Semaphorin signaling restricts neuronal regeneration in *C. elegans*

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Abstract

Extracellular signaling proteins serve as neuronal growth cone guidance molecules during development and are well positioned to be involved in neuronal regeneration and recovery from injury. Semaphorins and their receptors, the plexins, are a family of conserved proteins involved in development that, in the nervous system, are axonal guidance cues mediating axon pathfinding and synapse formation. The *Caenorhabditis elegans* genome encodes for three semaphorins and two plexin receptors: the transmembrane semaphorins, SMP-1 and SMP-2, signal through their receptor, PLX-1, while the secreted semaphorin, MAB-20, signals through PLX-2. Here, we determined the neuronal morphology and locomotion behavior of knockout animals missing each of the semaphorins and plexins; we described the expression pattern of all plexins in the nervous system of *C. elegans*; and we evaluated their effect on the regeneration of motoneuron neurites and the recovery of locomotion behavior following precise laser microsurgery.

Introduction

During neurodevelopment, growth factors and guidance cues regulate dendrite morphogenesis, axon growth cone initiation and navigation, axon elongation and target recognition, but their effects are less pronounced in the adult nervous system. Studying their role in the context of adult regeneration and recovery could provide insight into the molecular and cellular response to injury (Chen et al., 2011; Chisholm et al., 2016).

The semaphorins are a family of glycosylated proteins that were first characterized for their role in the development of the insect and avian nervous systems as axonal guidance cues but were later found in a variety of other tissues and organisms (Alto and Terman, 2017; Junqueira Alves et al., 2019). All semaphorins have a distinctive 500 residue long N-terminal domain, known as the Sema domain. This domain, which is a seven-blade beta-propeller, with each blade formed by four anti-parallel beta-strands (Gherardi et al., 2004), is exclusive to semaphorins and their receptors, the plexins,

where it mediates semaphorin dimerization and receptor binding. Eight classes of semaphorins are phylogenetically conserved in nematodes, flies, chick, mammals, and viruses, with three classes of smaller proteins that are secreted and five classes that are membrane-bound by a transmembrane domain or a glycosylphosphatidylinositol (GPI) link (Alto and Terman, 2017; Junqueira Alves et al., 2019). Correspondingly, four classes of plexins are conserved in invertebrates and vertebrates (Tamagnone et al., 1999; Negishi et al., 2005). All plexins are transmembrane proteins with an extracellular Sema domain that mediates semaphorin binding and signaling, either by themselves or with a neuropilin co-receptor, in the case of the secreted class 3 semaphorins in vertebrate (Negishi et al., 2005; Pascoe et al., 2015).

In mammals, semaphorins and their receptors, neuropilins and plexins, were originally described as guidance cues for neuronal growth cones aiding axons to their targets by acting as chemorepellents (Kolodkin and Tessier-Lavigne, 2011). More recently, semaphorins have been implicated in multiple key roles of neural circuit assembly during neurodevelopment (Yoshida, 2012; Koropouli and Kolodkin, 2014). For example, the mammalian secreted semaphorin, SEMA3A, is involved in various neurodevelopmental processes in the mouse, including repelling dorsal root ganglion sensory axons, promoting basal dendrite elaboration in cortical pyramidal neurons, and pruning of hippocampal axons (Bagri et al., 2003; Yaron et al., 2005; Mlechkovich et al., 2014; Danelon et al., 2020). Another well studied secreted semaphorin, SEMA3F, and its receptor Neuropilin-2, are also involved in axon guidance, synaptic plasticity, and refinement, as well as in restraining the excess of dendritic spines on apical dendrites of cortical neurons and regulating inhibitory interneuron numbers in the hippocampus (Tran et al., 2009; Riccomagno et al., 2012; Riccomagno and Kolodkin, 2015; Assous et al., 2019; Eisenberg et al., 2021). As the mediators of semaphorin signaling, the plexins are involved in axon guidance, synapse and dendrite formation, axonal pruning and synaptic stability (Shen and Cowan, 2010; Limoni, 2021).

In accordance with their role in neurodevelopment, semaphorins could be involved in axonal regeneration after injury (Fard and Tamagnone, 2021). For example, SEMA3A expression levels increase after injury in the spinal cord and cerebral cortex (de Winter et al., 2002; Hashimoto et al., 2004) and regenerating axons avoid areas with high SEMA3A expression (Pasterkamp and Verhaagen, 2001). Accordingly, a SEMA3A-specific inhibitor improved axon regeneration and spontaneous hind leg movement after spinal cord transection (Kaneko et al., 2006). Plexin expression and function in response to injury varies depending on the type. Plexin A family members increase their expression after axonal injury in facial motoneurons and rubrospinal neurons contributing to the role of semaphorins in restricting regeneration (Spinelli et al., 2007). On the other hand, PlexinB2 is upregulated after spinal cord injury in glial cells proximal to the injury site and is required for wound healing and recovery (Zhou et al., 2020).

The *Caenorhabditis elegans* genome encodes for only three semaphorin and two plexin homologues. Of those, PLX-1 binds the two membrane-bound semaphorins (SMP-1 and SMP-2), while PLX-2 binds the only secreted semaphorin (MAB-20; Figure 1A; (Ginzburg et al., 2002; Nakao et al., 2007). Both membrane-bound and secreted semaphorin-plexin systems are involved in development; semaphorins guide ventral enclosure (Ikegami et al., 2012), and regulate epidermal morphogenesis (Ginzburg et

al., 2002; Ikegami et al., 2012) as well as vulva and tail-rays morphogenesis in the hermaphrodite and males, respectively (Dalpe et al., 2012). In the nervous system, membrane-bound semaphorin signaling (the *plx-1/smp-1/smp-2* pathway) is necessary for synaptic tiling in two DA motoneurons in the tail (Mizumoto and Shen, 2013) and for guidance of the long axons of mechanosensory neurons (Ginzburg et al., 2002). Secreted semaphorin signaling (via the *plx-2/mab-20* pathway) contributes to motoneuronal axon guidance; eliminating this pathway, when not embryonic lethal, causes defasciculation of the ventral nerve cord (VNC; 17% of surviving *mab-20* knockout animals) and axon misguidance in DA and DB motoneuron classes (4% of surviving *mab-20* knockout animals; (Roy et al., 2000).

C. elegans is a well-established model for neuronal regeneration and most of its neurons are able to regenerate after precise laser microsurgery (60%) and in some cases reestablish functional connections (Yanik et al., 2004; Ghosh-Roy and Chisholm, 2010; Neumann et al., 2011; Harreguy et al., 2021). **Here we take advantage of the small number of plexins in *C. elegans* and the capability to precisely disconnect single neurites in intact animals, to investigate the role of semaphorin signaling in neuroregeneration *in vivo*. We describe the neuronal expression of the plexin receptors and the effect of their absence on neuronal regeneration and recovery of locomotion behavior.**

Methods

Strains and transgenics

We maintained *C. elegans* strains under standard laboratory conditions on nematode growth medium (NGM) agar plates with OP-50-1 *Escherichia coli* bacterial lawn at 15°C (Stiernagle, 2006). All animals used in the experiments were hermaphrodites.

We acquired semaphorin and plexin mutants from Caenorhabditis Genetics Center (CGC) or the *C. elegans* National Bioresource Project of Japan (NBRP): ev778 (*mab-20*, null), tm729 (*plx-2*, null), ev715 (*smp-1*, null), ev709 (*smp-2*, null), tm10697 (*plx-1*, null), and evls111 ([F25B3.3::GFP + dpy-20(+)], pan-neural GFP expression). To allow imaging and microsurgery, we crossed males of NW1229 (evls111), induced by 10 minute exposure of L4 larvae to 10% ethanol (Lyons and Hecht, 1997), with null-mutant hermaphrodites to obtain knockout animals expressing GFP in the entire nervous system: TOL55 (ev715, evls111), TOL57 (ev709, evls111), TOL59 (tm10697, evls111), and TOL62 (tm729, evls111).

We generated transgenic strains that express EGFP under the *plx-1p* promoter extrachromosomal array. We injected the gonads of adult wild type animals (N2) with a mixture consisting of 2.5 ng/μl pCFJ90 [*myo-2p::mCherry*], 10 ng/μl of pMS80 [*plx-1p::GFP(65C)*] and 87.5 ng/μl of 1Kb DNA Ladder (New England Biolabs). We identified insertion by selecting animals expressing mCherry and EGFP. The reporter construct was a generous gift from Dr Kota Mizumoto (University of British Columbia). The reporter strain for *plx-1p::EGFP* (NW2339) and *plx-2p::GFP* (NW1693) were shared by Dr Dr. Joseph Culotti (University of Toronto, Mt Sinai Hospital) and Dr Richard Ikegami (UC Berkeley), respectively.

Locomotion analysis

We tracked locomotion behavior of multiple animals over an agar surface (1.7% in NGM buffer), without food, as well as in liquid (NGM buffer). We recorded videos with a static multi-worm tracker, composed of three major parts, from top to bottom: (1) a CMOS camera (acA4024-29um, Basler) mounted with a fixed focal length lens (C Series 5MP 35 mm 2/3", Edmund Optics), and an infrared cut-off filter (SCOTT-KG3 M25.5x0.5, Edmund Optics); (2) a specimen stage for plates or slides; (3) a collimated Infrared LED light source (M850L3 and COP1-B, Thorlabs).

One day before the experiment, we transferred animals of the fourth larval stage (L4) onto a new plate with healthy OP-50-1 bacterial lawn. Immediately before tracking, animals were transferred onto a 30 mm agar plate with no food or a 150 ul drop of NGM buffer, placed on a microscope slide. During tracking, animals moved freely, and we recorded multiple 25 Hz 15-second videos using Pylon Viewer (Pylon Camera Software Suite, Basler). We analyzed the videos with Tierpsy worm-tracker (Javer et al., 2018) that can track multiple animals and extract up to 726 features for each tracked trajectory. We analyzed the HDF5 output file produced by the Tierpsy in Matlab (script code available upon request) to collect the mean speed and frequency values for each trajectory and then plotted the data and estimated confidence intervals among groups with a freely available software for Estimation Statistics (<https://www.estimationstats.com>; (Ho et al., 2019). We considered neurites regenerated when a new branch was observed extending from the proximal segment of the injury site (Harreguy et al., 2020). When the regenerating branch extended to the distal segment or the target of the pre-injury neurite we considered it reconnected. We tracked all the knockdown, transgenic, and wild type strains without injury to assess their baseline locomotion parameters. Further, 6 and 24 hours after microsurgery, we tracked locomotion to assess recovery. For comparison, we also quantified locomotion parameters of sham-surgery groups for each genotype and time point. We treated the sham-surgery groups through the same protocol (including cooling and immobilization, see below), except for the exposure to the laser beam.

Expression and neuronal morphology analysis.

We immobilized animals by placing them in a drop of cold, liquid 36% Pluronic F-127 (thermo-responsive material that gels at room temperature, Sigma-Aldrich) with 1 mM levamisole (a paralytic nicotinic agonist, Sigma-Aldrich), mounted on a microscope slide with a #1.5 coverslip and sealed with a room-temperature-hardening mixture (VALAP, equal weights of petroleum-jelly, lanolin, and paraffin). We used a laser scanning confocal microscope (Leica SP8; microscope: DM6000CS; objectives: Leica 40x/NA1.30 HC PL APO oil or Leica 63x/NA1.40 HC PL APO oil, with lateral resolutions of 223 nm and 207 nm respectively; laser lines: 405 nm, 561 nm, and 488 nm). We collected multiple optical slices (thickness optimized by the confocal software, ranging 0.343 - 0.345 μ m for the 63X objective, and 0.410 - 0.422 μ m for the 40X objective). To analyze morphology and expression we constructed the maximum intensity projections for at least 10 animals of each strain and, in some cases, processed images to reduce background noise via the Leica Application Suite (LASX) software.

Laser Microsurgery

For laser microsurgery and associated microscopy, we mounted *C. elegans* hermaphrodites at L4 stage by placing them in a drop of ice cold, liquid 36% Pluronic F-127 with 1 mM levamisole solution and pressed them between two #1 coverslips (Melentijevic et al., 2017). We brought the slides to room temperature, to solidify the Pluronic F-127 gel and immobilize the animals. We used a Yb-fiber laser (100 pulses at 10 kHz repetition rate) to cut a single neurite with submicron precision and no discernable collateral damage (Harreguy et al., 2020). We took images immediately before and after the lesion to visually verify the microsurgery. In some cases, multiple laser exposures were necessary to disconnect a neurite. We disconnected the ventral-dorsal commissures of all motoneurons that we were able to identify by their relative position, at about 45 μ m away from the VNC. We assessed neuronal regeneration 24 hours after microsurgery on the same microscope and imaging system in at least 6 neurons per animal in at least 15 animals for each condition. We classified neurite regeneration in three categories: no regeneration (gap at the injury site, no new branches), regeneration (a new branch extended from the proximal site of lesion), and reconnection (a new branch fused with the distal side of the lesion or with the dorsal nerve cord). We used ImageJ (FIJI v.1.52) and LASX (Leica) for image processing and visualization, and Prism (GraphPad v.9.2.0) for statistical analysis and plotting.

Results

***C. elegans* animals that do not express semaphorins or plexins exhibited altered locomotion patterns.**

We analyzed the contribution to locomotor behavior of each of *C. elegans* three semaphorins and two plexins (Fig. 1A) by observing knockout (ko) mutant strains. During crawling on agar (Fig. 1B), all strains except for *mab-20(ko)* translocated significantly slower and the undulation frequency of all mutant strains was reduced. The largest reduction of speed and frequency was in *smp-1(ko)* animals. During swimming (Fig. 1B), *plx-1(ko)* and *smp-1(ko)* animals translocated slower and at higher undulation frequency than wild type; *smp-2(ko)* animals translocated slower at lower undulation frequency. On the other hand, *plx-2(ko)* animals translocated faster than wild type but with a lower undulation frequency. We did not observe significant locomotion differences in swimming of *mab-20(ko)* animals.

We focused further analysis on the plexins (*plx-1* and *plx-2*), because as the only receptors, segregating membrane-bound and secreted pathways, they provide a comprehensive and specific manipulation of these pathways, as well as the identity of the cellular targets (Fujii et al., 2002).

Gross neuronal morphology was unaffected by the absence of PLX-1 and PLX-2.

We imaged intact young adult plexin-knockout animals expressing pan neuronal green fluorescent protein (GFP), with emphasis on neuron-rich areas around head, tail and vulva, and particularly at the commissures of motoneurons (Fig. 2). We did not observe

any morphological differences between mutant and wild type animals in any of these regions.

Motoneuronal expression of PLX-1 and PLX-2

We imaged transcriptional reporters for *plx-1* and *plx-2* in order to identify their neuronal expression in the ventral nerve cord (VNC). GFP under the *plx-1p* promoter (Figure 3AB) was mostly expressed in non-neuronal tissue including the body-wall muscle in the head and along the body, the lateral seam cells, and vulva cells. In the nervous system, *plx-1p::GFP* was expressed in a few neurons in the head and in two motoneurons at the base of the tail, namely DA8 and DA9 (Figure 3B inset; also reported in Mizumoto and Shen, 2013). GFP under the *plx-2p* promoter was expressed in neurons in the head and tail (Figure 3CD), as well as in four motoneuron classes in the VNC: DA, DB, and DD classes in first larval stage (L1), and VC class that develop post-embryonically in L2 and older animals. The nervous system of L1 includes only the DA, DB and DD classes of motoneurons (White et al., 1976, 1986) with commissures that extend from the VNC to the dorsal nerve cord on the opposite side of the animal. These commissures were the targets for microsurgery.

Neurites of plexin knockout mutants regenerate more than wild type after laser microsurgery.

We disconnected 156 commissural neurites of motoneurons of wild type and plexin knockout mutant animals with laser microsurgery (Harreguy et al., 2020). These lateral processes extend to connect the ventral and dorsal nerve cords and when multiple processes are disconnected, locomotion is impaired (Yanik et al., 2004). When we examine the same neurite after 24 hours, some regenerated by sprouting a growth cone from the proximal segment and some of those reconnected to the distal segment or the dorsal nerve cord (Fig. 4A). In the wild type, 38 of 73 neurites regenerated (0.52 ± 0.11) and only 5 of the 38 (13%) reconnected (Fig. 4B). The plexin knockout mutants exhibited significantly more regeneration ($p=0.049$, Fisher Exact on 2x3 contingency table), 33 of 47 (0.7 ± 0.11) for *plx-1(ko)* and 26 of 36 (0.72 ± 0.12) for *plx-2(ko)*. Reconnection happened significantly more ($p<0.0001$ Fisher Exact on 2x3 contingency table) in *plx-2(ko)*: 20 of the 26 regenerated neurites, which are 77%. In *plx-1(ko)*, 13 of 33 regenerated neurites reconnected (39%). Six hours after microsurgery, wild type and mutant animals moved slower than sham-treated animals of the same genotype. Subsequently, 24 hours after microsurgery, mean locomotion speed has recovered to levels comparable to sham-treated animals for all groups (Fig. 4C).

Discussion

Here we have demonstrated that the two plexins that mediate semaphorin signaling in *C. elegans* restrict neuronal regeneration and reconnection after injury. In their absence, injured neurons of plexin knockout mutants exhibit higher levels of regeneration (for both plexins) and reconnection (for *plx-2*).

By the nature of their ligands, the two plexins mediate different spatial signals. Paracrine interaction, such as those mediated by PLX-1 typically act at short-ranged by cell to cell interactions and conform subcellular resolution spatial information (Dalpé et

al., 2004, 2005; Gurrapu and Tamagnone, 2016). Because both ligand and receptor are transmembrane proteins, the flow of information could be bidirectional, such as in the case of reverse-signaling through semaphorins, in which plexins function as ligands (Yu et al., 2010; Battistini and Tamagnone, 2016). On the other hand, juxtacrine interactions, such as those mediated by PLX-2 are spatially more dispersed over tissue level where the ligand typically diffuses to set meaningful concentration gradients (Chen et al., 2007).

We demonstrated that neither the plexins nor the three semaphorins are necessary for gross neuromorphogenesis. However, at low penetrance their omission causes defasciculating and axon misguidance (Roy et al., 2000). In the nervous system, PLX-1 is only expressed in two motoneurons, namely DA8 and DA9, where it is involved in synaptic tiling during development by restricting the synaptic regions (Mizumoto and Shen, 2013). Because, to the most part, PLX-1 is expressed in muscle, seam cells, and other non-neuronal tissue (Fujii et al., 2002), we hypothesize that its restrictive effect on regeneration is achieved by interaction with the semaphorin SMP-1 presented by the motoneurons (Liu et al., 2005). The neurons could respond indirectly to the surrounding tissue via another signaling pathway, or SMP-1 could mediate a direct cellular response via reverse-signaling from plexins to semaphorins (Yu et al., 2010; Battistini and Tamagnone, 2016). The other membrane-bound semaphorin, SMP-2, might not be involved in motoneuronal regeneration because it is not expressed in VNC motoneurons, but in body wall muscle and some sensory neurons in the head (Ginzburg et al., 2002). PLX-2 is expressed in four classes of motoneurons and the most parsimonious hypothesis is that MAB-20 secretion from the laterally positioned seam cells generate a gradient that suppresses overgrowth of neurites in health and injury. A similar system was described for regenerating axons of murine spinal cord and brain, where SEMA3A and Plexin expression increases after injury and SEMA3A secreted by cells adjacent to the injury site create an exclusion zone which regenerating axons do not penetrate (Pasterkamp and Verhaagen, 2001; Pasterkamp et al., 2001; de Winter et al., 2002).

The phenotypes we describe for uninjured plexin and semaphorin knockout mutant animals are changes in speed and frequency of locomotion on agar surface and in liquid. To the most part, these effects are small in magnitude and include both increases and decreases compared to wild type animals. The largest effects were on the translocation speed of *smg-1(ko)* during swimming and even worse during crawling. Because the semaphorin signaling pathways are involved in several aspects of embryonic development and its components are expressed in neuronal and non-neuronal tissue in the embryo, the phenotypes are likely the product of an accumulation of effects on structure and function of different tissue, such as muscle, cuticle, or the nervous system. To remove the effect of these locomotion phenotypes from our study, we compare animals after laser microsurgery to sham-operated animals of the same genotype. Moreover, the laser microsurgery experiments included only plexin knockout mutants and *smg-1(ko)* animals were not included in that comparison.

Locomotion behavior was impaired 6 hours post-injury and recovered back to pre-injury parameters 24 hours post-injury in wild type animals and both plexin knockout mutant animals. Because less than half of the neurites in the wild type animals regenerated and

only 7% reconnected, we hypothesize that the recovery is due to reorganization of the locomotion circuit to produce a meaningful motor pattern that is indistinguishable from that of an uninjured animal (Haspel et al., 2021). Similarly, the recovery of plexin knockout mutants that exhibit much higher levels of regeneration (for both) and reconnection (for *plx-2*) can be due to reorganization. Full recovery of locomotion with only partial recovery of neurites and synapses has been described in other systems (Oliphint et al., 2010), but the underlying circuit mechanism is unknown.

The conserved but concise semaphorin-plexin system and readily available genetic and transgenic tools in *C. elegans*, together with accurate injury and quick neuroregeneration and recovery of behavior provide an attractive experimental model. The secreted and membrane-bound semaphorin signaling pathways both restrict regeneration but in distinct processes that likely include spatial specificity and recurrent signals. Further studies, including of the localization of each of the semaphorins, before and right after injury, as well as the spatiotemporal dynamics of related secondary messengers such as calcium and cGMP, will answer fundamental questions about the involvement of semaphorin signaling in neural recovery from injury.

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Figures

Figure 1. *C. elegans* semaphorin system comprises only three ligands and two receptors and omitting any one component affects locomotion. A) Semaphorin signaling system of *C. elegans*. The membrane bound semaphorins *smp-1* and *smp-2* signal through *plx-1*, while the secreted *mab-20* signals through *plx-2* (molecular diagrams adapted from Junqueira Alves et al., 2019). B) Mutant strains with knocked out semaphorins or plexins are significantly different from wild type when crawling (locomoting on agar) or swimming (locomoting in liquid media). The largest difference was in *smp-1*(ko) animals while *mab-20*(ko) animals are only different for lower undulatory frequency during crawling. Data points are mean absolute translocation speed or frequency to both direction of locomotion of analyzed trajectories; n.s. $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; two-sided permutation t-test; in parentheses are the number of analyzed trajectories from 20-25 animals.

Figure 2. Neuronal morphology of plexin knockout strains is comparable to wild type. We imaged pan-neuronal GFP in selected neuron-rich areas (vulva, nose, and tail) of wild type (WT) and knockout mutant animals (*plex-1(ko)* and *plex-2(ko)*), as well as the entire animals (not shown), to look for gross neuromorphological differences. We did not observe differences between wild type and mutant strains. N = 5 animals for each strain. Scale bar = 50 μ m.

Figure 3. PLX-1 is expressed in DA8, DA9, and in non-neuronal tissue, while PLX-2 is expressed in dorsal excitatory and inhibitory motoneurons. AB) Green fluorescent protein (GFP) driven by *plx-1p* promoter expressed in non-neuronal tissue such as body-wall muscle, seam cells and in two motoneurons close to the tail: DA8 and DA9 (inset, red arrowhead). C) GFP driven by *plx-1p* promoter expressed in all three motoneuron classes in the L1 larvae: DA, DB, and DD (two excitatory and one inhibitory dorsal motoneurons). D) In older animals, GFP was also expressed in VC neurons. Colored numbers in diagrams specify names of motoneurons at that location: DA (red), DB (green), DD (blue), and VC (black). All panel scale bars are 50 μ m; inset scale bar 5 μ m.

Figure 4. Neuronal regeneration and reconnection increased in the absence of plexins 24 hours after laser microsurgery, while locomotion speed fully recovers in all genotypes. A) We scored all commissural neurites 24 hours after microsurgery (yellow arrowhead) and scored them as exhibiting no-regeneration, regeneration, or reconnection (green diagrams, see methods). B) About half of wild type neurites regenerated 24 h post-injury and only 7% reconnected. Both plexin knockout mutant strains exhibited more regeneration (sum of colorful and empty bars) and *plx-2* exhibited more reconnection (colorful bars). C) Animals of all groups moved significantly slower 6 h post-injury and all recovered to levels equal to sham operated after 24 h. Data points are mean absolute translocation speed to both direction of locomotion; n.s. $p > 0.05$, * $p < 0.05$ *** $p < 0.001$, **** $p < 0.0001$; two-sided permutation t-test; in parentheses are the number of analyzed trajectories from 20-25 animals.