Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system

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SUMMARY

Neuropilin 1 is the specific receptor for Sema3A and plays a role in nerve fiber guidance. We report that *neuropilin 1* and *Sema3A* mutant mouse embryos, generated by targeted gene disruption, showed displacement of sympathetic neurons and their precursors and abnormal morphogenesis in the sympathetic trunk. We also show that Sema3A suppressed the cell migration activity of sympathetic neurons from wild-type but not *neuropilin 1* mutant embryos in vitro and instead promoted their accumulation into compact cell masses and fasciculation of their neurites. These findings suggest that the neuropilin 1-mediated Sema3A signals regulate arrest and aggregation of sympathetic neuron precursors and sympathetic neurons themselves at defined target sites and axon fasciculation to produce the stereotyped sympathetic nerve pattern.

Key words: Cell migration, Axon fasciculation, Neural crest, Sympathetic neurons, Sympathetic ganglia, Sympathetic trunk, *neuropilin 1* mutant mice, *Sema3A* mutant mice

INTRODUCTION

Neural crest cells leave the neural tube, migrate for long distances along defined paths, and subsequently settle at appropriate positions where they differentiate into diverse cell types (for reviews, see Bronner-Fraser, 1995; Anderson, 2000). Therefore, this cell population is a good experimental model to investigate mechanisms underlying cell migration, as well as cell differentiation.

Extensive analyses have been done to elucidate migratory patterns of neural crest cells in several vertebrate species, and have shown that progenitors of autonomic neurons of the sympathetic ganglia (neural crest cells of sympathetic neuron lineage) emerge from the neural crest at trunk level, and migrate ventrally (the ventral migratory pathway) through the rostral but not caudal halves of the sclerotomes to the dorsal aorta where they cease migration (Weston, 1963; Le Douarin and Teillet, 1974; Thiery et al., 1982; Rickmann et al., 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987; Lallier and Bronner-Fraser, 1988, Serbedzija et al., 1990). The neural crest cell descendants then proliferate and differentiate into neurons

that are aggregated into segmentally distinct groupings, the sympathetic ganglia. The sympathetic ganglia are interconnected by communicating rami to give rise to the sympathetic trunk.

Over the last decade, several molecules that influence the patterned migration of neural crest cells have been identified. Extracellular matrix molecules such as fibronectin and laminin are distributed along the neural crest cell migration routes and thought to be permissive substrates for cell migration (for a review, see Perris and Perissinotto, 2000). However, peanut agglutinin (PNA)-binding molecules (Oakley and Tosney, 1994; Krull et al., 1995), chondroitin sulfate proteoglycans (Henderson et al., 1997; Kubota et al., 1999; Perissinotto et al., 2000) (for a review, see Perris and Perissinotto, 2000), and ephrinB1 and ephrinB2, which are the ligands of Eph-related receptor tyrosine kinases (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997), are expressed in tissues lining the neural crest cell migratory pathways such as the caudal sclerotome and/or the perinotochordal mesenchyme and are thought to function as barriers that repel neural crest cells and limit their migration routes. However, as the influence of these

molecules on neural crest cell migration has mainly been elucidated in vitro, their roles in vivo are mostly unknown. Moreover, little is known about molecules that control the arrest of particular neural crest cell descendants at defined target sites, for example, the arrest of neural sympathetic neuron precursors at the dorsal aorta.

Sema3A is a member of the class 3 secreted semaphorin (Semaphorin Nomenclature Committee, 1999) and has been shown to be a potent neuronal chemorepellent that induces growth cone collapse or repels sensory axons of the dorsal root ganglia in vitro (Luo et al., 1993; Fan and Raper, 1995). Neuropilin 1 is a receptor for Sema3A (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Kitsukawa et al., 1997) (for a review, see Fujisawa and Kitsukawa, 1998), and propagates Sema3A signals into cells after forming receptor complexes with plexins (Takahashi et al., 1999; Rohm et al., 2000). Targeted inactivation of the *Sema3A* gene (Taniguchi et al., 1997) or the *neuropilin 1* gene (Kitsukawa et al., 1997) in mice induced disruption of the patterned pathways and projections of the peripheral nerve fibers, indicating the importance of neuropilin 1-mediated Sema3A signals to the directional guidance of nerve fibers in vivo.

Our previous study (Kawakami et al., 1996) showed that neuropilin 1 is expressed not only in the sensory neurons of the dorsal root ganglia but also in autonomic neurons of the sympathetic ganglia, both of which derive from a common progenitor, the trunk neural crest cells of the ventral migratory pathway. A recent study has shown that Sema3A suppresses migration of the avian trunk neural crest cells in vitro (Eickholt et al., 1999). Sema3A transcripts are limited to the caudal half of sclerotomes in avian embryos (Shepherd et al., 1996; Eickholt et al., 1999) and rodent embryos (Adams et al., 1996; Giger et al., 1996). Furthermore, Sema3A inhibits extension of neurites of sympathetic neurons in vitro (Püschel et al., 1995; Chen et al., 1998). These findings suggest that neuropilin 1-mediated Sema3A signals also play roles in the migration and/or arrest of neural crest cells of the sympathetic neuron lineage and the formation of the sympathetic nervous system.

We have closely examined the sympathetic nervous system of *neuropilin 1* mutant mouse embryos (Kitsukawa et al., 1997) and *Sema3A* mutant mouse embryos (Taniguchi et al., 1997), and report that, in these mutant embryos, many tyrosine hydroxylase (TH)-positive sympathetic neurons are displaced, and the pattern of the sympathetic nervous system is severely disrupted. We also report that, in these two mutant embryos, MASH1-positive sympathetic neuron precursors are not accumulated at their target sites around the dorsal aorta but dispersed widely, even though the migratory pathways of their progenitors within the sclerotomes are normal. To elucidate further the role of the neuropilin 1-mediated Sema3A signals in the formation of the sympathetic nervous system, we co-cultured sympathetic ganglia with Sema3A-secreting cells, and report that Sema3A suppresses cell migration activity of the sympathetic neurons from the wild-type but not *neuropilin 1* mutant mouse embryos, and instead promotes their aggregation into compact cell masses and fasciculation of their neurites into thick fiber bundles. These findings suggest that the neuropilin 1-mediated Sema3A signals function not only as a stop signal to prohibit migration of the neural crest cells of sympathetic neuron lineage into inappropriate regions of embryos but also as a signal to promote aggregation of sympathetic neurons and

probably their precursors into tightly packed cell masses, the sympathetic ganglia, at defined target sites and fasciculation of sympathetic neuron axons into a thick fiber bundle, the sympathetic trunk.

MATERIALS AND METHODS

Animals

Production of the *neuropilin 1* mutant mice and *Sema3A* mutant mice by targeted gene disruption and determination of genotypes by polymerase chain reaction was reported elsewhere (Kitsukawa et al., 1997; Taniguchi et al., 1997). The present study used *neuropilin 1* mutant mice created in the ICR strain, and *Sema3A* mutant mice in the C57BL/6 strain. Noon on the day on which a copulation plug was found was designated as embryonic day 0.5 (E0.5).

Immunohistochemistry

To detect sympathetic neurons in sections, embryos were fixed with 4% paraformaldehyde in 10 mM phosphate-buffered saline pH 7.4 (PBS) overnight at 4°C. Cryostat sections (14 µm thick) were thaw-mounted on poly-L-lysine (PLL: SIGMA)-coated glass slides and incubated with a rabbit anti-TH antibody (Nagatsu et al., 1977) (1:1,000 dilution with TBST (10 mM Tris-HCl pH 7.4, 130 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk). The bound antibodies were visualized with biotinylated goat anti-rabbit Ig antibody (Amersham Pharmacia Biotech; 1:500 dilution with TBST) and streptavidin-HRP complex (Vectastain ABC elite kit, Vector Labs). HRP activity was detected with 0.025% diaminobenzidine (DAB) and 0.03% H_2O_2 in TBST. In some specimens, the bound antibodies were visualized with Cy3-labeled anti-rabbit IgG antibody (Amersham Pharmacia Biotech; 1:500 dilution). To detect sympathetic neurons in whole-mounted specimens, embryos were fixed with paraformaldehyde, internal organs were removed, and they were then processed for immunohistochemistry following the procedures reported (Klymkowsky and Hanken, 1991; Kitsukawa et al., 1997). The specimens were treated with the anti-TH-antibody (1:100 dilution with TBST containing 5% skimmed milk, 5% DMSO and 0.1% NaN₃) for 2 days at room temperature. Immunostaining of sections with a rabbit anti-neuropilin 1 antibody (Kawakami et al., 1996) and a rat anti-neurofilament monoclonal antibody 2H3 (Dodd et al., 1988) was carried out using the Cy3-labeled anti-rabbit antibody and anti-rat Ig antibody as secondary antibodies, respectively. To detect neural crest cells within the sclerotomes, embryos were fixed with 100% methanol containing 0.1% formaldehyde for 5-10 minutes at 4°C. Cryostat sections were incubated with a rat anti-mouse monoclonal antibody 4E9R (Kubota et al., 1996) (1:5 dilution of hybridoma culture supernatant) and biotin-labeled isolectin-B4 (Sigma; 10 µg/ml in TBST) overnight at 4°C. The bound 4E9R and isolectin-B4 were detected by treatment of sections with Cy3-labeled anti-mouse Ig antibody and fluorescein-conjugated streptavidin (Amersham Pharmacia Biotech; 1:200 dilution with TBST) each for 30 minutes at room temperature.

Production of HEK293T-EBNA cells secreting Sema3A

The sequence encoding the mouse Sema3A protein (amino acids 26-773) (Püschel et al., 1995) was amplified and a his tag added at the 5′ end by PCR. The sequence encoding the signal sequence of the mouse Sema3A (amino acids 1-25) was amplified and a myc tag added at the 3′ end by PCR. The PCR products were fused and cloned into the expression vector pCEP4 (Invitrogen). The plasmid was used to obtain a stable HEK293T-EBNA cell line (Invitrogen) that secretes the Sema3A-myc-his fusion protein (Sema3A-secreting cells), as described (Shirasaki et al., 1996). The empty pCEP4 vector was used to produce a control HEK293T-EBNA cell line (control cells). HEK293T cells were cultured in Dulbecco's modified Eagle's

medium (DMEM; Nissui, Japan) containing 10% FCS (FBS; JRH Bioscience).

Explant culture

Small fragments of the sympathetic ganglia and dorsal root ganglia from E12.5 embryos were cultured in a medium containing 45% DMEM, 45% Ham's F12 (Nissui, Japan), 10% FBS and 2.5S NGF (30 ng/ml; Wako, Japan), in culture dishes (30-mm: Nunc) which had been incubated with a mixture of PLL (100 μg/ml) and mouse laminin (Gibco-BRL; 20 μg/ml) for 2 hours at room temperature. The cultures were fixed with paraformaldehyde and processed for immunostaining with anti-TH antibody and/or 2H3.

In situ hybridization

In situ hybridization with digoxigenin-labeled rat *Mash1* cRNA probe (a gift from Dr Kageyama) and mouse *Sema3A* cRNA on cryostat sections (10 μm thick) was performed, following procedures reported previously (Schaeren-Wiemers and Gerfin-Moser, 1993). The bound probes were visualized with alkaline phosphatase-conjugated anti-digoxigenin Fab fragment (Boehringer Mannheim) and its color-indicated substrate.

RESULTS

Dislocation of sympathetic neurons in the *neuropilin 1* and *Sema3A* mutant embryos

Sympathetic neurons contain catecholamines and are detected by immunoreactivity for the catecholamine synthetic enzyme, tyrosine hydroxylase (TH). In the wild-type ($Nrp\text{-}1^{+/+}$) and heterozygous *neuropilin 1* mutant ($Nrp\text{-}1^{+/-}$) embryos at E12.5, TH-positive cells accumulated to form sympathetic ganglia at positions dorsolateral to the dorsal aorta (Fig. 1A,D). In contrast, in the homozygous *neuropilin 1* mutant ($Nrp\text{-}1^{-/-}$) embryos, TH-positive cells did not accumulate but distributed widely within embryos. Several TH-positive cells were detected in regions more medial to the original sites of the sympathetic ganglia (Fig. 1B,E), in shoulder (Fig. 1B,F), upper arm, axilla, and sometimes the abdominal wall. As most of the ectopic TH-positive cells possessed axon-like processes (Fig. 1E,F), we assume these cells to be sympathetic neurons. Dislocation of sympathetic neurons was also observed in the homozygous *Sema3A* mutant ($Sema3A^{-/-}$) embryos (Fig. 1C) but not the wild-type ($Sema3A^{+/+}$) or heterozygous mutant ($Sema3A^{+/-}$) embryos. A double immunostaining with anti-TH antibody and anti-neurofilament antibody 2H3 (Dodd et al., 1988) revealed that the ectopic sympathetic neurons were always associated with spinal nerve fibers and sprouted cell processes along the nerves (Fig. 1G).

Immunostaining of whole-mounted embryos with anti TH-antibody clearly demonstrated the overall pattern of the sympathetic nervous system. In $Nrp\text{-}1^{+/+}$ (Fig. 1H), TH-positive neurons formed ganglia and sympathetic trunks bilaterally to the vertebra. In contrast, in the $Nrp\text{-}1^{-/-}$ embryos, many TH-positive neurons were located at ectopic positions, formed irregular cell masses and extended cell processes in various directions (Fig. 1I). As a whole, the sympathetic trunk was severely disorganized.

Quantitative analysis revealed that the total number of TH-positive neurons (an average number of neurons per section) in the $Nrp\text{-}1^{-/-}$ embryos was 60% of that in the $Nrp\text{-}1^{+/+}$ or $Nrp\text{-}1^{+/-}$ embryos, and 51.2% of the neurons were located at the original sites of the sympathetic ganglia (Fig. 2A). In the $Sema3A^{-/-}$ embryos, the total number of TH-positive neurons was 86.3% of that in the wild-type embryos, and 35.9% of the neurons were located at the original sites of the sympathetic ganglia (Fig. 2B).

Migratory pathways of neural crest cells within the sclerotomes in the *neuropilin 1* and *Sema3A* mutant embryos

Neuropilin 1 is expressed in cultured mouse trunk neural crest cells (Fig. 3A). Furthermore, Sema3A is expressed in the caudal half of the sclerotomes in the mouse embryos (Adams et al., 1996). These findings suggest that the neuropilin 1-expressing neural crest cells avoid Sema3A expressed in the caudal half of each sclerotome to give rise to the metameric migratory pattern. Therefore, we examined whether the migratory pathways of neural crest cells within the sclerotomes are disorganized in the *neuropilin 1* and *Sema3A* mutant embryos.

As progenitors of sympathetic neurons start to emigrate from the neural tube at around E8.5 to E9.5 (Serbedzija et al., 1990), we made serial sagittal sections through the sclerotomes in embryos at E9.0 and detected neural crest cells by immunohistochemistry using a monoclonal antibody 4E9R, which is a marker for neural crest cells (Kubota et al., 1996) (Fig. 3A). As 4E9R cross-reacts with endothelial cells, we performed a double-immunostaining using 4E9R and isolectin B4, which binds to endothelial cells (Kawasaki et al., 1999) but not neural crest cells. We divided each sclerotome into 9 areas (Fig. 3B), and counted the number of 4E9R-positive and isolectin B4-negative cells in each area.

As shown in Fig. 3C-E, there was no clear difference in the distribution pattern of neural crest cells within the sclerotome between the mutant and wild-type embryos; neural crest cells in both the *neuropilin 1* and *Sema3A* mutant embryos were mainly distributed in the rostral sclerotome, as in the wild-type embryos. These results indicate that the migratory pathways of neural crest cells within the sclerotomes are mostly normal in the neuropilin 1-deficient and Sema3A-deficient mouse embryos, and suggest that the displacement of the sympathetic neuron progenitors would occur after they have left the sclerotomes.

Distribution of sympathetic neuron precursors in the *neuropilin 1* and *Sema3A* mutant embryos

To examine whether sympathetic neuron progenitors that left the sclerotomes are displaced in the *neuropilin 1* and *Sema3A* mutant embryos, we performed in situ hybridization analysis using *Mash1* cRNA as a probe. *Mash1* encodes a basic helix-loop-helix protein that is a mammalian homologue of the *Drosophila* achaete-scute proteins and is expressed in the precursors of sympathetic neurons that have arrived at the dorsal aorta but not migrating neural crest cells within the sclerotomes (Johnson et al., 1990; Lo et al., 1991). As neural crest cells of the sympathetic neuron lineage arrive at the dorsal aorta by E9 to E10 (Serbedzija et al., 1990), we analyzed the embryos at E9.25 (10-20 somites stage).

In the $Nrp\text{-}1^{+/+}$ (Fig. 4A) and $Nrp\text{-}1^{+/-}$ embryos (4 embryos each), most cells with *Mash1* transcripts accumulated at the dorsal aorta. In contrast, in the $Nrp\text{-}1^{-/-}$ embryos (8 embryos), Mash1-positive cells distributed widely within embryos, not only at the dorsal aorta but also in the proximal part of the
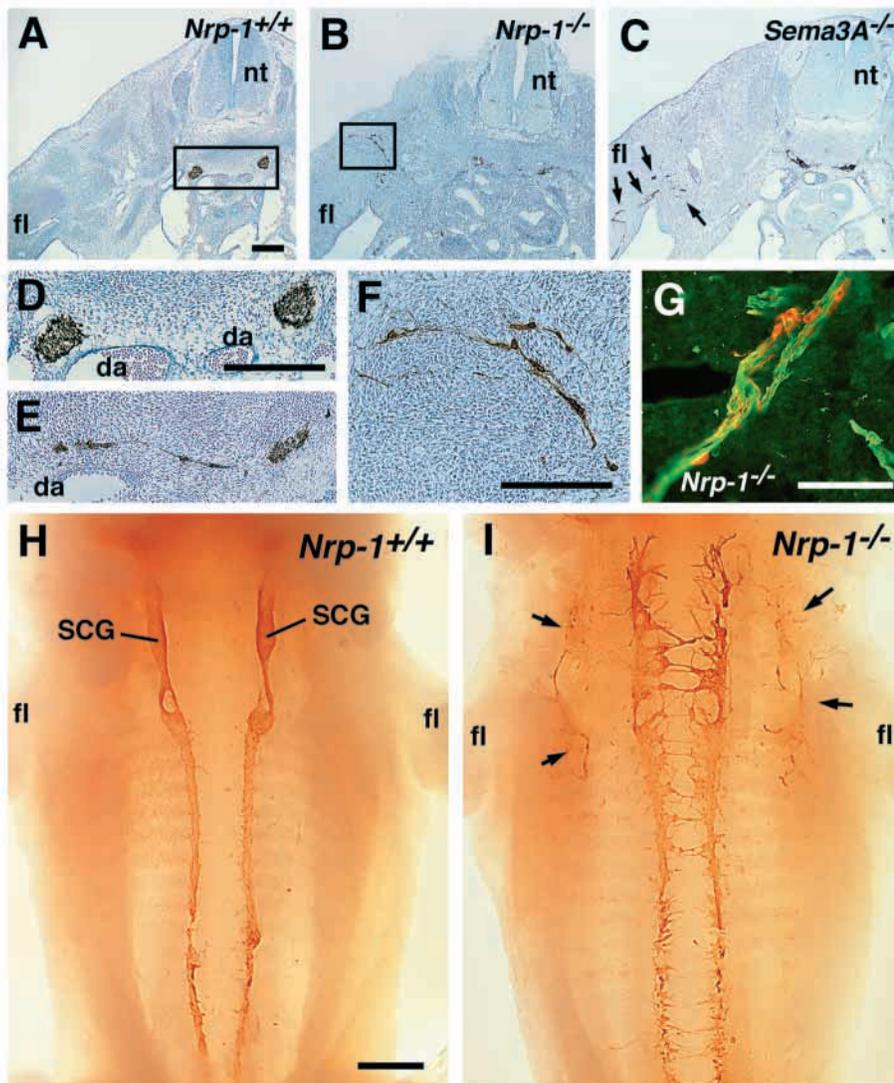
Fig. 1. Dislocation of TH-positive sympathetic neurons in the *neuropilin 1* and *Sema3A* mutant embryos. (A-F) Frontal sections of the wild-type (A), *neuropilin 1* homozygous mutant (B) and *Sema3A* homozygous mutant (C) embryos at E12.5, immunostained with anti-TH antibody. (D,F) High-magnification views of the boxed areas in A and B, respectively. (E) A high-magnification view of the medial part of an embryo in a section adjacent to B. Arrows in C indicate TH-positive neurons in the upper arm and axilla. (G) A double immunostaining of a section from the *Nrp-1⁻/⁻* embryo at E12.5, with anti-TH antibody (red) and anti-neurofilament antibody 2H3 (green). (H,I) The wild-type (H) and homozygous *neuropilin 1* mutant (I) embryos at E12.5, immunostained in whole-mount with anti-TH antibody. Arrows indicate TH-positive neurons at ectopic positions. nt, the neural tube; da, the dorsal aorta; SCG, the superior cervical ganglion; fl, the forelimb. Scale bar: 200 μm (A-C); 200 μm (D,E); 100 μm (F,G); 500 μm (H,I).

vicinity of the neural tube in the *Nrp-1⁺/⁺* (Fig. 4C), *Nrp-1⁺/⁻* and *Nrp-1⁻/⁻* (Fig. 4D) embryos. The findings indicate that the emigration of sympathetic neuron precursors from the neural tube to the dorsal aorta precedes the sprouting of the spinal nerve fibers, and suggest that sympathetic neuron precursors arrive at the dorsal aorta independently of spinal nerve fibers. We further examined expression patterns of *Sema3A* by in situ hybridization, and observed strong signals for the Sema3A transcripts in the forelimb and the dermamyotome (Fig. 4E). These results indicate that, in the neuropilin 1-deficient embryos, sympathetic neuron precursors have migrated into regions where Sema3A is expressed, such as the forelimbs (compare Fig. 4B,E).

forelimbs and regions more medial to the original sites of the sympathetic ganglia (Fig. 4B). In the *Sema3A* mutant embryos Mash1-positive cells were also displaced in a similar pattern to that in the *neuropilin 1* mutant embryos (data not shown). Immunohistochemistry with 2H3 in adjacent sections revealed that 2H3-positive spinal nerve fibers were limited to the

Effect of Sema3A on cell migration activity

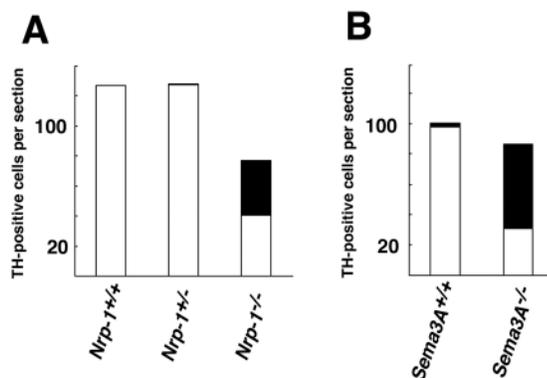Next, we examined whether Sema3A suppress cell migration

Fig. 2. Quantitative analysis on the distribution of TH-positive sympathetic neurons. The average number of TH-positive neurons per section was calculated from around 20 serial frontal sections that were made at the cervical level of embryos at E12.5. Open and filled columns indicate the number of TH-positive neurons located at the original sites of the sympathetic ganglia and ectopic positions, respectively. (A) *neuropilin 1* mutant embryos. 100%, 99.4% and 51.2% of TH-positive neurons are located at the original sites of the sympathetic ganglia in the wild-type embryo (1 embryo), heterozygous mutant embryos (2 embryos) and homozygous mutant embryos (3 embryos), respectively. (B) The *Sema3A* mutant embryos. 97.6% and 35.9% of TH-positive neurons are located at the original sites of the sympathetic ganglia in the wild-type embryos (3 embryos) and homozygous mutant embryos (2 embryos), respectively.
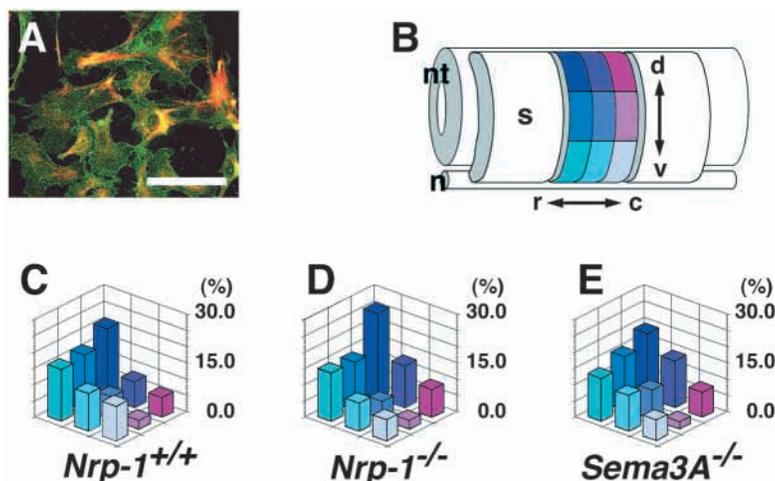
Fig. 3. Migratory pathways of neural crest cells within the sclerotome. (A) Neural crest cells from the wild-type embryo at E8.5 in vitro, immunostained with a neural crest cell-specific monoclonal antibody 4E9R (red) and anti-neuropilin 1 antibody (green). (B-E) Distribution of neural crest cells within the sclerotomes at the cervical level of E9.0 embryos. (B) A diagram indicating 9 regions of the sclerotome defined by different colors. (C-E) Histograms showing relative number (percentages) of neural crest cells contained within each region of the sclerotome shown in B, in the wild-type (C), *neuropilin 1* homozygous mutant (D) and *Sema3A* homozygous mutant (E) embryos. d, dorsal; v, ventral; r, rostral; c, caudal; nt, neural tube; n, notochord; s, sclerotome. Scale bar: 50 μm in A.

in vitro. Because isolation of sympathetic neuron precursors was technically difficult, we co-cultured small explants of the sympathetic ganglia (SG explant) from E12.5 embryos or dorsal root ganglia (DRG explant) from E12.5 embryos with Sema3A-secreting HEK293T cells generated by the EBNA system using an expression vector pCEP4, and examined the effect of Sema3A on neuronal cell migration. HEK293T cells transfected with the empty vector were used as a control (control cells). In the present culture conditions, TH-positive sympathetic ganglion neurons strongly expressed neuropilin 1 (see Fig. 5E).

First, we analyzed cell migration activity in the presence of a relatively high concentration of Sema3A; we seeded the Sema3A-secreting cells or control cells (2,000 cells/cm^2 each) 12 hours before inoculation of the SG explants or DRG explants, and cultured then for 12 hours. In these culture conditions, neurite outgrowth was completely blocked by Sema3A in the DRG explants from the *Nrp-1$^{+/+}$* embryos (Fig. 5A,B) but not *Nrp-1$^{-/-}$* embryos (Fig. 5C,D), even though several non-neuronal cells grew out from the explants. Neurite outgrowth and migration of TH-positive neurons from the SG explants of the *Nrp-1$^{+/+}$* embryos occurred in the co-culture with the control cells (Fig. 5F) but was completely blocked in the co-culture with the Sema3A-secreting cells (Fig. 5G). In contrast, neurite outgrowth and migration of TH-positive neurons was not suppressed in the co-culture of the SG explants from the *Nrp-1$^{-/-}$* embryos with the control cells (Fig. 5H) and Sema3A-secreting cells (Fig. 5I).

In the explant culture of the sympathetic ganglia, outgrowth of neurites preceded migration of neuronal cells. TH-positive neurons always migrated from the explants along with the preformed neurites. As neurite outgrowth from the SG explants was completely suppressed with Sema3A under the above co-culture conditions (see Fig. 5G), we cannot say whether Sema3A primarily suppresses cell migration activity of sympathetic neurons. Therefore, we then examined migration activity of sympathetic neurons in the presence of a lower concentration of Sema3A that does not inhibit neurite outgrowth; we inoculated the SG explants from the wild-type embryos with Sema3A-secreting cells or control cells (800 cells/cm^2 each) simultaneously and cultured then for 2 days. In the co-culture with the control cells, many TH-positive neurons migrated from the SG explants along with neurites
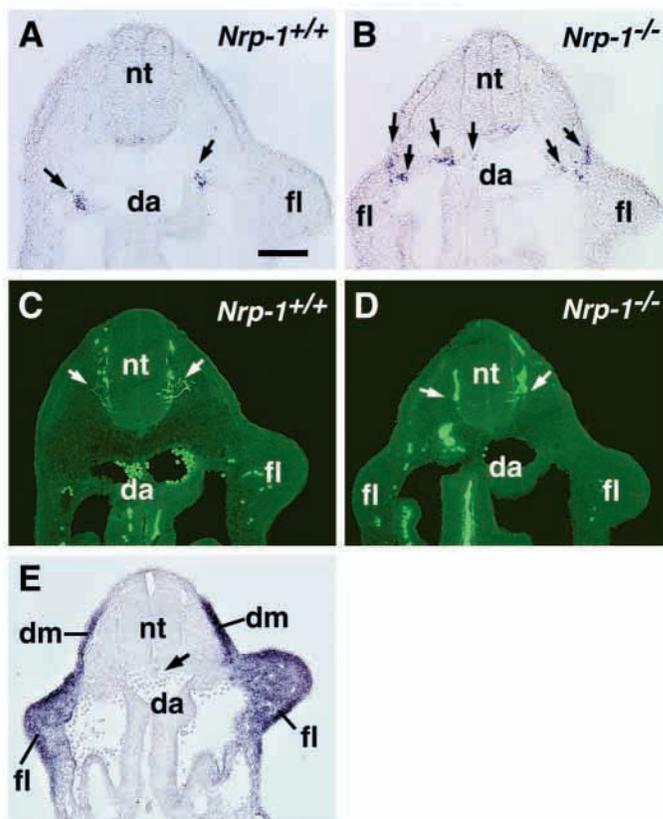

Fig. 4. Distribution of sympathetic neuron precursors, spinal nerve fibers, and *Sema3A* transcripts in E9.25 embryos. (A,B) In situ hybridization analysis on frontal sections of the wild-type (A) and *neuropilin 1* homozygous mutant (B) embryos, using an antisense cRNA probe for *Mash1*. Arrows indicate cells with the *Mash1* transcripts. (C,D) Detection of spinal nerve fibers by immunostaining with 2H3 in sections adjacent to A and B. 2H3-positive fibers (arrows) are limited around the neural tube (nt). The staining in blood cells and the internal surface of the digestive tracts is non-specific. (E) In situ hybridization analysis on a frontal section of a wild-type embryo, using an anti-sense cRNA probe for *Sema3A*. An arrow indicates the notochord. da, dorsal aorta; fl, the forelimb; dm, dermamyotome; nt, neural tube. Scale bar: 100 μm (A-E).

(Fig. 5J,L). In contrast, no TH-positive neurons migrated from the SG explants in the co-culture with the Sema3A-secreting cells, even though several neurites grew from the explants (Fig. 5K,M). These results suggest that neuropilin 1-mediated Sema3A signals suppress the cell migration activity of sympathetic neurons.

It has been reported that Sema3A induces neuronal apoptosis in cultured sympathetic neurons (Shirvan et al., 1999). Therefore, we performed TUNEL staining and Hochst dye staining of the SG explants, but found no apparent increase in apoptotic cells in the explants co-cultured with Sema3A-secreting cells (data not shown), suggesting that the prevention of neuron migration from the SG explants is not due to cell death.

Effect of Sema3A on the morphology of sympathetic ganglion explants and neurites

In addition to the suppression of sympathetic neuron migration, Sema3A influenced the morphology of SG explants and neurites. When the SG explants from the wild-type embryos were co-cultured with the control cells, the explants were gradually spread out, and neurites sprouted without apparent fasciculation (Fig. 6A-C, also see Fig. 5F,J,L). In contrast, when the SG explants were co-cultured with the Sema3A-secreting cells (800 cells/cm^2), cells in the explants were gradually aggregated into compact cell masses (Fig. 6D-F, also see Fig. 5K,M). In addition, neurites that had initially been dispersed became fasciculated into thick fiber bundles (Fig. 6D-F, also see Fig. 5K,M). The Sema3A-mediated cell aggregation and neurite fasciculation were observed in the SG explants from the wild-type embryos (23 explants form 3 independent experiments) but not neuropilin 1 mutant embryos (see Fig. 5I).

DISCUSSION

Ectopic sympathetic neurons arise as a result of the displacement of sympathetic neurons but not phenotypic change of neural crest cells

The present analysis showed that many sympathetic neurons are located at ectopic positions in the neuropilin 1 and Sema3A mutant embryos at E12.5. The neural crest cells of the ventral migratory pathway are multipotent, and become restricted in phenotype either during their migration or at their sites of localization into sensory neurons of the dorsal root ganglia, Schwann cells, neurons in the sympathetic ganglia, chromaffin

cells of the adrenal medulla, or enteric neurons of the digestive tracts (Stern et al., 1991; Bronner-Fraser and Fraser, 1991; Durbec et al., 1996) (for reviews, see Le Douarin et al., 1993; Selleck et al., 1993; Bronner-Fraser, 1995; Anderson, 1993; Anderson, 2000). Therefore, it is possible that the sympathetic neurons located at ectopic positions were produced by the change of phenotype of neural crest cells into sympathetic neurons. However, the reduction of TH-positive sympathetic neurons, in particular, the marked reduction of the neurons at the original sites for the sympathetic ganglia in the neuropilin 1 and Sema3A mutant embryos (see Fig. 2) suggests that the distribution of sympathetic neurons at ectopic positions accounted for the displacement of the sympathetic neuron progenitors and/or sympathetic neuron themselves but not for the phenotypic conversion of neural crest cells, which would differentiate other cell types into bona fide sympathetic neurons.

It is well documented that factors in the local environment derived from the dorsal aorta such as BMP2/4/7 are required for the expression of the sympathetic neuron phenotype in neural crest cells (Reissmann et al., 1996; Shah et al., 1996; Varley and Maxwell, 1996). However, the localization of TH-
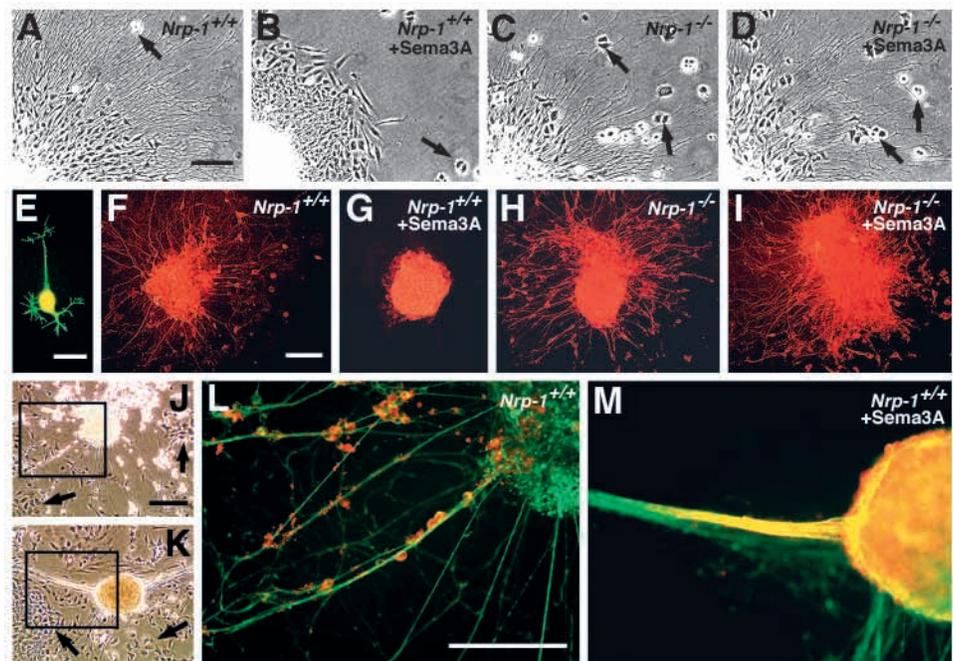

Fig. 5. Effect of Sema3A on migration of dorsal root ganglia neurons and sympathetic neurons in vitro. (A-D) Co-cultures of the dorsal root ganglia explants from the wild-type (A,B) and homozygous neuropilin 1 mutant (C,D) embryos at E12.5 with the control HEK293T cells (A,C) and the Sema3A-secreting HEK293T cells (B,D) (2,000 cells/cm^2 each). (E) A cultured sympathetic ganglion neuron, immunostained with anti-neuropilin 1 antibody (green) and anti-TH antibody (originally red but yellow after merging). (F-I) Co-culture of the sympathetic ganglion explants from the wild-type (F,G) and homozygous neuropilin 1 mutant (H,I) embryos at E12.5 with the control HEK293T cells (F,H) and the Sema3A-secreting HEK293T cells (G,I) (2,000 cells/cm^2 each). Cultures were immunostained with anti-TH antibody. (J-M) Co-culture of the sympathetic ganglion explants from the wild-type embryos at E12.5 with the control HEK293T cells (J,L) or the Sema3A-secreting HEK293T cells (K,M) (800 cell/cm^2 each). The cultures were immunostained with anti-TH antibody (red) and 2H3 (green). J and K are phase-contrast micrographs. L and M correspond to the boxed regions in J and K, respectively. Arrows in A-D, J and K indicate the HEK293T cells. Scale bar: 100 μm (A-D); 25 μm (E); 200 μm (F-I); 200 μm (J,K); 200 μm (L,M).
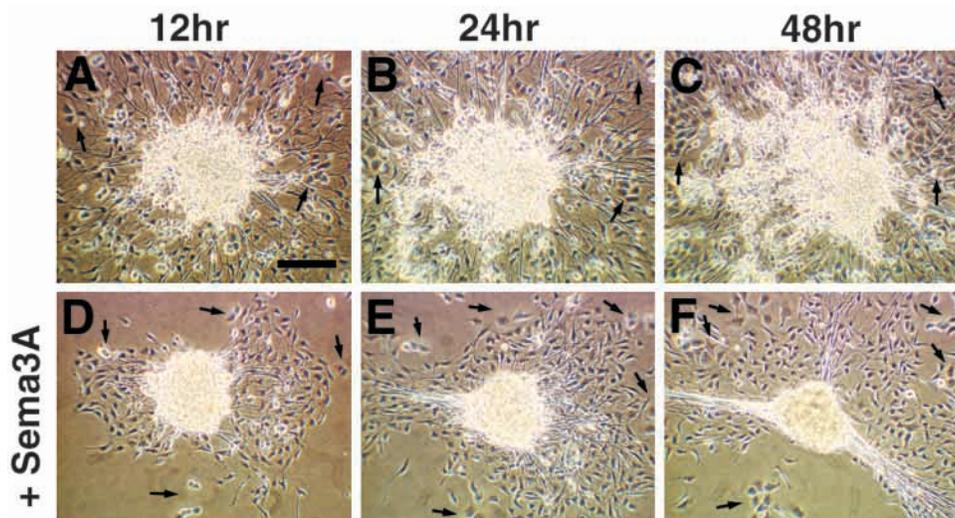
positive neurons at ectopic positions such as the limb bud and abdominal wall in the *neuropilin 1* and *Sema3A* mutant embryos suggests that neural crest cells can express the sympathetic neuron phenotype, the expression of TH, at sites far from the dorsal aorta. The number of TH-positive cells located at ectopic positions in the *neuropilin 1* mutant embryos was smaller than that in the *Sema3A* mutant embryos. This may be attributable primarily to the regression of embryonic vessel formation in the *neuropilin 1* mutant embryos (Kawasaki et al., 1999). The vascular defect may result in the reduction of cell proliferation of sympathetic neuron precursors or neuronal cell death.

Which steps of neural crest cell migration are disrupted in the *neuropilin 1* and *Sema3A* mutant embryos?

The selective expression of Sema3A in the caudal half of each sclerotome in the avian embryos (Shepherd et al., 1996; Eickholt et al., 1999) and rodent embryos (Adams et al., 1996; Giger et al., 1996) has led us to speculate that Sema3A affects the metameric migratory patterns of neural crest cells (Adams et al., 1996; Eickholt et al., 1999). The present study, however, showed no apparent abnormal distribution of neural crest cells within the sclerotomes in the *neuropilin 1* and *Sema3A* mutant embryos. As several extracellular matrix molecules and the Eph family transmembrane ligands, which would inhibit neural crest cell migration, are redundantly expressed in the caudal half of the sclerotomes (for reviews, see Robinson et al., 1997; Perris and Perissinotto, 2000) and work in concert, the effect of deprivation of a single inhibitory molecule may be negated. We previously reported that cell packaging in the dorsal root ganglia was slightly loose in the *neuropilin 1* mutant embryos, even though the number of dorsal root ganglion and their location within embryos were nearly normal (Kitsukawa et al., 1997). Therefore, we cannot exclude the possibility that the migratory pathways of

the neural crest cells at somite level are slightly disorganized in the mutant embryos.

The in situ hybridization analysis indicates that MASH1-positive sympathetic neuron precursors were distributed widely in the *neuropilin 1* and *Sema3A* mutant embryos, suggesting that displacement of the progenitors of sympathetic neurons occurs after they have left the sclerotomes. It has been reported that, in avian embryos, neural crest cells leave the sclerotomes and then closely associate with newly emerging ventral root fibers (Rickmann et al., 1985; Loring and Erickson, 1987). As reported previously (Kitsukawa et al., 1997; Taniguchi et al., 1997), pathways of the spinal nerves in the *neuropilin 1* and *Sema3A* mutant embryos are severely disorganized. In addition, in the *neuropilin 1* and *Sema3A* mutant embryos at E12.5, displaced sympathetic neurons were always associated with spinal nerve fibers (see Fig. 1G). These findings raised the possibility that the displacement of sympathetic neuron precursors in the mutant mouse embryos is a side effect of the disorganization of the spinal nerve fiber pathways. However, the present finding that MASH1-positive sympathetic neuron precursors arrive at their final target sites before the sprouting of the spinal nerve fibers (see Fig. 4) excludes this possibility.

Progenitors of sympathetic neurons that arrive at the dorsal aorta migrate 2 segments rostrally and 3 segments caudally
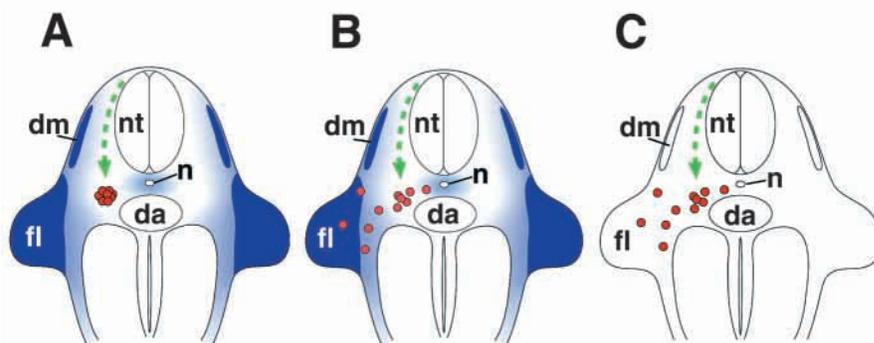

Fig. 7. Possible roles of Sema3A in migration and arrest of sympathetic neuron precursors. (A) In the wild-type embryo, Sema3A (blue) production in the dermamyotome (dm), forelimb (fl) and paraxial mesenchymal tissue forms a concentration gradient. Sympathetic neuron precursors (red spots), which have migrated through the ventral migratory pathway (a green arrow), are arrested at the bottom of the Sema3A gradient, the lateral part of the dorsal aorta (da). (B,C) Sympathetic neuron precursors in the neuropilin 1-deficient embryo (B) and the Sema3A-deficient embryo (C) do not stop at the lateral part of the dorsal aorta but migrate further along the permissive route. nt, neural tube; n, notochord.

along the aorta and then accumulate to form ganglia (Yip, 1986), suggesting the importance of the dorsal aorta in migration and/or accumulation of sympathetic neuron precursors. However, the present study showed that the displacement of sympathetic neurons occurs to nearly the same extent in the *neuropilin 1* mutant embryos in which segments of the dorsal aorta are often regressed (Kawasaki et al., 1999) and the *Sema3A* mutant embryos in which the morphogenesis of the dorsal aorta is apparently normal (unpublished). Therefore, the displacement of sympathetic neuron precursors in these mutant embryos seems to be induced independently of the dorsal aorta.

Taken collectively, the results obtained by histological analyses on the *neuropilin 1* and *Sema3A* mutant embryos suggest that the progenitors of sympathetic neurons migrate along normal paths within the sclerotomes but fail to arrive at their target sites and disperse widely within embryos.

Putative roles of Sema3A in migration and arrest of sympathetic neuron precursors and patterning of the sympathetic nervous system

Why do sympathetic neurons and their precursors in the neuropilin 1- and Sema3A-deficient embryos not accumulate at the dorsal aorta but are displaced, even though their migratory pathways within the sclerotomes are normal?

A most likely answer is that Sema3A functions as a stop signal for the migration of sympathetic neurons and their precursors. The co-culture of the sympathetic ganglions with Sema3A-secreting cells showed that Sema3A suppressed neuron migration from the sympathetic ganglion explants. Though we did not directly test the effect of Sema3A on the sympathetic neuron precursors, for technical reasons, the precursors may also be responsible to Sema3A and suppressed their migration activity. In the mouse embryos at E9.5 when the sympathetic neuron precursors have just arrived at the dorsal aorta (Serbedzija et al., 1990), Sema3A transcripts are expressed in the dermamyotome and the forelimb, as well as the caudal sclerotome (see Fig. 4E). In addition, several studies have shown that, in the mouse embryos at E10 (Adams et al., 1996) and rat embryos at corresponding developmental stages (Wright et al., 1995), Sema3A is also expressed in paraxial mesenchymal tissues. As Sema3A is a secreted protein, the Sema3A produced in these tissues may diffuse within embryos and make a concentration gradient with the lowest point at the lateral part of the dorsal aorta. On this assumption, we speculate that, in the wild-type embryos, the diffused Sema3A suppresses the migration activity of sympathetic neuron precursors and arrests them at the bottom of the Sema3A gradient, the lateral part of the dorsal aorta (Fig. 7A). In contrast, in the *neuropilin 1* and *Sema3A* mutant embryos, sympathetic neuron precursors may migrate further following the permissive routes for these cells into diverse regions, even into the areas where Sema3A is originally expressed (Fig. 7B,C).

The co-culture of the sympathetic ganglions with Sema3A-secreting cells also suggests a function of Sema3A other than as the stop signal, that is, Sema3A promotes aggregation of sympathetic neurons into compact cell masses. The cell aggregation activity of Sema3A may play a role in the aggregation of differentiated sympathetic neurons into ganglia and probably the aggregation of sympathetic neuron precursors into compact cell masses at the dorsal aorta. In the *Sema3A* and *neuropilin 1* mutant embryos, sympathetic neuron precursors

may fail to be aggregated but disperse widely and give rise to sympathetic neurons at ectopic positions. The differentiated sympathetic neurons may migrate further or be pulled by growing spinal nerve fibers into places far from the original target sites, such as the upper arm and the abdominal wall.

The present in vitro study also showed that Sema3A promotes neurite fasciculation. This result led us to speculate that Sema3A, expressed in paraxial mesenchymal tissues surrounding the sympathetic trunk, functions not only as a signal to inhibit sprouting of sympathetic neuron axons into the medial part of embryos but also as a signal to promote their fasciculation into a thick fiber bundle, the sympathetic trunk, along the rostro-caudal axis of embryos. The lack of Sema3A from the tissues surrounding sympathetic neurons or neuropilin 1 from the sympathetic neurons may cause defasciculation of axons and their sprouting in random directions, as found in the *neuropilin 1* (see Fig. 1I) and *Sema3A* mutant embryos.

It is open to question how Sema3A suppresses cell migration activity and promotes cell aggregation in the sympathetic neurons and their precursors. Sema3A paralyses growth cones and stops their migration, probably through depolymerization of actin (Fan et al., 1993). A similar molecular mechanism might be exerted in the suppression of cell migration. We have previously reported that neuropilin 1 mediates cell adhesion after interacting with unknown ligands (Takagi et al., 1995; Shimizu et al., 2000). More recently, neuropilin 1 has been shown to form a complex with a neuronal cell adhesion molecule, L1 (Castellani et al., 2000). Therefore, Sema3A may modify the interaction of neuropilin 1 with L1 or other cell adhesion molecules and increase cell adhesiveness. Several studies have shown that repulsive signals regulate axon fasciculation (Bray et al., 1980; Winberg et al., 1998; Ringstedt et al., 2000; Yu et al., 2000), and suggested that axon fasciculation versus defasciculation appears to be determined by a balance of attractive (or adhesive) and repulsive forces on the axons relative to their surrounding environment (Tessier-Lavigne and Goodman, 1996). The repulsive function of Sema3A expressed in the tissues surrounding sympathetic neurons might promote fasciculation of their axons. It is also likely that Sema3A directly increases the adhesiveness of the axons.

Whatever the mechanisms, the results obtained in the present in vivo and in vitro studies emphasize the central role of neuropilin 1-mediated Sema3A signals in the migration of neural crest cells of sympathetic neuron lineage, their arrest at defined target sites and accumulation into the sympathetic ganglia, and fasciculation of sympathetic neuron axons into the sympathetic trunk.

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