Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 315 (2008) 448–458
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Plexin-A3 and plexin-A4 restrict the migration of sympathetic neurons but not their neural crest precursors

Kathryn E. Waimey [a,1], Pei-Hsin Huang [a,1,2], Maggie Chen [a], Hwai-Jong Cheng [a,b,*]

[a] *Center for Neuroscience, University of California, Davis, CA 95618, USA*
[b] *Section of Neurobiology, Physiology and Behavior, College of Biological Sciences, and Department of Pathology and Laboratory Medicine, School of Medicine, University of California, Davis, CA 95618, USA*

Received for publication 7 August 2007; revised 21 December 2007; accepted 2 January 2008
Available online 16 January 2008

Abstract

During development, the semaphorin family of guidance molecules is required for proper formation of the sympathetic nervous system. Plexins are receptors that mediate semaphorin signaling, but how plexins function during sympathetic development is not fully understood. Using phenotypic analyses of mutant mice *in vivo*, expression pattern studies, and *in vitro* assays, we show that plexin-A3 and plexin-A4 are essential for normal sympathetic development. This study confirms our previous *in vitro* findings that the two plexins differentially regulate the guidance of sympathetic axons. In addition, we find that semaphorin signaling through plexin-A3 and plexin-A4 restricts the migration of sympathetic neurons, but these two plexins function redundantly since migration defects are only observed in plexin-A3/-A4 double mutants. Surprisingly, our analysis also indicates that plexin-A3 and plexin-A4 are not required for guiding neural crest precursors prior to reaching the sympathetic anlagen. Immunoprecipitation studies suggest that these two plexins independently mediate secreted semaphorin signaling. Thus, *plexin-A3* and *plexin-A4* are expressed in newly-differentiated sympathetic neurons, but not their neural crest precursors. They function cooperatively to regulate the migration of sympathetic neurons and then differentially to guide the sympathetic axons.

Keywords: Plexin; Semaphorin; Sympathetic; Axon guidance; Neuron migration; Neural crest

Introduction

During the development of the nervous system, neurons migrate to appropriate locations and project axons to make connections with targets. Environmental cues are required to guide these events. The semaphorin family of axon guidance molecules is one such cue that has been implicated in both neuron migration and axon guidance (Bagri and Tessier-Lavigne, 2002; Dickson, 2002; He et al., 2002; Tessier-Lavigne and Goodman, 1996). The class 3 semaphorins are a unique subset of this family as they are secreted and signal through receptor complexes consisting of neuropilins and plexins (Fujisawa, 2004; Huber et al., 2003; Tran et al., 2007; Pasterkamp and Kolodkin, 2003; Waimey and Cheng, 2006). In the past decade, specific interactions between class 3 semaphorins and neuropilins have been established (Chen et al., 1997; Giger et al., 1998, 2000; He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997; Nakamura et al., 1998; Taniguchi et al., 1997). Particularly, Sema3A and Sema3F have been shown to bind neuropilin-1 and neuropilin-2, respectively. While plexins and neuropilins form co-receptors for the class 3 semaphorins, most plexins do not directly bind to the secreted semaphorins (Gu et al., 2005; He et al., 2002; Takahashi et al., 1999; Tamagnone et al., 1999). Consequently, it is challenging to understand how each plexin specifically mediates the function of class 3 semaphorins in the developing nervous system.

The development of the sympathetic nervous system has been well documented in multiple species (Rubin, 1985; Francis and Landis, 1999; Le Douarin and Kalcheim, 1999; Kuan et al.,

* Corresponding author. Center for Neuroscience, University of California, Davis, 1544 Newton Court, Davis, CA 95618, USA. Fax: +1 530 754 4159.
E-mail address: hjcheng@ucdavis.edu (H.-J. Cheng).
[1] These authors contributed equally to the study.
[2] Current address: Department of Pathology, College of Medicine, National Taiwan University and University Hospital, Taipei, Taiwan.

0012-1606/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2008.01.002

2004; Young et al., 2004; Kasemeier-Kulesa et al., 2005). During sympathetic development, neural crest cells from the dorsal neural tube migrate ventrally to aggregate near the dorsolateral aspect of the developing aorta, forming the sympathetic anlagen. This process occurs by embryonic day 10.5 (E10.5) in the mouse. Sympathetic precursors then differentiate into neurons and undergo a period of rostral–caudal intermixing migration (Le Douarin and Kalcheim, 1999). This intermixing migration segregates sympathetic neurons into the discrete ganglia of the mature sympathetic nervous system by E13.5, including the superior cervical ganglia (SCG), stellate ganglia, and sympathetic chain ganglia. Finally, to function properly, young sympathetic neurons also must send their axons to make connections with appropriate targets.

Several families of molecules have been implicated in regulating sympathetic migration and axon guidance, including semaphorins and their neuropilin receptors (Bron et al., 2004; Durbec et al., 1996; Gammill et al., 2006; Kawasaki et al., 2002; Krull et al., 1997; Young et al., 2004). Sema3F and neuropilin-2 are required for the segmental migration of neural crest cells through the somites (Gammill et al., 2006). In addition, mutant mouse analysis and expression pattern studies show that Sema3A and neuropilin-1 are involved in the migration of sympathetic cells and axon guidance (Behar et al., 1996; Kawasaki et al., 2002). Although plexin-A3 and plexin-A4 are required for sympathetic axon guidance *in vitro* (Cheng et al., 2001; Suto et al., 2005; Yaron et al., 2005), how they mediate semaphorin signaling *in vivo* is still unclear. Here, we confirm that plexin-A4 preferentially regulates sympathetic axon projections *in vivo*. Unexpectedly, we also find that plexin-A3 and plexin-A4 redundantly guide sympathetic migration and that these receptors are required to restrict the migration of sympathetic neurons but not neural crest precursors.

Materials and methods

Animals

Plexin-A3 and *plexin-A4* mutant mice were generated by gene targeting (Cheng et al., 2001; Yaron et al., 2005). Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Immunohistochemistry

Whole-mount anti-TH immuno-staining was performed as reported (Cheng et al., 2001). After fixation in 4% paraformaldehyde (PFA) and removal of internal organs, embryos were treated with a rabbit anti-TH-antibody (1:200 dilution; Chemicon International Inc., Temecula, CA) in Tris buffered saline containing 1% Tween-20, 5% milk, 5% DMSO and 0.1% sodium azide for 2 days at 4 °C. After washing with phosphate buffered saline (PBS), HRP-conjugated goat anti-rabbit antibody (1:200 dilution; Vector Labs, Burlingame, CA) was added. Embryos were developed in diaminobenzidine (DAB) with 0.1% H_2O_2.

Immunohistochemistry on sections was performed as follows. Embryos were fixed with 4% PFA, cryo-sectioned (10 μm thick), and permeablized with 0.1% Triton-X-100 for 5 min, blocked with 3% bovine serum albumin (BSA) in PBS for 1 h, and incubated with primary antibody overnight at 4 °C. A biotinylated secondary antibody was added and incubated for 1 h. After several washes in PBS, sections were developed with an ABC kit (Vector Labs). Images were acquired with a Zeiss microscope.

Detection of sympathetic precursors was performed with antibodies against rabbit anti-p75 NGF (nerve growth factor) receptor antibody (1:200 dilution; Chemicon International Inc.) and mouse anti-MASH1 (1:100 dilution; Becton Dickinson, Franklin Lakes, NJ). The signals were detected with appropriate Alexa 488-conjugated secondary antibodies.

Quantification of ectopic cells

Quantification of ectopic sympathetic neurons was performed by examining serial 10 μm sections at the level of the SCG in each embryo, as described by Nishino et al. (1999) in quantifying cell number at the ganglia. Cells were considered to be ectopic sympathetic neurons if they were 1) TH-positive, 2) displayed the smooth, rounded morphology consistent with cell somas, and 3) more than 20 μm away from the normally located superior cervical ganglia. To quantify the ectopic cells, the number of all ectopic TH-positive cells in each section was counted and averaged. A minimum of 10 sections was examined for each embryo, and two or more animals were quantified per embryonic stage and genotype.

BrdU labeling

Proliferating cells were detected by labeling with 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO). Pregnant dams were intra-peritoneally injected 2 h before sacrifice with BrdU (50 μg/gm body weight). Cryo-sections were permeablized, treated in 2 N HCl for 30 min at 37 °C, and blocked with 3% BSA in PBS. Sections were stained with primary mouse anti-BrdU antibody (1:50 dilution; Becton Dickinson) and secondary anti-mouse Alexa 594-conjugated antibodies (Molecular Probes, Eugene, OR). Nuclei were counter-stained with 4′, 6-Diamidino-2-phenylindole (DAPI; Molecular Probes). During double labeling for BrdU and TH, additional anti-TH staining was performed as described above except that an anti-rabbit Alexa 488-conjugated secondary antibody was used. The mitotic index was calculated as the ratio of BrdU-positive cells within TH-positive sympathetic neurons for each sympathetic ganglion and ectopic TH-positive cells.

Cell death detection

Cell death was assessed using a TUNEL *in Situ* Cell Death Detection Kit (Roche Diagnostics Corporation, Indianapolis, IN). Cryo-sections were permeablized in 10 mM Tris, pH 7.5, containing 0.1% Triton-X-100 and 3% proteinase K, incubated with 0.1% sodium citrate in PBS for 30 min, and blocked with 0.2% BSA in PBS for another 30 min. DNA strand breaks were visualized by labeling free 3′-OH DNA ends with a labeling mixture containing TdT and fluorescent-labeled nucleotides for 1 h at 37 °C. Nuclei were counterstained with 4′, 6-Diamidino-2-phenylindole (DAPI; Molecular Probes) and subsequent double labeling of TH by immuno-fluorescence was performed as described. The percentage of sympathetic cells undergoing apoptosis was determined in wild-type and *plexin-A3/-A4* double mutant mice.

RNA in situ hybridization

In situ hybridization of tissue sections with digoxigenin-labeled mouse Sema3A, Sema3F, plexin-A3 and plexin-A4 riboprobes on cryo-sections (20 μm thick) was performed as described (Cheng et al., 2001). Whole mount *in situ* hybridization was performed as follows: 32 somite embryos were fixed in 4% paraformaldehyde in PBS and dehydrated in a graded series of PBT (PBS with 0.1% Tween-20) in methanol ending with 100% methanol. Embryos were rehydrated and bleached with 6% hydrogen peroxide and treated with 10 mg/ml proteinase K for 10 min. Hybridization was carried out at 68 °C in hybridization buffer for 24 h. Alkaline phosphatase development was carried out in the presence of 10% poly vinyl alcohol (Sigma-Aldrich) in CT buffer (100 mM Tris–HCl, pH 9.5, 150 mM NaCl, 25 mM MgCl2, 0.5% Tween-20 and 2 mM levamisol) for 3–6 h at room temperature.

In vitro migration assay

Sympathetic ganglia were dissected from E12.5 embryos in ice-cold L15 media containing 5% heat-inactivated horse serum. SCGs were washed in 1×

Hank's Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA) and dissociated with 0.05% Trypsin–EDTA and 0.5 mg/ml DNase I (Sigma-Aldrich) at 37 °C for 6 min, followed by trituration with 1 mg/ml DNase I in HBSS. Cell debris was removed with centrifugation at 1000 rpm for 4 min, and neurons were resuspended in growth medium (1 mg/ml BSA, 20 mM L-glutamine, 1000 units/ml Penicillin/Streptomycin, and 50 ng/ml NGF in F12: DMEM). Of the cells dissected from the sympathetic ganglia, more than 95% were TH-positive sympathetic neurons. Four-thousand cells per well were plated on the upper chambers of poly-D-lysine and laminin-treated Corning Transwell permeable membranes, with Sema3A-, Sema3F-, or control-conditioned media added into the upper chambers. Migration was stopped after 15 h by fixation and post-migratory cells on the lower side of the filters were visualized and quantified with DAPI. The chemotactic index was determined by counting the number of post-migratory cells in control and experimental wells. The number of cells repelled by each semaphorin-added well was divided by the number of repelled neurons in control media in a minimum of 5 experiments; thus, the index equals 1.0 if no repulsion occurs.

Sema3A- or Sema3F-conditioned media were prepared as follows: COS cells were transfected with plasmids encoding full-length Sema3A or Sema3F cDNA using Lipofectamine 2000 (Invitrogen). After 16 h of transfection, the medium was replaced with sympathetic growth media. The transfected COS cells were further incubated for 48 h before the medium was collected and filtered using a Centriplus YM-1000 filter device (Millipore, Bedford, MA).

Immunoprecipitation

COS cells at 90% confluency were subjected to Lipofectamine 2000 transfection (Invitrogen) with plasmids containing cDNAs. Transfected cells were harvested in lysis buffer containing 50 mM HEPES pH 6.9, 1 mM $MgCl_2$, 5 mM EGTA, 10% glycerol, and 0.2% NP-40 with a cocktail of protease inhibitors (Roche). Insoluble material was removed by centrifugation at 4 °C for 15 min at 10,000×*g*, and recovered supernatant was immunoprecipitated by primary antibodies. The immune complexes were then immobilized on protein G- or protein A-Sepharose beads (Roche), washed 6 times with lysis buffer, resuspended and boiled in SDS-PAGE sample buffer, separated by SDS-PAGE, and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore) for Western blotting.

Results

Plexin mutants exhibit sympathetic axon guidance and neuron migration defects

To determine the specific roles of plexin-A3 and plexin-A4 in sympathetic development, we set out to examine the morphology of the sympathetic ganglia in mouse mutants lacking these plexin receptors. Using anti-tyrosine hydroxylase (TH) whole-mount staining we examined the overall structure of the ganglia at E13.5 (Figs. 1A, B). At this stage, cervical-level sympathetic neurons normally project axons along the ganglia in rostral–caudal directions, which will innervate the eyes, head, and neck. Mutant mice lacking *plexin-A3* also exhibit this normal phenotype (Fig. 1C). In contrast, *plexin-A4* mutants show obvious axon guidance defects (Fig. 1D). Numerous abnormal axons in *plexin-A4* mutants project medially from the sympathetic ganglia at this stage (arrows in Fig. 1D). In *plexin-A3/-A4* double mutants more severe axon guidance defects are observed (Fig. 1E). When examined in sections, the TH-positive signals in *plexin-A4* mutants exhibit a thin filamentous morphology (Figs. 2C, E). In *plexin-A3/-A4* double mutants numerous large bundles of TH-positive filamentous fibers are seen medial to the sympathetic ganglia (Figs. 2D, F), consistent with more severe axon guidance

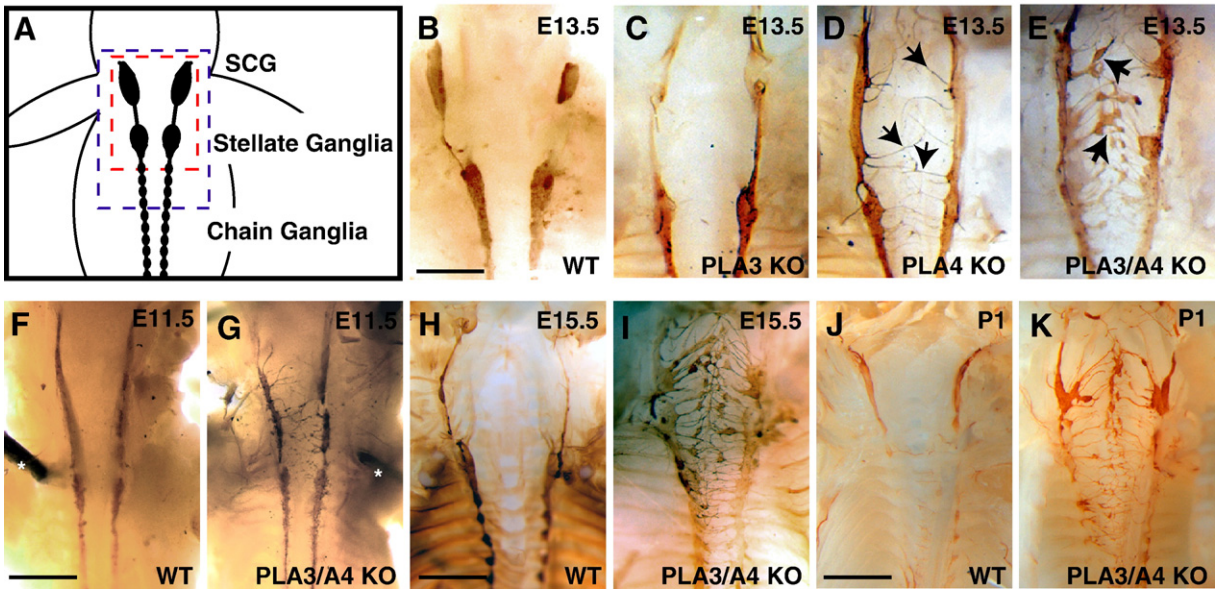


Fig. 1. Abnormalities of the developing sympathetic nervous system in plexin mutant mice. (A) A schematic diagram of the sympathetic nervous system in an E13.5 embryo indicating the normal placement of ganglia, which includes the superior cervical ganglia (SCG), stellate ganglia, and sympathetic chain ganglia. The dashed red line shows the area of images B–E. The dashed blue line shows the area of images in panels F–K. (B) A whole-mount image of an E13.5 wild-type (WT) mouse stained with an antibody against tyrosine hydroxylase (TH). (C) The *plexin-A3* knockout (PLA3 KO) shows no obvious defects. (D) The *plexin-A4* mutant (PLA4 KO) exhibits aberrant axons that project medially (arrows). (E) The *plexin-A3/-A4* double mutant (PLA3/A4 KO) exhibits abnormally located axons and cell aggregates (arrows) medial to the normal ganglia. (F–G) Whole-mount staining of WT and PLA3/A4 KO show defects as early as E11.5. Asterisks indicate pins used to splay the embryos for viewing. (H–I) Sympathetic defects of PLA3/A4 KO at E15.5. (J–K) Defects are still apparent at postnatal day 1 (P1) in PLA3/A4 KO mice. Scale bars: B–E, 200 μm; F–G, 150 μm; H–I, 275; J–K, 400 μm.

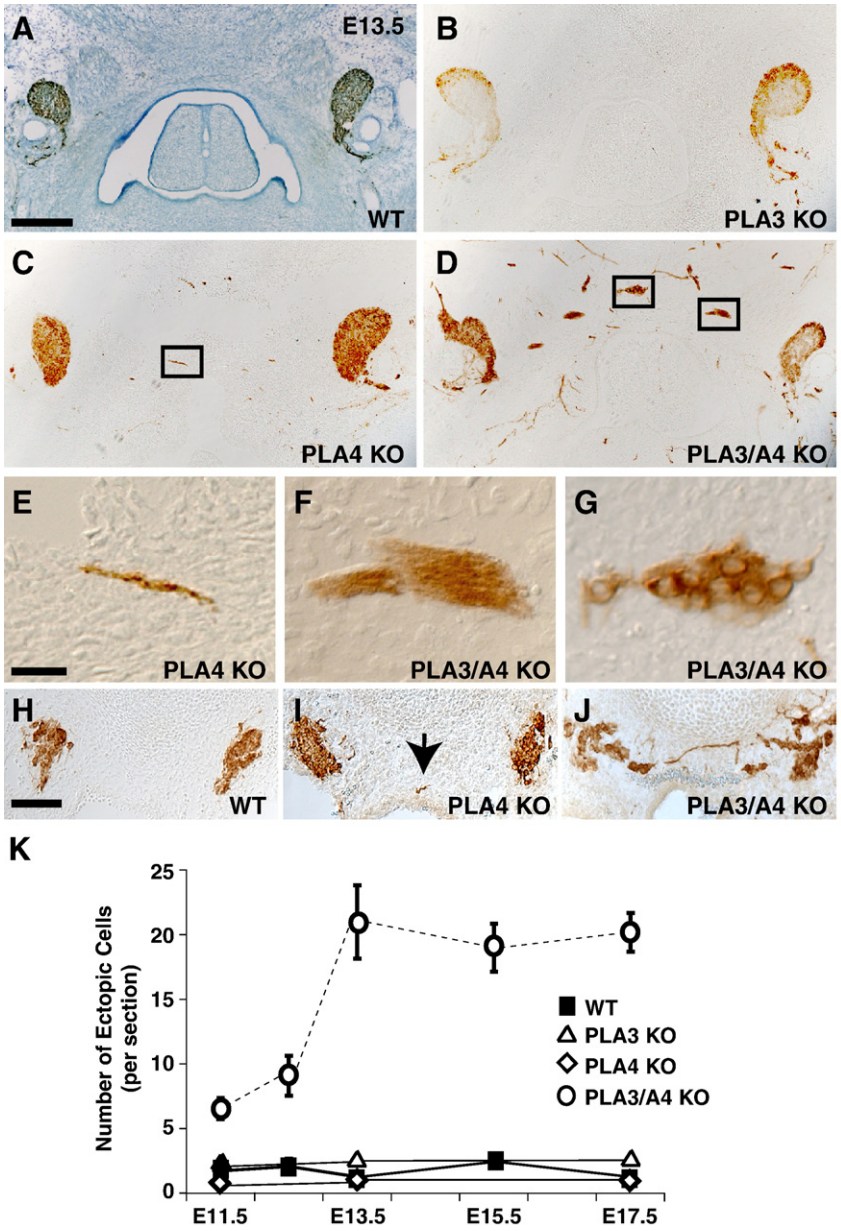


Fig. 2. Axon guidance and neuronal migration defects in the sympathetic nervous system of *plexin-A3*, *plexin-A4* and *plexin-A3/-A4* mutants. (A–D) Cross sections of E13.5 mice at the level of superior cervical ganglia (SCG) stained with anti-tyrosine hydroxylase (TH) antibodies. No ectopic staining is seen in panel A wild-type mice (WT, with Toluene Blue counter-staining to show surrounding tissues) and in panel B *plexin-A3* mutants (PLA3 KO). *Plexin-A4* mutants (PLA4 KO) exhibit only axon guidance defects (C). Numerous ectopic axons and neurons are seen in *plexin-A3/-A4* double mutants (PLA3/A4 KO) (D). (E) A higher magnification of the boxed area in panel C to highlight the TH-positive axons. (F) A higher magnification of the right boxed area in panel D showing greater numbers of fasciculating axons in PLA3/A4 KO than in PLA4 KO. (G) A higher magnification of the left boxed area in panel D showing ectopic cells that aggregate medially to the ganglia. (H–J) Sample images of the sympathetic chain ganglia from E13.5 WT or mutant embryos at trunk levels. Defects observed at cervical level are also seen at this level in PLA4 KO (arrow in panel I) and in PLA3/A4 KO (J). (K) Quantification of dislocated sympathetic neurons from WT, PLA3 KO, PLA4 KO, and PLA3/A4 KO mice. The line graph shows the average number (mean ± S.E.M.) of ectopic sympathetic neurons for each phenotype in the cervical region ($n = 15$ for WT; $n = 3$ for PLA3 KO; $n = 3$ for PLA4 KO; $n = 17$ for PLA3/A4 KO). See Materials and methods for quantification details. From E11.5 to E17.5, WT, PLA3 KO, and PLA4 KO mice exhibit few dislocated cells. In contrast, PLA3/A4 KO mice at E11.5 have already shown significantly increased dislocated neurons medial to the ganglia ($p < 0.0001$, Student's *t* test). This difference is dramatically increased between E12.5 and E13.5, and plateaus after E13.5. Scale bars: A–D, 200 μm; E–G, 50 μm; H–J, 100 μm.

defects in double mutants. The defects are first detected at E11.5 in *plexin-A3/-A4* double mutants (Fig. 1G), and persist through postnatal day 1 (P1) (Fig. 1K). These *in vivo* results confirm *in vitro* findings that plexin-A3 and plexin-A4 differentially regulate axon guidance in sympathetic neurons (Yaron et al., 2005).

While plexin-A3 and plexin-A4 have been implicated in the axon guidance of sympathetic neurons, their role in sympathetic cell migration has not been determined. In addition to abnormal axons, *plexin-A3/-A4* double mutants exhibit ganglion-like TH-positive staining. In whole mount preparations, they are located medially to the normal sympathetic ganglia (arrows in Fig. 1E).

In cross sections, this TH-positive staining is consistent with neurons that form ectopic ganglia, as they form aggregates with smooth and rounded appearances (Figs. 2D, G). Neither *plexin-A3* nor *plexin-A4* single mutant exhibits ectopic ganglia. The finding that ectopic cells are only seen in the double mutants indicates that plexin-A3 and plexin-A4 are redundantly required for cell migration, as a lack of both of these receptors is necessary for the development of migration defects in the sympathetic nervous system.

Although neither single plexin mutant exhibits ectopic sympathetic cells, we did note that *plexin-A4* single mutants are morphologically different from wild type mice. After reaching the dorsal aorta, differentiated sympathetic neurons undergo rostral–caudal intermixing migration that results in the differentiated ganglia. This process forms the superior cervical ganglia and stellate ganglia by E13.5 (Fig. 1B). This segregation seems incomplete in *plexin-A4* and *plexin-A3/-A4* mutants (Figs. 1D, E), indicating that the rostral–caudal phase of sympathetic migration may require plexin-A4. Since this subtle phenotype is hard to quantify, migration defects in this report focus on ectopic neurons. Taken together, we conclude that, while plexin-A4 may contribute to the segregation between the SCG and the stellate ganglia, both plexin-A3 and plexin-A4 act redundantly to restrict migrating neurons to the sympathetic ganglia during development.

Plexin-A3 and plexin-A4 are not required for neural crest migration

The migration defects seen in *plexin-A3/-A4* double mutants suggest that abnormal migration occurs prior to or during E13.5 in the mouse. Since sympathetic neurons have undergone both neural crest migration and rostral–caudal migration by this time, we decided to determine which of these processes require plexin-A3 and plexin-A4 signaling. To address the possibility that plexin-A3 or plexin-A4 may guide sympathetic precursors prior to reaching the dorsal aorta, we studied the expression of *plexin-A3* and *plexin-A4* in migrating neural crest cells and the sympathetic anlagen. *Plexin-A3* and *plexin-A4* are not detected in neural crest cells during migration through the somites (Figs. 3A, B). Instead, these plexins are only upregulated after neural crest cells reach the sympathetic anlagen. *Plexin-A3* expression is not evident in the sympathetic ganglia until E11.5 (Fig. 3G). In contrast to *plexin-A3*, *plexin-A4* is expressed earlier. In whole mount preparations, *plexin-A4* is first detected at the sympathetic anlagen at E9.5 (Fig. 3B). This expression pattern has been observed in other genes that are specific to sympathetic anlagen (For example: Britsch et al., 1998; Durbec et al., 1996). The expression of *plexin-A4* in the sympathetic ganglia is more evident at E10.5 (Figs. 3D, F).

Even low expression levels of plexins may be able to guide neural crest cells. As neural crest cells migrate ventrally, they undergo segmental migration through the rostral somites and avoid the caudal somites. Previous analyses show that segmental neural crest migration through the somites is disrupted in *Sema3F* and *neuropilin-2* mutants (Gammill et al., 2006). However, it has not been fully addressed whether the later development of the sympathetic nervous system is altered in these mutants (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). Since plexins form co-receptors with neuropilins to mediate class 3 semaphorins, we set out to determine the roles of plexin-A3 and plexin-A4 in this context despite possible low expression levels. We first investigated the requirement of plexin-A3 and plexin-A4 in neuropilin-2-mediated segmental migration through the somites by viewing p75-positive neural crest cells. As reported, *neuropilin-2* mutants exhibit a loss of this segmental migration (Fig. 3J). However, *plexin-A3/-A4* double mutants show normal migration through the rostral somites, as seen in wild-type mice (Figs. 3I, K), indicating that plexin-A3 and plexin-A4 are not required for Sema3F/neuropilin-2-mediated neural crest migration through the somites.

To further examine the roles of plexin-A3 and plexin-A4 during ventral neural crest migration, we used markers for p75, MASH1, and TH, to label sympathetic precursors and neurons as they aggregate at the dorsal aorta to form the sympathetic anlagen. In wild-type mice, sympathetic precursors and neurons are located in aggregates at the dorsal aorta by E10.5 (Figs. 3L, N, P). This aggregation is also seen in *plexin-A3/-A4* double mutants, with no ectopic sympathetic precursors or neurons observed (Figs. 3M, O, Q). That no ectopic cells are seen by this stage indicates that plexin-A3 and plexin-A4 are not required for the ventral migration of sympathetic precursors. The absence of ectopic neurons at E10.5 in *plexin-A3/-A4* double mutants is different from what has been reported in *neuropilin-1* or *Sema3A* mutants, in which ectopic sympathetic precursors can be seen as early as E9.25 (Kawasaki et al., 2002). Instead, migration defects in *plexin-A3/-A4* double mutants are not seen until E11.5, when both plexins are first expressed.

Ectopic neurons in plexin-A3/-A4 double mutants develop with the rostral–caudal migration of sympathetic neurons

Since the migration defects in *plexin-A3/-A4* double mutants are not due to aberrant migration of sympathetic neural crest precursors, we next characterized the migration of differentiated sympathetic neurons in plexin mutant embryos. *Plexin-A3* and *plexin-A4* are first coexpressed in sympathetic neurons at E11.5. Starting at E11.5, we quantified and compared the average number of ectopic sympathetic neurons at the level of the SCG. This analysis shows that, from E11.5 to E17.5, *plexin-A3* or *plexin-A4* single mutants exhibit only small numbers of dislocated neurons that are not significantly different from the numbers observed in wild-type mice. However, in *plexin-A3/-A4* double mutants, ectopic neurons are evident by E11.5 (Fig. 2K). More importantly, instead of increasing linearly over time, a more than 2-fold increase in ectopic neurons is observed between E12.5 and E13.5 (Fig. 2K). After E13.5, the number of ectopic cells in double mutants plateaus through E17.5. The rapid increase in ectopic cells through E13.5 correlates with the rostral–caudal migration of sympathetic neurons (Nishino et al., 1999), suggesting that plexin-A3 and plexin-A4 act to restrict the migration of sympathetic neurons during this phase.

During sympathetic development, the different ganglia of the sympathetic nervous system are thought to be derived from

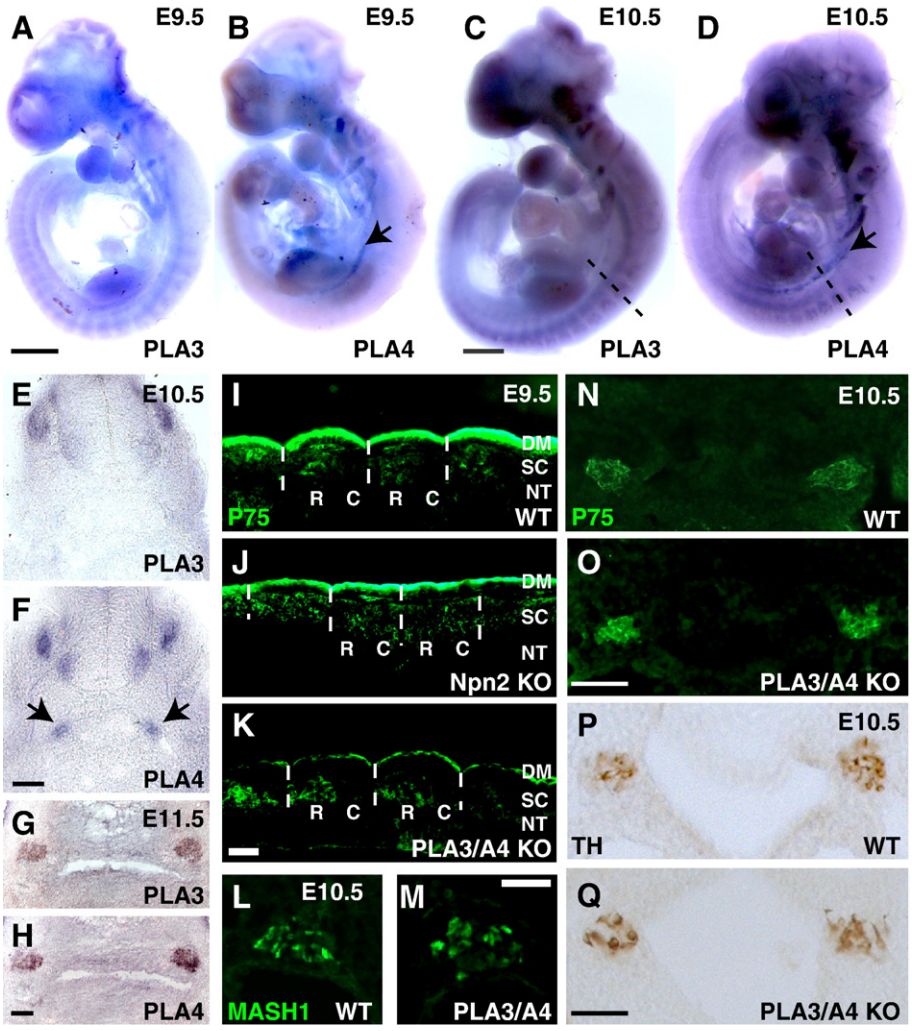


Fig. 3. Ventral migration of neural crest cells is unaffected in *plexin-A3/-A4* double mutants. (A–D) *In situ* hybridization on whole mount embryos shows that *plexin-A3* and *plexin-A4* are expressed after sympathetic precursors have reached the sympathetic anlagen. *Plexin-A3* and *plexin-A4* are not expressed in segmentally migrating neural crest cells in the somites at E9.5 (A and B) but initial *plexin-A4* expression can be seen at the sympathetic anlagen at this stage (arrow in panel B). At E10.5, *plexin-A4* expression is seen in the sympathetic anlagen (arrow in panel D). Additionally, both *plexin-A3* and *plexin-A4* expression is seen in the dorsal root ganglia (C and D). (E–F) Sections of embryos from C and D again show *plexin-A4* expression in the sympathetic ganglia, but not *plexin-A3* expression (Plane of section is indicated with dashed lines on the whole mount view). The expression of *plexin-A3* at the sympathetic ganglia is not seen until E11.5 (G). (I–K) Coronal sections of E9.5 WT and mutant embryos immuno-stained with anti-p75 antibodies to show the migration patterns of neural crest cells in the somites. Somites are delineated by dotted lines. Neural crest cells migrate segmentally through the rostral sclerotomes (R), and avoid the caudal (C) in wild-type mice (WT) (I). The rostral migration pattern is lost in *neuropilin-2* mutants (Npn2 KO) (J). *Plexin-A3/-A4* double mutants (PLA3/A4 KO) do not exhibit neural crest migration defects (K). (L–M) Cross sections of sympathetic precursors at E10.5 in WT and PLA3/A4 KO mice immuno-stained with a MASH1 antibody. A subpopulation of sympathetic precursors expresses MASH1 at this stage in WT embryos (L). Similar aggregation and expression are also seen in PLA3/A4 KO mutants (M). (N–Q) Cross sections of E10.5 WT and mutant embryos immuno-stained with antibodies against p75 and tyrosine hydroxylase (TH) to show the aggregation patterns of the sympathetic precursors in the anlagen. Cells in the WT embryos form discrete aggregates at the dorsal aorta (N and P). Normal aggregation also occurs in the PLA3/A4 KO embryos, as no ectopic cells are seen (O and Q). DM, dermamyotome; SC, sclerotomes; NT, neural tube. Scale bars: A–B, 200 μm; C–D, 500 μm; E–H, 100 μm; I–K, N–Q, 50 μm; L–M, 25 μm.

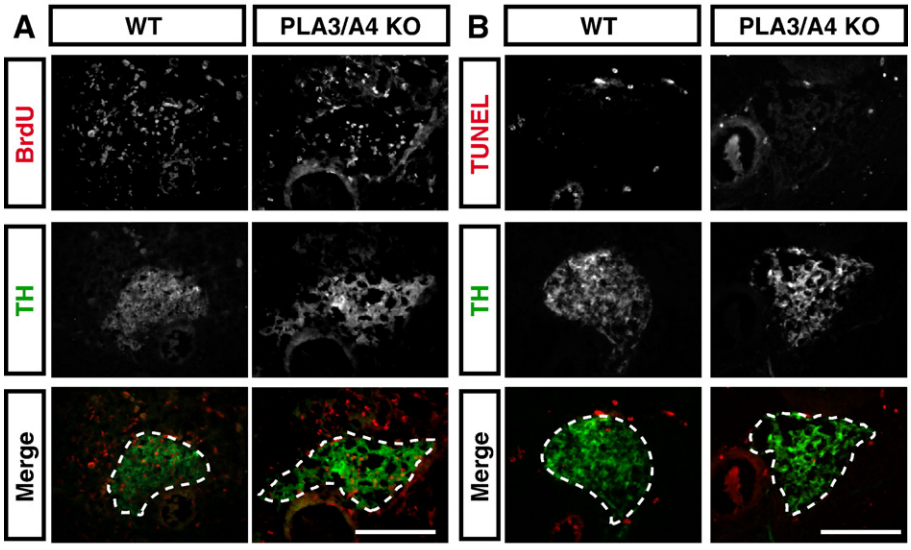
different axial levels of the neural crest. Particularly, the vagal neural crest is thought to contribute primarily to the SCG while the trunk neural crest develops mainly into the more caudal stellate ganglia and sympathetic chain ganglia (Durbec et al., 1996). Interestingly, defects in *plexin-A3/-A4* double mutants were found at all axial levels examined (Figs. 2H, I, J). Since the SCG and stellate ganglia contain the largest numbers of sympathetic neurons, defects were most obvious at these levels. This indicates that plexin-A3 and plexin-A4 are required to restrict migrating sympathetic neurons regardless of neural crest origin.

One can argue that the increase in ectopic cells between E12.5 and E13.5 in *plexin-A3/-A4* double mutants may be due to increased cell division or decreased cell death, rather than due to aberrant cell migration. Cell division occurs during this period of sympathetic development and contributes to the increased size of the SCG (Rubin, 1985). However, very little cell death occurs between E11.5 and E13.5 in sympathetic

neurons (Enomoto et al., 2001). To rule out these possibilities, we first calculated the mitotic index, defined as the ratio of BrdU-positive cells within TH-positive sympathetic neurons, in the sympathetic ganglia and in ectopic neurons. The mitotic index is similar between SCG cells in wild-type and double mutant mice. This index is also the same in ectopic neurons and neurons of the SCG in double mutant mice (Figs. 4A, C). Second, between E11.5 and E13.5, cell death (TUNEL) assays show that less than 2% of sympathetic cells are both TH- and TUNEL-positive (Figs. 4B, D). Such low frequency of cell death is consistent with previous reports (Enomoto et al., 2001). In addition, no detectable differences in cell death are seen between wild-type and *plexin-A3/-A4* double mutants (Figs. 4B, D). These data suggest that plexin-A3 and plexin-A4 confine sympathetic neurons to the sympathetic anlagen during rostral–caudal migration and that ectopic cells in *plexin-A3/-A4* double mutants develop due to abnormal migration rather than changes in the cell cycle.

Sympathetic neurons from plexin-A3/-A4 double mutants lose migratory responsiveness to class 3 semaphorins

To address how plexins mediate sympathetic neuronal migration, we investigated the migration of sympathetic neurons in a modified Boyden chamber chemotactic assay. Since Sema3A and Sema3F have previously been determined to guide sympathetic axon guidance, we investigated their roles in sympathetic migration. We found that both Sema3A and Sema3F are able to repel dissociated neurons from wild-type sympathetic SCGs *in vitro* (Fig. 5A). In contrast to previous results for sympathetic axon guidance (Yaron et al., 2005), we found that Sema3A and Sema3F can indistinguishably repel



C

Mitotic Index

	E11.5	E12.5	E13.5
WT-SCG	0.275±0.09	0.21±0.09	0.323±0.06
PLA3/A4 KO-SCG	0.271±0.12	0.179±0.04	0.268±0.1
PLA3/A4 KO-Ectopic	0.138±0.02	0.196±0.04	0.154±0.08

D

Apoptosis (%)

	E11.5	E12.5	E13.5
WT-SCG	1.35±0.48	1.02±0.3	1.13±0.24
PLA3/A4 KO-SCG	1.03±0.52	0.86±0.17	1.16±0.34
PLA3/A4 KO-Ectopic	1.5±0.69	1.3±0.62	1.34±0.73

Fig. 4. Rates of sympathetic neuron mitosis and apoptosis are normal in *plexin-A3/-A4* double mutant embryos. (A–B) Representative images of the SCG showing the colocalization of tyrosine hydroxylase (TH) with BrdU or TUNEL staining. Individual channels are shown separately and in a merged image below. In each merged image, the SCG is outlined with a white dashed line. The colocalization of TH (green) and BrdU (red) staining in WT and PLA3/A4 KO mice is seen in panel A. Colocalization of TH and TUNEL-positive (red) cells in the SCG of WT and PLA3/A4 KO mice (B). (C) Quantification of mitotic indices (the ratio of BrdU-positive cells to TH-positive sympathetic cells) in the superior cervical ganglia (SCG) shows no significant differences between wild-type (WT) and *plexin-A3/-A4* double mutant (PLA3/A4 KO) embryos. In addition, ectopic cells (Ectopic) and SCGs from *plexin-A3/-A4* double mutants show similar indices. Numbers are in mean±S.E.M. cells for each phenotype from E11.5 to E13.5 ($n=6$ for WT; $n=7$ for PLA3/A4 KO). (D) The percentage of apoptosis in the superior cervical ganglia at E11.5, E12.5, and E13.5 are not changed between WT and PLA3/A4 KO mice. Numbers are in mean±S.E.M. as determined by TUNEL analysis. Scale bars: A–B, 100 μm.

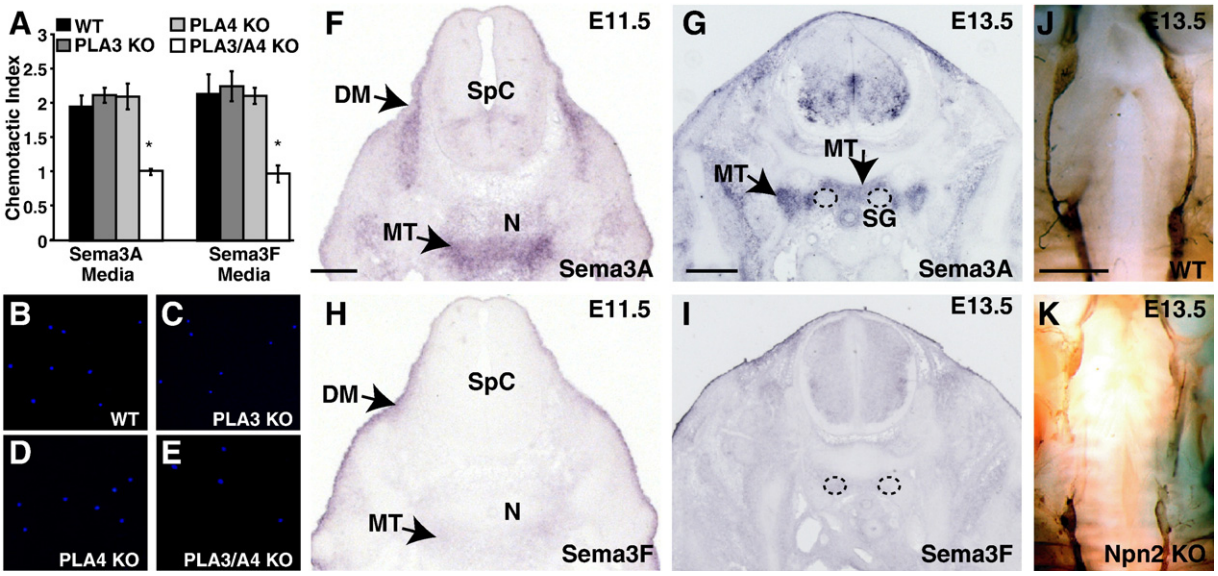


Fig. 5. Sympathetic neurons from *plexin-A3/-A4* double mutants lose their migratory responsiveness to Sema3A and Sema3F *in vitro*. (A) Responses of dissociated sympathetic neurons from E12.5 wild-type (WT), *plexin-A3* (PLA3 KO), *plexin-A4* (PLA4 KO), and *plexin-A3/-A4* (PLA3/A4 KO) mutant mice to a gradient of Sema3A or Sema3F in a modified Boyden chamber chemotaxis assay. The chemotactic index is determined by the number of neurons repelled by semaphorins divided by the number of repelled neurons treated with control media ($n=3$ to 6 for each experiment). See Materials and methods for detail. Both Sema3A and Sema3F repel migrating sympathetic neurons from WT, PLA3 KO and PLA4 KO mice. In contrast, sympathetic neurons from PLA3/A4 KO animals lose responsiveness to both Sema3A and Sema3F (asterisks indicate $p<0.01$ and $p<0.05$, for Sema3A and Sema3F, respectively, Student's *t* test). (B–E) Representative images of DAPI-stained sympathetic neurons after migration from a gradient of Sema3A. (F–I) Expression patterns of *Sema3A* and *Sema3F* with *in situ* hybridization. Cross sections of E11.5 embryos show that *Sema3A* is expressed in the dermamyotome and paraxial mesenchymal tissues (F). By E13.5, *Sema3A* is highly expressed in the mesenchymal areas medial to and surrounding the sympathetic ganglia (G). The sympathetic ganglia are circled in panels G, I. *Sema3F* is not expressed in tissues surrounding the developing sympathetic ganglia from E11.5 to E13.5 (H–I). (J–K) Tyrosine hydroxylase (TH) staining of wild type and neuropilin-2 mutant (Npn2 KO) mice. Npn2 KO mice exhibit no obvious defects. DM, dermamyotome; SG, sympathetic ganglia; SpC, spinal cord; N, notochord; DRG, dorsal root ganglia. Scale bars; F, H, 100 μm; G, I, J–K 200 μm.

migrating neurons from either *plexin-A3* or *plexin-A4* single mutants. However, sympathetic neurons from *plexin-A3/-A4* double mutants do lose their ability to migrate away from both of these semaphorins *in vitro* (Figs. 5A–E). These data are consistent with *in vivo* results showing that the presence of either plexin-A3 or plexin-A4 in sympathetic neurons is sufficient to restrict these cells to the ganglia.

Previous studies have implicated Sema3A and neuropilin-1 in regulating the migration of sympathetic neurons (Behar et al., 1996; Kawasaki et al., 2002) but it is not clear whether Sema3F and neuropilin-2 are also involved. To elucidate which semaphorins may be involved in plexin-A3/-A4-mediated sympathetic migration, we examined the expression of *Sema3A* and *Sema3F*. During neural crest cell migration, *Sema3A* mRNA is strongly expressed in dermamyotomes and forelimbs, and mildly in paraxial mesenchymal tissues (Kawasaki et al., 2002) (data not shown). The expression of *Sema3A* in paraxial mesenchymal tissues becomes stronger when sympathetic neurons began the stage of rostral–caudal migration. By E11.5, strong *Sema3A* expression is seen in the dermamyotome, surrounding dorsal root ganglia, and in paraxial mesenchymal tissues at cervical and trunk levels (Figs. 5F) (data not shown). By E13.5, continued expression of *Sema3A* is noted in the dermamyotome and mesenchymal tissues medial to and surrounding the sympathetic ganglia (Fig. 5G). In contrast, *Sema3F* transcripts are undetectable during this period (Figs. 5H–I). Since neuropilin-2 is required for Sema3F signaling, we also examined mutant mice lacking neuropilin-2, and found that *neuropilin-2* mutants do not exhibit any detectable sympathetic defects at E13.5, demonstrating that Sema3F/neuropilin-2 signaling is not required to restrict sympathetic neurons to the ganglia *in vivo* (Figs. 5J–K). Taken together, these results indicate that, in response to Sema3A signals, plexin-A3 and plexin-A4 function redundantly to restrict the migration of sympathetic neurons during the rostral–caudal phase of sympathetic migration.

Plexin-A3 and plexin-A4 form independent receptor complexes with neuropilins

Previous biochemical data have shown that plexin co-immunoprecipitates with neuropilin to form a receptor complex in transiently transfected cells (Rohm et al., 2000; Takahashi et al., 1999), but it is not known whether these two different plexins interact with each other. We report that plexin-A3 and plexin-A4 are differentially and redundantly required for axon guidance and neuron migration in the developing sympathetic nervous system *in vivo*. It is likely that these two plexins form separate receptor complexes with neuropilin on the cell surface to mediate semaphorin signals but there is some data to suggest that plexins can also associate with each other (Usui et al., 2003). To examine what associations plexin-A3 and plexin-A4 can make, we performed co-immunoprecipitation experiments in COS cells. As expected, plexin-A4 associates with neuropilin-1 and

neuropilin-2 (Fig. 6A). We then tested the interactions between plexin-A3 and plexin-A4 and found that the two plexins do not associate with each other when over-expressed (Fig. 6B). The presence of neuropilin-1 or neuopilin-2 does not lead to the association of these two plexins (Fig. 6C). These data indicate that when plexin-A3, plexin-A4, neuropilin-1 and neuropilin-2 are all present on the cell surface, as seen in sympathetic neurons, each plexin and neuropilin forms a distinct receptor complex to mediate specific semaphorins.

Discussion

Proper neuron migration and axon guidance are important for the formation of a functional nervous system. We have examined the roles of plexin-A3 and plexin-A4 in sympathetic development, and found that both plexins redundantly restrict sympathetic neurons to the ganglia during rostral–caudal migration, and differentially guide sympathetic axons *in vivo*. However, these two plexins are not required during ventral neural crest migration. Immunoprecipitation assays suggest that plexin-A3 and plexin-A4 form independent receptor complexes to mediate semaphorin signaling. Our results have identified the specific roles of plexin-A3 and plexin-A4 in regulating sympathetic axon guidance and neuron migration *in vivo*.

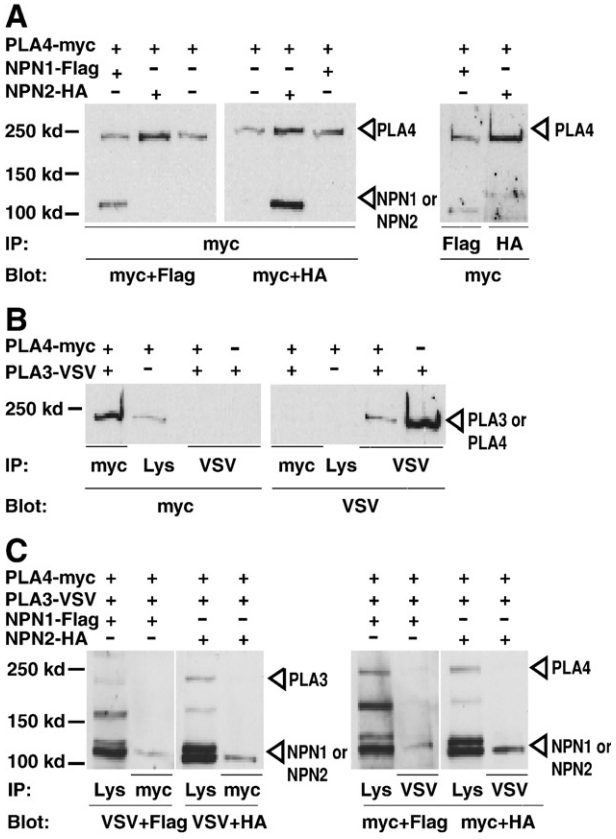


Fig. 6. Plexin-A3 and plexin-A4 do not associate with each other. (A) Western blot analysis shows that plexin-A4 (PLA4) immunoprecipitates with neuopilin-1 (Npn1) or neuropilin-2 (Npn2) when over-expressed in COS cells. (B) Plexin-A3 (PLA3) does not associate with PLA4 when both plexins are expressed. (C) The presence of either neuropilin does not promote the association of PLA3 and PLA4 in COS cells over-expressing these proteins. Each protein is fused with a tag as indicated. For each lane, "+" indicates the proteins that are expressed. Specific antibodies against tags that are used in the immunoprecipitation (IP) and western blot (Blot) are listed. The bands corresponding to the size of plexin or neuropilin are also indicated. Lys, cell lysates.

Both peripheral sensory and sympathetic neurons are derived from neural crest cells. During the period when these two types of neurons are actively sending their axons to make connections, sensory neurons express only neuropilin-1, which associates with Sema3A. In contrast, sympathetic neurons express both neuropilin-1 and neuropilin-2, allowing them to respond to both Sema3A and Sema3F, respectively (Chen et al., 1997; Kawakami et al., 1996). Among all nine plexin receptors, only plexin-A3 and plexin-A4 are expressed in these neurons at this time point (Cheng et al., 2001). We previously used an *in vitro* sympathetic axon repulsion assay to test the specificity of plexins in mediating different semaphorin signals and found that plexin-A3 is preferentially used in Sema3F/neuropilin-2 signaling while plexin-A4 primarily signals downstream of Sema3A/neuropilin-1 (Yaron et al., 2005). However, this preferential usage does not exclude the involvement of plexin-A3 in Sema3A/neuropilin-1 signaling or plexin-A4 in Sema3F/neuropilin-2 signaling *in vivo*. Sema3A/neuropilin-1 signaling guides peripheral sensory axons via both plexin-A3 and plexin-A4, although plexin-A4 plays a more significant role than plexin-A3 (Yaron et al., 2005). In this report, we examined the guidance of sympathetic axons in *plexin-A3* and *plexin-A4* mutants and found that the defects are very similar to what has been observed in sensory axons: sympathetic axon guidance is defective in *plexin-A4* single knockout animals but not in the *plexin-A3* mutants. In addition, these defects are more severe in double mutants. Although neuropilin-2 is expressed in sympathetic neurons, we showed that the *neuropilin-2* knockout embryos have normal sympathetic axon guidance and that no detectable Sema3F transcripts can be seen in the neighboring tissues when sympathetic projections are being formed. Taken together, we conclude that, similar to sensory axons, sympathetic neurons use Sema3A signals to guide their axons *in vivo*. Both plexin-A3 and plexin-A4 are the signaling receptors for this process, with a preference for the latter in *vivo*. In addition, the presence of the neuropilin-2 receptor on sympathetic axons does not seem to interfere with the differential usage of the two plexins for Sema3A/neuropilin-1 signaling.

Suto et al. (2005) have recently suggested that two transmembrane semaphorins, Sema6A and Sema6B, may also contribute to the guidance of sympathetic axons through plexin-A4. They show that plexin-A4 directly associates with Sema6A and Sema6B, and that these two semaphorins are expressed in the anterior vertebrate muscle near the sympathetic ganglia. We have preliminarily examined the development of the sympathetic nervous system in *Sema6A* mutant embryos and did not observe any neuronal migration or axon guidance defects (data not shown). Further examination is required to determine if Sema6B regulates sympathetic development. As *plexin-A4* mutant embryos exhibit axon guidance defects that are not restricted to areas where *Sema6B* is expressed, it is likely that plexin-A4 may mediate both long-range (secreted) and short-range (transmembrane) semaphorin signals to guide sympathetic

axons. Since plexin-A3 receptors may also play a role in sympathetic axon guidance, further studies will be required to determine whether plexin-A3 can also mediate short-range semaphorin signals in developing sympathetic neurons.

Semaphorins and neuropilins are known to regulate the migration of neural crest cells and sympathetic neurons *in vivo* (Young et al., 2004). Sema3F signaling through neuropilin-2 restricts migrating neural crest cells to the rostral halves of somites as they migrate from the neural tube (Gammill et al., 2006). As these neural crest cells leave the somites and continue migrating ventrally to form the sympathetic anlagen near the aorta, Sema3A signaling through neuropilin-1 is required to regulate this migration (Kawasaki et al., 2002). Although plexin-A3 and plexin-A4 are known signaling receptors for Sema3A and Sema3F, it is surprising to find that the ventral migration of neural crest cells is intact in *plexin-A3/-A4* double mutants. By studying expression patterns, we found that this is due to the lack of *plexin-A3* and *plexin-A4* in migrating neural crest cells. This finding is consistent with the observations that 1) sympathetic migration defects in *plexin-A3/-A4* double mutants are not detected until the beginning of sympathetic rostral–caudal migration (E11.5) and that 2) the extent of the defects in *plexin-A3/-A4* double mutants is more limited than those in *Sema3A* or *neuropilin-1* mutants. These results also suggest that other plexins or non-plexin receptors are required for the Sema3A- and Sema3F-mediated ventral migration of sympathetic neural crest precursors.

Plexin-A3 and plexin-A4 are required to restrict the migration of sympathetic neurons during the rostral–caudal intermixing phase of migration. Unexpectedly, we find that migration defects are observed only in *plexin-A3/-A4* double mutants but not in either of the single mutants. Consistent with this finding, sympathetic neurons from *plexin-A3/-A4* double mutants, but not those from single mutants, lose their responsiveness to Sema3A and Sema3F in the *in vitro* migration assay. These somewhat surprising results indicate that, in contrast to the guidance of sympathetic axons, both plexin-A3 and plexin-A4 are utilized to mediate the sympathetic neuronal migration caused either by Sema3A/neuropilin-1 signaling or Sema3F/neuropilin-2 signaling. Since we did not observe any sympathetic neuron migration defects in *neuropilin-2* mutants and did not detect *Sema3F* transcripts near the migrating sympathetic neurons, it is most likely that plexin-A3 and plexin-A4 redundantly mediate Sema3A/neuropilin-1 signals *in vivo* to restrict migrating sympathetic cells to the ganglia during the rostral–caudal phase of migration.

In the presence of neuropilin-1, neuropilin-2, plexin-A3, and plexin-A4, why do the two plexins act differently in mediating the neuronal migration and axon guidance of developing sympathetic neurons? In our attempt to address this issue, we asked whether plexin-A3 and plexin-A4 are both present in large receptor complexes with neuropilins on the cell surface. Previous studies have focused on the cell surface interactions between neuropilins and plexins, and have found that the interactions are quite promiscuous (Rohm et al., 2000; Takahashi et al., 1999; Tamagnone et al., 1999), although there is evidence that plexins may associate with each other (Usui et al., 2003). We show that, plexin-A3 and plexin-A4 did not associate with each other, even in the presence of neuropilins. The biochemical data suggest that these two plexins may separately form receptor complexes with neuropilin to mediate semaphorin signaling in sympathetic neurons. Apparently, more experiments are required to further address this issue. For example, the components of cytoplasmic signaling molecules that mediate plexin signaling can be changed when sympathetic neurons shift developmental stages, such as from migration to axonal projection. Alternatively, the number of receptors expressed on the cell surface or the expression level of semaphorins in the surrounding tissues can also change with development. Despite these additional factors, this current hypothesis is consistent with the observations that the presence of neuropilin-2 does not affect the preferential requirement of plexin-A4 for the guidance of the sympathetic axons and that plexin-A3 and plexin-A4 are redundantly required in mediating the migration of sympathetic neurons.

Many factors have been reported to affect the development of the sympathetic nervous system. These factors include other axon guidance molecules that have been implicated in neural crest migration, such as ephrins, and neurotrophins that guide sympathetic migration and axon innervation (Honma et al., 2002; Krull et al., 1997; Kuruvilla et al., 2004). It seems clear that these factors are required at multiple specific points of development to ensure the completion of sympathetic development but how all these signals are integrated during development remains to be addressed. Our results indicate that plexin-A3 and plexin-A4 are upregulated after sympathetic differentiation has occurs to specifically mediate semaphorin signals. Since many of the *plexin-A3/-A4* double mutant mice survive into adulthood, it will also be interesting to know what the consequence of the sympathetic abnormality is and whether these abnormalities are corrected later in development.

Acknowledgments

We thank C. Erickson, A. Yaron and members of the Cheng and Erickson laboratories for valuable comments. This work was supported by grants from the National Institutes of Health (HD045757) and the Department of Pathology and Laboratory Medicine, UC Davis, and by fellowships from the Esther A. and Joseph Klingenstein Fund, the Alfred P. Sloan Foundation, and the UC Davis Molecular and Cell Biology Training Grant.

References

Bagri, A., Tessier-Lavigne, M., 2002. Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance. Adv. Exp. Med. Biol. 515, 13–31.

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., Fishman, M.C., 1996. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature 383, 525–528.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C., Riethmacher, D., 1998. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes Dev. 12, 1825–1836.

Bron, R., Eickholt, B.J., Vermeren, M., Fragale, N., Cohen, J., 2004. Functional knockdown of neuropilin-1 in the developing chick nervous system by

siRNA hairpins phenocopies genetic ablation in the mouse. Dev. Dyn. 230, 299–308.
Chen, H., Chedotal, A., He, Z., Goodman, C.S., Tessier-Lavigne, M., 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19, 547–559.
Chen, H., Bagri, A., Zupicich, J.A., Zou, Y., Stoeckli, E., Pleasure, S.J., Lowenstein, D.H., Skarnes, W.C., Chedotal, A., Tessier-Lavigne, M., 2000. Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron 25, 43–56.
Cheng, H.J., Bagri, A., Yaron, A., Stein, E., Pleasure, S.J., Tessier-Lavigne, M., 2001. Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. Neuron 32, 249–263.
Dickson, B.J., 2002. Molecular mechanisms of axon guidance. Science 298, 1959–1964.
Durbec, P.L., Larsson-Blomberg, L.B., Schuchardt, A., Costantini, F., Pachnis, V., 1996. Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. Development 122, 349–358.
Enomoto, H., Crawford, P.A., Gorodinsky, A., Heuckeroth, R.O., Johnson Jr., E.M., Milbrandt, J., 2001. RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development 128, 3963–3974.
Francis, N.J., Landis, S.C., 1999. Cellular and molecular determinants of sympathetic neuron development. Annu. Rev. Neurosci. 22, 541–566.
Fujisawa, H., 2004. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. J. Neurobiol. 59, 24–33.
Gammill, L.S., Gonzalez, C., Gu, C., Bronner-Fraser, M., 2006. Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. Development 133, 99–106.
Giger, R.J., Urquhart, E.R., Gillespie, S.K., Levengood, D.V., Ginty, D.D., Kolodkin, A.L., 1998. Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron 21, 1079–1092.
Giger, R.J., Cloutier, J.F., Sahay, A., Prinjha, R.K., Levengood, D.V., Moore, S.E., Pickering, S., Simmons, D., Rastan, S., Walsh, F.S., Kolodkin, A.L., Ginty, D.D., Geppert, M., 2000. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron 25, 29–41.
Gu, C., Yoshida, Y., Livet, J., Reimert, D.V., Mann, F., Merte, J., Henderson, C.E., Jessell, T.M., Kolodkin, A.L., Ginty, D.D., 2005. Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. Science 307, 265–268.
He, Z., Tessier-Lavigne, M., 1997. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90, 739–751.
He, Z., Wang, K.C., Koprivica, V., Ming, G., Song, H.J., 2002. Knowing how to navigate: mechanisms of semaphorin signaling in the nervous system. Sci. STKE RE1.
Honma, Y., Araki, T., Gianino, S., Bruce, A., Heuckeroth, R., Johnson, E., Milbrandt, J., 2002. Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons. Neuron 35, 267–282.
Huber, A.B., Kolodkin, A.L., Ginty, D.D., Cloutier, J.F., 2003. Signaling at the growth cone: ligand–receptor complexes and the control of axon growth and guidance. Annu. Rev. Neurosci. 26, 509–563.
Kasemeier-Kulesa, J.C., Kulesa, P.M., Lefcort, F., 2005. Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia. Development 132, 235–245.
Kawakami, A., Kitsukawa, T., Takagi, S., Fujisawa, H., 1996. Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. J. Neurobiol. 29, 1–17.
Kawasaki, T., Bekku, Y., Suto, F., Kitsukawa, T., Taniguchi, M., Nagatsu, I., Nagatsu, T., Itoh, K., Yagi, T., Fujisawa, H., 2002. Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. Development 129, 671–680.
Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T., Fujisawa, H., 1997. Neuropilin–semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron 19, 995–1005.
Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J., Ginty, D.D., 1997. Neuropilin is a semaphorin III receptor. Cell 90, 753–762.
Krull, C.E., Lansford, R., Gale, N.W., Collazo, A., Marcelle, C., Yancopoulos, G.D., Fraser, S.E., Bronner-Fraser, M., 1997. Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. Curr. Biol. 7, 571–580.
Kuan, C.Y., Tannahill, D., Cook, G.M., Keynes, R.J., 2004. Somite polarity and segmental patterning of the peripheral nervous system. Mech. Dev. 121, 1055–1068.
Kuruvilla, R., Zweifel, L.S., Glebova, N.O., Lonze, B.E., Valdez, G., Ye, H., Ginty, D.D., 2004. A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118, 243–255.
Le Douarin, N., Kalcheim, C., 1999. The Neural Crest. Cambridge University Press, Cambridge, UK.
Nakamura, F., Tanaka, M., Takahashi, T., Kalb, R.G., Strittmatter, S.M., 1998. Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. Neuron 21, 1093–1100.
Nishino, J., Mochida, K., Ohfuji, Y., Shimazaki, T., Meno, C., Ohishi, S., Matsuda, Y., Fujii, H., Saijoh, Y., Hamada, H., 1999. GFR alpha3, a component of the artemin receptor, is required for migration and survival of the superior cervical ganglion. Neuron 23, 725–736.
Pasterkamp, R.J., Kolodkin, A.L., 2003. Semaphorin junction: making tracks toward neural connectivity. Curr. Opin. Neurobiol. 13, 79–89.
Rohm, B., Ottemeyer, A., Lohrum, M., Puschel, A.W., 2000. Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A. Mech. Dev. 93, 95–104.
Rubin, E., 1985. Development of the rat superior cervical ganglion: ganglion cell maturation. J. Neurosci. 5, 673–684.
Sahay, A., Molliver, M.E., Ginty, D.D., Kolodkin, A.L., 2003. Semaphorin 3F is critical for development of limbic system circuitry and is required in neurons for selective CNS axon guidance events. J. Neurosci. 23, 6671–6680.
Suto, F., Ito, K., Uemura, M., Shimizu, M., Shinkawa, Y., Sanbo, M., Shinoda, T., Tsuboi, M., Takashima, S., Yagi, T., Fujisawa, H., 2005. Plexin-a4 mediates axon-repulsive activities of both secreted and transmembrane semaphorins and plays roles in nerve fiber guidance. J. Neurosci. 25, 3628–3637.
Takahashi, T., Fournier, A., Nakamura, F., Wang, L.H., Murakami, Y., Kalb, R.G., Fujisawa, H., Strittmatter, S.M., 1999. Plexin–neuropilin-1 complexes form functional semaphorin-3A receptors. Cell 99, 59–69.
Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G.I., Song, H., Chedotal, A., Winberg, M.L., Goodman, C.S., Poo, M., Tessier-Lavigne, M., Comoglio, P.M., 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99, 71–80.
Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mishina, M., Yagi, T., 1997. Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. Neuron 19, 519–530.
Tessier-Lavigne, M., Goodman, C.S., 1996. The molecular biology of axon guidance. Science 274, 1123–1133.
Tran, T.S., Kolodkin, A.L., Bharadwaj, R., 2007. Semaphorin regulation of cellular morphology. Annu. Rev. Cell Dev. Biol. 23, 263–292.
Usui, H., Taniguchi, M., Yokomizo, T., Shimizu, T., 2003. Plexin-A1 and plexin-B1 specifically interact at their cytoplasmic domains. Biochem. Biophys. Res. Commun. 300, 927–931.
Waimey, K.E., Cheng, H.J., 2006. Axon pruning and synaptic development: how are they per-plexin? Neuroscientist 12, 398–409.
Yaron, A., Huang, P.H., Cheng, H.J., Tessier-Lavigne, M., 2005. Differential requirement for Plexin-A3 and -A4 in mediating responses of sensory and sympathetic neurons to distinct class 3 Semaphorins. Neuron 45, 513–523.
Young, H.M., Anderson, R.B., Anderson, C.R., 2004. Guidance cues involved in the development of the peripheral autonomic nervous system. Auton. Neurosci. 112, 1–14.