display VNC axon guidance defects**

Collagen P4Hs are required for the maturation of collagen and their subsequent assembly into diverse types of extracellular matrices (Mylllyharju, 2003). Such matrices also contain a diverse array of signaling molecules, which in combination guide axons to their target sites (Varela-Echavarria and Guthrie, 1997). To ask whether P4Hs are important for axon guidance, we analyzed knock-out strains on the four P4H genes in the C. elegans genome: dpy-18/phy-1, phy-2, phy-3, and phy-4 (Fig. 1; Friedman et al., 2000; Hill et al., 2000; Keskiario et al., 2008). We focused our analysis on the development of the HSNs, which are well studied neurons known to be regulated by multiple conserved molecular pathways and by environmental factors (Desai et al., 1998; Rhiner et al., 2005; Boulin et al., 2006; Pocock and Hobert, 2008; Pedersen et al., 2013). The left and right HSN (HSNL/R) neuronal cell bodies migrate to the midbody region during embryogenesis (Desai et al., 1988). During larval development, HSNL/Rs extend axons in a highly stereotypical manner around the vulval region, before entering the left and right fascicles of the VNC, extending anteriorly and terminating at the nerve ring (Desai et al., 1988). The HSNL/R axon pairs are separated into the left and right fascicles of the VNC by a midline structure initially made of embryonic motor neuron cell bodies and later by a hypodermal ridge (Boulin et al., 2006). After analyzing the four P4H genes, we found that loss of dpy-18 (using two independent alleles, ok162 and e364) caused VNC axon guidance defects, whereas deletion of the other P4H enzymes had no detectable effect on axon guidance (Fig. 1; data not shown).

**dpy-18 mutant animals are short and fat (Dpy) due to defects in cuticular collagen formation** (Hill et al., 2000; Mylllyharju et al., 2002). We found that in addition to defective body size, loss of dpy-18 causes HSNL axons to erroneously extend via the right axon track in 94% of defective animals, suggesting an asymmetric fault in axon guidance (Fig. 1). To further establish the importance of dpy-18 for neuronal development, we analyzed other axon-guidance and cell-migration events in dpy-18 mutant animals (Fig. 2; Table 1). We found that those axons that navigate the VNC in a posterior-to-anterior direction (HSN, PVP, and PVQ) are severely defective in dpy-18 mutant animals, whereas the AVK axons, which navigate in the opposite direction, are unaffected by loss of dpy-18 (Fig. 2; Table 1). As seen with the HSN neurons (Fig. 1), the vast majority of VNC defects occur where the axon in the left fascicle crosses over to the right. To ask whether the axonal defects observed in dpy-18 mutant animals are developmental cross-over events rather than neuronal maintenance flip-over events (Aurelio et al., 2002; Pocock et al., 2008), we analyzed PVQ neurons of freshly hatched L1 larvae. We found that the penetrance of defects was comparable to that of adult animals, suggesting that the defects observed are developmental in nature (data not shown). In addition to the VNC midline defects, we found that the left/right choice of commissural D-type motor neuron axons were defective in dpy-18 mutant animals, whereas the DA and DB motor neurons were less affected (Table 1). In addition, we found that the longitudinal processes of DA-type, DB-type, and D-type motor neurons exhibit defasciculation defects in dpy-18 mutant animals (Table 1; data not shown). Other neurodevelopmental paradigms of cell migration and axon guidance we studied were unaffected by loss of dpy-18 (Table 1). Together, our neuroanatomical data indicate that dpy-18 plays a major role in the regulation of specific axon guidance events.

**Left VNC axons are misplaced in the hypodermal cleft**

To gain a better understanding of why left VNC axons cross over to the contralateral fascicle in dpy-18 mutants, we analyzed the VNC by electron microscopy (EM). For technical reasons, we studied electron micrographs in the posterior of L1 larvae (Fig. 2H, I). In wild-type animals, the PVQL, PVPR, and AVKR axons are deeply embedded in the hypodermal cleft (zfr;Fig. 2H). In dpy-18 mutant animals, however, we found that the left VNC axons are located more toward the top of the hypodermal cleft, or have crossed over to the right VNC, potentially because of altered adhesion in this environment (Fig. 2I). We could not detect any marked changes in the structure of the hypodermal ridge in dpy-18 mutant animals in the EM study, suggesting that specific molecules or molecular pathways are dysregulated in dpy-18 mutant animals that cause the VNC defects.

**dpy-18 is expressed in multiple tissues**

Previous studies have shown that dpy-18 is predominantly expressed in the hypodermis and in some head and tail neurons (Hill et al., 2000). However, this study did not use the full-length dpy-18 promoter to drive expression of fluorescent protein (Hill et al., 2000). To confirm and potentially extend this analysis, we...
obtained transgenic strains expressing a full-length transcriptional GFP reporter construct to visualize the expression of dpy-18 during development (Fig. 3). Fluorescence in the transcriptional dpy-18prom::GFP reporter strain was first detected at the bean stage of embryogenesis in hypodermis and muscle, and gfp continued to be expressed in these tissues, as well as in motor neurons and in other unidentified neurons, during embryonic development (Fig. 3C, E, G). Postembryonically, dpy-18prom::GFP is expressed in a similar pattern and is more expansive than observed previously (Hill et al., 2000; Fig. 3H). dpy-18 expression is therefore detected at the correct time (bean stage onwards) and in tissues known to be sources of axon guidance regulation along the ventral midline (hypodermis, muscle, neurons).

dpy-18 acts in multiple tissues to regulate axon guidance

As dpy-18 is expressed in multiple tissues, we performed transgenic rescue experiments to assess the spatial requirement for dpy-18 regulation of axon guidance. To confirm that we could rescue the dpy-18(ok162)-induced defects in neuronal development and body size, we transgenically expressed a fosmid containing the entire genomic locus of dpy-18 (Fig. 4A). We found that this fosmid rescued both the Dpy phenotype and HSN axon guidance defects of dpy-18 mutant animals (Fig. 4A; data not shown). Based on our expression data, which showed that the dpy-18 promoter drives expression in multiple tissues, we used a panel of tissue-specific promoters in an attempt to ascertain the dpy-18 focus of action in neuronal development. First, we drove dpy-18 expression in the hypodermis using the dpy-7 promoter. Even though expression of dpy-18 in the hypodermis fully rescued the Dpy phenotype, we obtained poor rescue of the axon guidance defects (Fig. 4B; data not shown). This suggests that dpy-18 regulates body size and axon guidance via independent mechanisms and/or from different tissues. Indeed, we found that Dpy phenotypes are not a prerequisite of axon guidance phenotypes as mutant worms deficient in the collagens dpy-7 and dpy-13 (McMahon et al., 2003) result in a Dpy phenotype but have no detectable defects in axon guidance (data not shown). We next tested whether transgenic expression of dpy-18 in other tissues shown to express dpy-18prom::GFP could rescue the HSN axon guidance defects. We drove dpy-18 cDNA under the control of the unc-120 (body wall muscle), unc-47 (D-type motor neurons), and unc-129 (DA-type and DB-type motor neurons) promoters and found that we could partially rescue the HSN guidance defects in each case. We also used an HSN promoter (egl-6) to drive dpy-18 expression and found that this also rescued the dpy-18 defects in HSN axon guidance. These data suggest that dpy-18 possibly controls HSN axon guidance from multiple tissues, where it could regulate distinct targets in each tissue or the same target from multiple tissues.

\[ \text{dpy-18 HS} \text{axonal defects are partially caused by defective collagen} \]

DPY-18 is required for the correct expression, secretion, and stabilization of the type IV collagens EMB-9 and LET-2 in the BM.
phenotype (Table 2). We found the 98% penetrant defect of dpy-18 mutant animals was reduced to 61 and 69% in the emb-9 and let-2 mutants respectively at 20°C. These data suggest that incorrectly folded type IV collagen is deposited on the BM in dpy-18 mutant animals and that this defective collagen is at least partially responsible for the HSN axon guidance defects observed.

The type XV/XVIII collagen CLE-1 has previously been shown to be required for cell migration and axon guidance (Ackley et al., 2001). Therefore, we tested whether defective CLE-1 synthesis is also responsible for the dpy-18 mutant defects in HSN axon guidance. cle-1(cg120) mutant animals exhibit weakly penetrant HSN axon guidance defects (20%). However, we found that loss of cle-1 partially suppresses the dpy-18 mutant axonal defects from 98 to 76% (Fig. 5A; Table 2). Next, we tested whether defective collagens generated in dpy-18 mutant animals may act through proteins known to bind collagens to direct aberrant axon guidance. We tested DDR-1 and DDR-2 (discoidin domain receptors) and NID-1 (nidogen/entactin), mutants of which cause defects in the nervous system (Kim and Wadsworth, 2000; Unsoeld et al., 2013). We found that loss of ddr-1 or nid-1 partially suppressed the HSN axon guidance defects of dpy-18 mutant animals (Table 2). Together, these data indicate that dpy-18 mutant axon guidance defects are partially dependent on the expression of type IV and type XV/XVIII collagens. Defective deposition of these collagens in the ECM may interfere with the function of DDR-1-mediated and NID-1-mediated axon guidance.

dpy-18 axonal defects are dependent on ephrin expression

Our experiments showing that the dpy-18 HSN axonal defects are only partially dependent on collagens suggested that modification of other guidance pathways might suppress the highly penetrant dpy-18 axon guidance defects. We therefore generated double-mutant combinations between the dpy-18(ok162) mutant and loss-of-function mutants of genes that encode conserved axon guidance cues and receptors (Table 3). We found that mutations in netrin, FGF, and Slit/Robo signaling components were unable to suppress the HSN axon guidance defects of dpy-18 mutant animals (Table 3). Next, we tested whether the ephrin system plays a role, as it had previously been shown to be important for VNC axon guidance (Boulin et al., 2006; Pocock and Hobert, 2008). First, we found that the VAB-1 Eph receptor acts in a partially kinase-independent manner to direct HSN axon guidance as the kinase-dead allele, e2, exhibits weaker penetrant defects to the dx31-null allele (Fig. 5A). We then assayed ephrin expression in dpy-18 mutant and studied the effect of reduced type IV collagen production on the HSN guidance (Topf and Chiquet-Ehrismann, 2011). Therefore, we tested whether HSN axon guidance is affected in emb-9 and let-2 mutants. As emb-9 and let-2 mutants are lethal, we used the temperature-sensitive alleles emb-9(hc70) and let-2(b246) for this analysis (Guo et al., 1991; Sibley et al., 1994). Both alleles exhibit embryonic lethality at 25°C, whereas at 20°C they are able to grow. We found that at this permissive temperature, HSN axons extend normally in emb-9 and let-2 mutant animals (Table 2). This suggests that either type IV collagens are not required for HSN axon guidance or that at 20°C sufficient type IV collagen is produced to enable guidance. We next combined the emb-9 and let-2 mutant alleles with the dpy-18 mutant and studied the effect of reduced type IV collagen production on the HSN guidance.
Table 2. Double-mutant analysis between dpy-18(ok162) collagen/collagen-interacting protein mutants

<table>
<thead>
<tr>
<th>Mutants</th>
<th>HSN guidance defect (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpy-18(ok162)</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>ddr-1(ek874)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>ddr-1(ek874); dpy-18(ok162)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>ddr-2(ek574)</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>ddr-2(ek574); dpy-18(ok162)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>nid-1(1c119)</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>nid-1(1c119); dpy-18(ok162)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>dpy-18(ok162); vab-2(ju1)</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>dpy-18(ok162); vab-2(ju1)</td>
<td>76</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>emb-9(hc90) 15°C</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>emb-9(hc90); dpy-18(ok162)</td>
<td>72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>emb-9(hc90) 20°C</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>emb-9(hc90); dpy-18(ok162)</td>
<td>61</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>let-2(b246) 15°C</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>let-2(b246); dpy-18(ok162)</td>
<td>95</td>
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<td>let-2(b246) 20°C</td>
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<td>let-2(b246); dpy-18(ok162)</td>
<td>20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>let-2(b246); dpy-18(ok162)</td>
<td>20</td>
<td>&lt;0.0001</td>
</tr>
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</table>

VNC defects of HSN axons in dpy-18(ok162) animals are suppressed by mutations in genes encoding type IV and type XV/XVIII collagens (EMB-9, LET-2, and CLE-1) and in collagen-interacting proteins (DDR-1, DDR-2, and NID-1). Data are expressed as mean ± SD and statistical significance was assessed by ANOVA followed by Dunnet’s multiple-comparison test. n > 50, ****p < 0.0001, n.s., Not significant.

Discussion

Our study has identified a requirement for proline hydroxylation in neuronal guidance that is supplied by a specific P4H α-subunit, DPY-18 (Fig. 5F). Using tissue-specific rescue experiments, we found that DPY-18 acts from multiple tissues, including VNC motor neurons, to regulate VNC axon guidance. This suggests that DPY-18 may either act through a variety of pathways to drive axon guidance or that it provides a convivial substratum for axons to navigate through. Interestingly, we observed that the VNC defects in dpy-18 mutant animals have a strong left–right bias where left axons cross over to the right fascicle. This left–right bias in neuronal patterning has also been reported recently where loss of Eph signaling causes asymmetric defects in amphibian neuron guidance (Grossman et al., 2013). Such findings suggest the asymmetric expression of particular guidance cues and postulate a role for DPY-18 in their localization.

To identify the pathways in which DPY-18 acts, we performed double-mutant analysis and found that reduction of either collagen or ephrin expression alleviated the axon guidance phenotypes caused by DPY-18 loss. In wild-type animals, the type IV collagen EMB-9 and LET-2, together with the type XV/XVIII collagen CLE-1, normally play a minor role in regulating HSN axon guidance (our data; Ackley et al., 2001). In dpy-18 mutant animals, however, the presence of defective collagen presumably interferes with collagen-interacting proteins or with other axon guidance signaling pathways to cause aberrant HSN axon guidance. In a similar way, individual loss of any of the ephrins (VAB-2, EFN-2, or EFN-3) weakly affects HSN axon guidance in wild-type animals, yet their dysregulation in the molecular landscape of the dpy-18 mutant causes HSN axon guidance defects. We found that the expression levels of the ephrins are altered in mutants of dpy-18 and emb-9, suggesting that they are able to respond to perturbations in the extracellular environment, as has been shown after brain injury in other systems (Goldshmit et al., 2017).
Figure 5. Dysregulated ephrin expression causes dpy-18 defects in axon guidance. A. Ephrin ligands act redundantly via the VAB-1 Eph receptor to regulate HSN axon guidance. The triple knock-out of the ephrins (vab-2, efn-2, efn-3) phenocopies the vab-1(dx31)-null mutant. Single and double ephrin mutant combinations result in a partially penetrant HSN axon guidance phenotype compared with the vab-1(dx31)-null mutant. The kinase-dead vab-1(e2) allele causes HSN axon guidance defects, though at lower penetrance compared with the vab-1-null mutant, suggesting that VAB-1 plays partially kinase-independent functions in HSN development. Data are expressed as mean ± SD and statistical significance was assessed by ANOVA followed by Dunnett’s multiple-comparison test. n > 50. n.s., Not significant when comparing single and double ephrin mutant strains and the vab-1(dx31)-null strain and the ephrin triple mutant. B. HSN axon guidance defects of dpy-18(ok162) animals are partially suppressed by mutations in the ephrin receptor (VAB-1) and the ephrin ligands (VAB-2, EFN-2, and EFN-3). Data are expressed as mean ± SD and statistical significance was assessed by ANOVA followed by Dunnett’s multiple-comparison test. ****p < 0.0001. Statistical test compares dpy-18(ok162) animals to each double mutant. C, qRT-PCR showing the expression levels of vab-1, vab-2, efn-2, and efn-3 in wild-type, dpy-18(ok162), and emb-9(hc70) mutant animals. **p < 0.01, ****p < 0.0001. n.s., Not significant. D, The suppression of dpy-18(ok162) HSN axon guidance defects by loss of vab-2 is reversed with transgenic expression of vab-2 cDNA in the D-type motor neurons, under the control of the unc-47 promoter. Expression of the same transgenes in wild-type animals results in a 10% penetrant defect in HSN axon guidance that is comparable to background levels (data not shown). Data are expressed as mean ± SD and statistical significance was assessed by ANOVA followed by Dunnett’s multiple-comparison test. ****p < 0.0001 when transgenic rescue strains are compared with the dpy-18(ok162); vab-2(mu1) double mutant. #, Independent transgenic lines. E, Transgenic expression of efn-3 cDNA in DA and DB motor neurons, under the control of the unc-129 promoter, suppresses the HSN axon guidance defects in dpy-18(ok162) mutant animals. Data are expressed as mean ± SD and statistical significance was assessed by ANOVA followed by Dunnett’s multiple-comparison test. ****p < 0.0001 when transgenic rescue strains are compared with the dpy-18(ok162). #, Independent transgenic lines. F, Model for the role of the collagen P4H DPY-18 during axon guidance of the C. elegans VNC. DPY-18 is required for faithful maturation and folding of collagen IV (EMB-9/LET-2), which ensures that the ephrins (VAB-2/EFN-2/EFN-3) are correctly expressed via unidentified ECM-mediated signaling pathway(s).
The outcome of ephrin dysregulation in dpy-18 mutant animals is VNC axon guidance defects. However, the reason for this ephrin response to ECM perturbations is not understood. To conclude, our study shows that DPY-18 is required to generate the appropriate molecular landscape to direct VNC axon midline navigation. When dpy-18 is removed, axons fail to respect the structural and molecular barriers at the ventral midline, leading to axonal cross-over events. These axonal defects are normally prevented by the correct expression of ephrins that repel axons from the midline (Boulin et al., 2006). In the absence of dpy-18 and collagen IV (EMB-9), ephrin expression levels are dysregulated, which may cause disruption of multiple signaling pathways required for axon guidance in the VNC. The prevalence of prolyl 4-hydroxylation in mammalian systems suggests that this modification may also play important roles in neuronal guidance in higher organisms.

References


