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RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons

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SUMMARY

Sympathetic axons use blood vessels as an intermediate path to reach their final target tissues. The initial contact between differentiating sympathetic neurons and blood vessels occurs following the primary sympathetic chain formation, where precursors of sympathetic neurons migrate and project axons along or toward blood vessels. We demonstrate that, in *Ret*-deficient mice, neuronal precursors throughout the entire sympathetic nervous system fail to migrate and project axons properly. These primary deficits lead to mis-routing of sympathetic nerve trunks and accelerated cell death of sympathetic neurons later in development. Artemin is expressed in blood vessels during periods of early sympathetic differentiation, and can promote and attract axonal growth of the sympathetic ganglion in vitro. This analysis identifies RET and artemin as central regulators of early sympathetic innervation.

Key words: Ret, Knockout mouse, Sympathetic neuron, Axon growth, Axon guidance, Migration

INTRODUCTION

Proper development of the sympathetic nervous system involves determination of neuronal cell identity, as well as establishment of complex neuronal circuits that are exquisitely controlled by intrinsic and extrinsic signals. Recent genetic evidence, together with elegant in vivo experiments, has lead to molecular identification of such signals. For example, in the mouse, primary formation of the sympathetic ganglia first relies on proper ventral migration of the sympathoadrenal lineage of the neural crest cells, which depends partly, but not exclusively, on neuregulin 1/ErbB2 ErbB3 signaling (Britsch et al., 1998). After migration, these neural crest cells coalesce immediately adjacent to the dorsal aorta to form the primary sympathetic chain. Subsequently, transcriptional regulators including *Mash1* (*Ascl1* – Mouse Genome Informatics) a mouse homolog of the *Drosophila* achaete scute proneural gene, and *Phox2a* (*Arix* – Mouse Genome Informatics) and *Phox2b* (*Pmx2b* – Mouse Genome Informatics) paired homeodomain genes, are expressed in these sympathetic progenitors (Ernsberger et al., 2000; Groves et al., 1995; Guillemot et al., 1993; Pattyn et al., 1997; Tiveron et al., 1996). In the chicken, expression of these transcription factors has been shown to require signals provided by bone morphogenetic proteins (BMPs) that are secreted from the dorsal aorta (Schneider et al., 1999). Mash1 and Phox2b in turn play crucial roles in the determination of neuronal fate and induction of expression of pan-neuronal genes as well as lineage-specific markers such as tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) (Guillemot et al., 1993; Pattyn et al., 1999). Precursors in the primary sympathetic chain migrate at subsequent developmental stages to generate the primordia of the superior cervical sympathetic ganglion (SCG) and prevertebral ganglia as well as to give rise to chromaffin cells in the adrenal medulla (LeDouarin, 1986). During this period, some sympathetic precursors begin to extend axonal processes, while others undergo mitotic division. The migration and axonal outgrowth of these sympathetic precursors proceed in close contact with blood vessels, and most sympathetic trunks travel along blood vessels thereafter. Based on this close relationship between differentiating sympathetic neurons and blood vessels, it has long been postulated that a biological cue originating from blood vessels regulates motility and growth cone guidance of these cells. To date, however, the molecular identity of this signal has been unclear.

Neurotrophic factors play essential roles in the proper development of a wide variety of neuronal populations. Neurotrophins are a family of neurotrophic factors that are required for the survival of sensory and sympathetic neurons during development (Snider and Wright, 1996). Accumulating evidence has also revealed the importance of these factors in promoting nerve growth. For example, injection of nerve

growth factor (NGF) to the brain leads to the abnormal increase of sympathetic axon ingrowth in this region (Menesini Chen et al., 1978). In line with these classical experiments, tissue engineered to express high levels of NGF or NT3 harbor increased sympathetic and sensory fibers (Albers et al., 1996; Albers et al., 1994; Hassankhani et al., 1995). In slice cultures, sensory neurons project axons towards neurotrophin-impregnated beads that are placed in ectopic positions (Tucker et al., 2001). Furthermore, sensory neurons in mice with disruption of *Ngf* or *TrkA* (*Ntrk1* – Mouse Genome Informatics) fail to innervate the target properly, even when cell death of these neurons is prevented by deficiency of the pro-apoptotic gene, *Bax* (Patel et al., 2000). Despite these observations, however, neurotrophins do not appear to be physiologically required for initial sympathetic axonal growth. First, recent genetic evidence has revealed both NGF and NT3 influence sympathetic neurons only late in development (Francis et al., 1999), presumably exerting their effects via TRKA receptors (Wyatt et al., 1997). Second, expression of *TrkA* is barely detectable in sympathetic neuron precursors early in development (Wyatt et al., 1997). Finally, previous genetic evidence has demonstrated that initial projections of sympathetic axons occur normally in *TrkA*-deficient ($TrkA^{-/-}$) embryos (Fagan et al., 1996).

The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), a newly identified family of neurotrophic factors, includes GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) (Baloh et al., 1998; Kotzbauer et al., 1996; Lin et al., 1993; Milbrandt et al., 1998). GFLs signal through a receptor complex composed of a signaling subunit, the RET tyrosine kinase, and a binding subunit, the GFRα (GDNF family receptor α) family which are glycosylphosphatidylinositol (GPI) linked cell surface proteins. To date, there are four members of GFRα family, GFRα1, GFRα2, GFRα3 and GFRα4, which serve as preferential receptors for GDNF, NRTN, ARTN and PSPN, respectively (Baloh et al., 1997; Buj-Bello et al., 1997; Jing et al., 1996; Jing et al., 1997; Klein et al., 1997; Sanicola et al., 1997; Suvanto et al., 1997; Treanor et al., 1996).

Although recent genetic experiments have established GFLs as neurotrophic factors essential for proper development of enteric, sympathetic, parasympathetic, sensory and motoneurons (Cacalano et al., 1998; Enomoto et al., 1998; Enomoto et al., 2000; Heuckeroth et al., 1999; Laurikainen et al., 2000; Marcos and Pachnis, 1996; Moore et al., 1996; Pichel et al., 1996; Rossi et al., 1999; Rossi et al., 2000; Sanchez et al., 1996; Schuchardt et al., 1994; Airaksinen et al., 1999; Baloh et al., 2000; Rosenthal, 1999), the biological roles of the GFLs and their receptors in sympathetic ganglion development have been enigmatic. In mice, it has been reported that RET deficiency leads to elimination of all SCG neurons by birth (Durbec et al., 1996). In contrast to the profound deficit in the SCG of $Ret^{-/-}$ mice, neonatal mice lacking GDNF or GFRα3, a cognate receptor for ARTN, only harbor a decrease of 30% of SCG neurons (Moore et al., 1996; Nishino et al., 1999). Moreover, no sympathetic deficits are found in $Nrtn^{-/-}$ and $GFR\alpha 2^{-/-}$ mice (Heuckeroth et al., 1999; Rossi et al., 1999). It is also unclear why RET deficiency affects only the SCG, given the high level of *Ret* expression in sympathetic precursors throughout the entire primitive sympathetic chain (Durbec et al., 1996). To address these questions, we have re-examined the development of sympathetic ganglia in $Ret^{-/-}$ mice. Our analysis has revealed that RET deficiency affects proper migration as well as initiation/promotion of axonal growth of neurons and their precursors throughout the entire sympathetic ganglia. During periods of sympathetic precursor migration and axonal projection, ARTN, but not other GFLs, is expressed in various blood vessels. ARTN induces profuse neurite outgrowth from sympathetic ganglion explants and has an ability to direct growing axons. Our analysis identifies RET and ARTN as the receptor and ligand essential for an early phase of sympathetic neuron differentiation that occurs in close association with blood vessels.

MATERIALS AND METHODS

Generation of Ret-TGM (tau-EGFP-myc) mice

A 9.0 kb *Kpn*I fragment containing the first coding exon of *Ret* was used to construct the targeting vector. The TGM cDNA followed by Tn5 neo cassette flanked with loxP sequences was inserted into the first coding exon by bacterial homologous recombination (Zhang et al., 1998), removing 39 bp nucleotides that encompass the first methionine and the following six amino acids of the signal peptide. The TGM cDNA encodes a fusion protein composed of N-terminal region of bovine tau, full-length EGFP and three repeats of human Myc tag. The vector was transfected by electroporation into RW-4 embryonic stem cells and homologous recombinants were identified by Southern blot hybridization. Properly targeted ES cell clones were injected into blastocysts to generate chimeric mice that successfully achieved germline transmission of the targeted allele. Heterozygous animals grew normally and were further crossed with β-actin Cre transgenic mice (Meyers et al., 1998) to remove the Tn5 neo cassette that could potentially interfere with the efficient transcription of TGM cDNA and thereby generate the knock-in allele referred to as Ret^{TGM}. Genotype of Ret^{TGM} mice was determined either by Southern blotting or PCR using primers P1(5′-CAGCGCAGGTCTCTCATCAGT-ACCGCA-3′), P2(5′-ACGTCGCTTTCGCCATCGCCCGTGCGC-3′) and P3(5′-CCCTGAGCATGATCTTCCATCACGTCG-3′) (94°C 30 seconds, 65°C 30 seconds, 72°C 60 seconds; 35 cycles), where wild-type and mutant alleles were amplified with P1-P2 (228 bp PCR product) and P1-P3 (289 bp PCR product) primer pairs, respectively.

Histological analysis

Immunohistochemical detection for RET, Phox2a, Phox2b, caspase 3, TH, TUNEL and BrdU and Phox2b double labeling, as well as in situ hybridization are described elsewhere (Enomoto et al., 1998; Enomoto et al., 2000). The antibodies for GFP (Abcom), class III β tubulin (TUJ1: BAbCO) and neurofilament (2H3: Developmental Studies Hybridoma Bank) were used at 1:200, 1:2000 and 1: 200 dilution, respectively. GFP signals of TGM could also be detected directly under fluorescent microscopy. For estimation of ganglion volume, ganglion area of the SCG was measured in every fifth section of consecutive parasagittal sections using Image Analysis Software. The volume was calculated by multiplying the total ganglion area by the distance between two sections (30 μm). Neuronal cell death was quantified as number of cells with pyknotic nuclei per 1000 counted neurons. At least 2000 neurons were counted on randomly selected parasagittal sections of every ganglion for each genotype (n=3).

For whole-mount TOH immunohistochemistry, 4% paraformaldehyde-fixed embryos or newborn mouse tissues were dehydrated by methanol series (50-80%) and incubated overnight in 20% dimethylsulfoxide (DMSO)/80% methanol solution containing 3% H_2O_2 to quench endogenous peroxidase activity. Tissues were then re-hydrated, blocked overnight in blocking solution (4% BSA/1% Triton X-100 in PBS) and incubated for 48-72 hours at 4°C with sheep anti-TH antibodies (1:200 in blocking solution). The signal

was detected using diaminobenzidine after successive treatment of the tissues with horseradish peroxidase (HRP)-conjugated anti-sheep Ig antibodies. Tissues were re-fixed, dehydrated by methanol series and cleared with benzyl benzoate/benzyl alcohol (2:1 mixture) to allow visualization of staining inside the tissue.

For GFP and activated caspase 3 double staining, anti-GFP and anti-activated caspase 3 antibodies (Cell Signaling) were used at 1: 12800 and 1: 100 dilutions, respectively. A more detailed protocol for the double labeling using antibodies raised in the same species is described elsewhere (Shindler and Roth, 1996).

Explant culture of mouse sympathetic ganglion

Sympathetic ganglia (SCG and STG) were dissected out from E13.5 mouse embryos. Ganglia were cut into pieces of appropriate size and cultured in 1 mg/ml type I collagen (rat) in DMEM/F12 media containing 5% horse serum. For neurite outgrowth assay, GDNF, NRTN, ARTN or NT-3 was applied at concentrations ranging from 5 to 100 ng/ml. For chemotaxis assay, a NRTN- or ARTN- impregnated heparin bead was placed at a 300-400 μm distance from the explant in a collagen gel. For both assays, explants were cultured for 40 hours in 5% CO_2 at 37°C. The SCG and STG were examined separately, and almost identical results were obtained from both cultures. Representative results from the STG cultures of three independent experiments are described in the text. To quantify axonal growth, axons were visualized by immunostaining with anti-Class III β tubulin antibodies and axon-covering area was measured using NIH Image software.

RESULTS

Generation of Ret-null mice expressing EGFP

Previous studies in mice with a kinase-deficient form of *Ret* (*Ret*$^{kinase/kinase}$ mice) have shown that RET is required for proper development of the kidney, enteric neurons and the SCG (Durbec et al., 1996; Schuchardt et al., 1994). However, detailed characterization of neural development in *Ret*$^{kinase/kinase}$ mice is often compromised by the inability to detect population of neurons that would normally express RET. Identification of these neurons is extremely important, as RET is expressed in only a subset of neurons in a variety of neuronal structures. To address this issue, we inserted a cDNA encoding tau-EGFP-myc (TGM) into the first coding exon of the *Ret* gene by homologous recombination (Fig. 1A,B). Mice heterozygous for *Ret*-TGM mutation (*Ret*$^{TGM/+}$ mice) grew normally and displayed no obvious deficits either macroscopically or microscopically. In these mice, the expression pattern of TGM completely overlapped that of RET in a variety of locations, including sensory (trigeminal and spinal), sympathetic, parasympathetic, enteric and motoneurons (spinal and cranial), verifying that TGM serves as a reliable reporter for *Ret* expression (Fig. 1C). *Ret*$^{TGM/+}$ mice were interbred to generate *Ret*$^{TGM/TGM}$ mice. No RET protein expression was detected in *Ret*$^{TGM/TGM}$ homozygotes (spinal motoneurons shown in Fig. 1D), and these mice died shortly after birth owing to the absence of the kidney and enteric neurons, a phenotype identical to that of *Ret*$^{kinase/kinase}$ mice. In this paper, we have used this mouse to analyze the role of RET in sympathetic neuron development. As identical phenotypes of the sympathetic nervous system were observed in both *Ret*$^{TGM/TGM}$ and *Ret*$^{kinase/kinase}$ mice, we hereafter refer to both mice as *Ret*-deficient (*Ret*$^{-/-}$ mice) except for situations where the distinction is necessary.

RET deficiency affects the entire sympathetic nervous system

Previous studies have shown that RET deficiency leads to

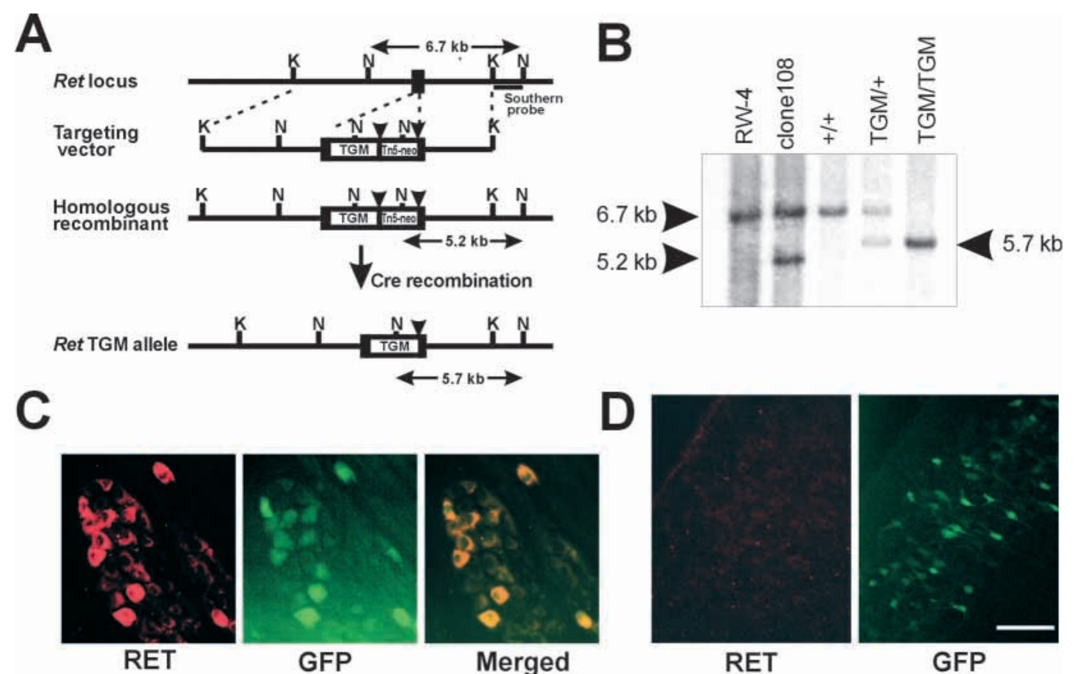

Fig. 1. Generation of *Ret*-TGM mice. (A) Homologous recombination between *Ret* gene (top) and the targeting vector (second line) resulted in insertion of TGM and floxed-Tn5-neo (third line). Tn5-neo was successively removed in mice to generate *Ret*-TGM allele (bottom). Arrowheads indicate loxP sites. (B) Southern blot analysis for *Ret*^{TGM} mutation using genomic DNA from RW-4 ES cells (control), homologous recombinant ES clone (clone 108), and tails of wild-type (+/+), heterozygous (TGM/+) and homozygous (TGM/TGM) mice. The DNA samples were digested with *Nco*I and hybridized with radiolabeled probe, indicated in Fig. 1A. (C) Representative pictures of TGM expression in the nervous system. Signals obtained by RET immunohistochemistry (left) completely overlaps that of GFP fluorescent signal provided by TGM (middle) in trigeminal ganglion of newborn *Ret*$^{TGM/+}$ mice (merged image, right). (D) No RET protein expression is detected in spinal motoneurons (left) that are identified by GFP fluorescence (right) in *Ret*$^{TGM/TGM}$ newborn mice. Abbreviations: K, *Kpn*I; N, *Nco*I. Scale bar: 55 μm in C; 100 μm in D.

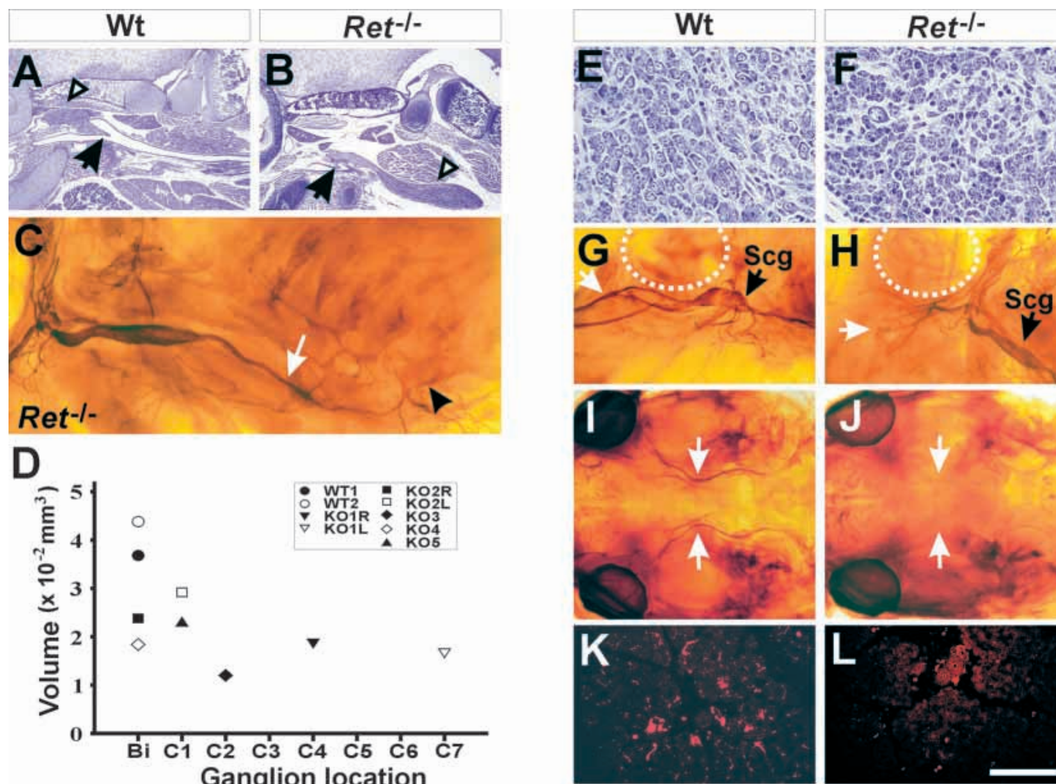

Fig. 2. Aberrant position and impaired innervation of the SCG in newborn *Ret*$^{-/-}$ mice. (A,B) Parasagittal section of newborn mouse head stained with Hematoxylin and Eosin (rostral part is on the left). The SCG (white arrowheads) is located far caudal to its normal location near the bifurcation of the carotid artery (black arrows) in *Ret*$^{-/-}$ mouse. (C) Whole-mount TH immunohistochemistry showing connection of the SCG to the STG (arrowhead) by the cervical sympathetic trunk (white arrow). (D) Ganglion volume of the SCG. Closest vertebral level to the center of the ganglion is shown by *x*-axis. WT1,2, wild type; KO, *Ret*$^{-/-}$ mice; R, right SCG; L, left SCG. Note that the location and the volume vary between the right and left side of the same animal (KO1R versus KO1L, KO2R versus KO2L). (E,F) SCG neurons of *Ret*$^{-/-}$ mice are often small and display immature morphology. (G-J) The internal carotid nerve (white arrows) is abnormally branched and fails to travel rostrally in *Ret*$^{-/-}$ mice. Dotted circles depict the inner ear. (I,J) Dorsal view of the newborn head, showing total absence of the internal carotid nerve. The brain was removed in this preparation. (K,L) Absence of TH-positive fibers in the peripheral lobe of the submandibular gland of *Ret*$^{-/-}$ mice. Scale bar: 2 mm in A,B; 600 μm in C; 550 μm in E,F; 1 mm in G,H; 1.7 mm in I, J.

complete absence of SCG neurons but leaves the other sympathetic ganglia apparently intact. Surprisingly, however, our examination of consecutive parasagittal sections of newborn mouse head revealed the presence of the SCG-like ganglion in *Ret*$^{-/-}$ mice (Fig. 2A,B). The SCG-like ganglion of *Ret*$^{-/-}$ mouse was consistently found in a position caudal to where the SCG is normally located. Whole-mount TH immunohistochemistry of the newborn *Ret*$^{-/-}$ mouse head verified the SCG-like ganglion to be authentically the SCG as the ganglion expressed TH and was connected to the stellate ganglion by cervical sympathetic trunk (Fig. 2C). The location of the SCG in *Ret*$^{-/-}$ mouse was variable among animals and even between right and left sides of the same animal. The SCG in *Ret*$^{-/-}$ mice also displayed severe, but again variable, reductions in size (Fig. 2D). Thionine staining of the SCG revealed that the sympathetic neurons are variably reduced in size, suggesting that the ganglion contains neurons at various stages of maturation (Fig. 2E,2F; *n*=8). In addition, more pyknotic cells were observed in the SCG of *Ret*$^{-/-}$ than in their wild-type counterpart (see below). We also observed severe deficits in the axonal projections from the SCG in *Ret*$^{-/-}$ mice. For example, the internal carotid nerve (ICN) displayed aberrant branching and was attenuated on the course of its projection, even when the SCG was found in an almost normal position (Fig. 2G-J). As a consequence, sympathetic fibers in the rostral facial structures including the eye, nasal mucosa and skin were almost completely depleted (*n*=7). Although sympathetic fibers were detectable in the submandibular gland in many *Ret*$^{-/-}$ animals, their density and distribution never reached normal levels (*n*=7), often leading to complete absence of sympathetic innervation in the peripheral lobes of the gland (Fig. 2K,L, observed in five out of seven examined). Thus, disruption of the *Ret* gene does not result in total elimination of SCG neurons by birth but does lead to the mis-location of the ganglion and accompanying dramatic loss of target innervation.

The deficits in the SCG of *Ret*$^{-/-}$ mice prompted us to examine other sympathetic ganglia for abnormalities. This analysis revealed a reduction in ganglion size of thoracic and lumbar sympathetic chain ganglia including the stellate ganglion (STG) in *Ret*$^{-/-}$ mice (Fig. 3A-F). The STG also displayed severe innervation deficits as exemplified by an attenuation of the vertebral nerve, a major trunk of the STG which projects along the vertebral artery (Fig. 3A,B). Finally in the prevertebral division, sympathetic ganglia were often aberrantly located, reduced in size (Fig. 3G,H) and displayed decreased target innervation (Fig. 3I,J). These deficits in the thoracic and lumbar chain as well as the prevertebral ganglia were consistently observed in all mice examined (*n*=6). Taken together, this analysis reveals that RET deficiency affects the proper formation of sympathetic ganglion throughout the entire sympathetic nervous system.

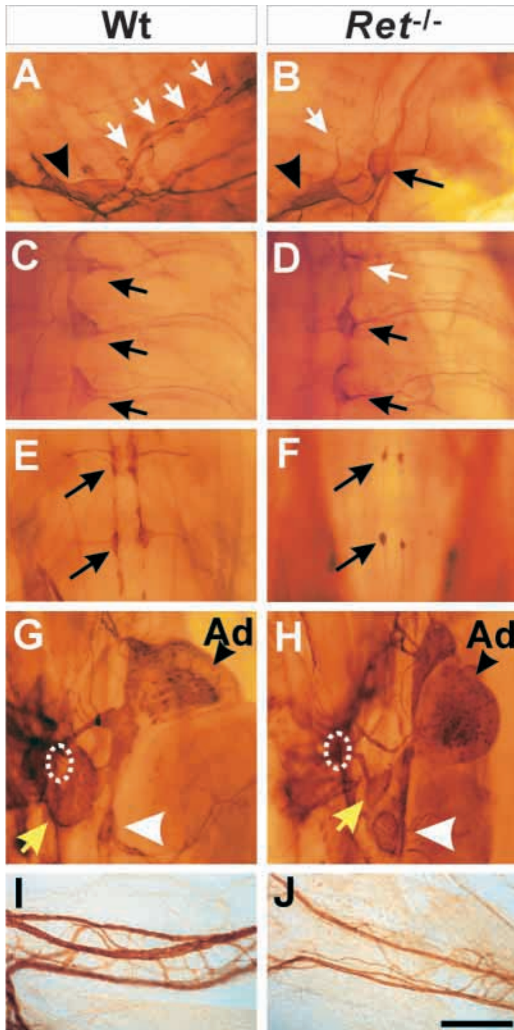

Fig. 3. Newborn *Ret*$^{-/-}$ mice display deficits in the thoracic and lumbar sympathetic chain and prevertebral sympathetic ganglia. (A-J) Whole mount TH staining of the mouse body. (A,B) The STG (arrowheads) is small, and displays a defective axonal projection pattern in *Ret*$^{-/-}$ mice. Note the projection of the vertebral nerve (white arrows) is attenuated in *Ret*$^{-/-}$ mice. Black arrow indicates the SCG, which is located abnormally close to the STG. (C-F) Individual chain ganglia (black arrows) are small or missing (white arrow in D) at both thoracic (C,D) and lumbar (E,F) levels in *Ret*$^{-/-}$ mice. (G,H) Prevertebral sympathetic ganglion complex. The celiac superior mesenteric ganglion complex (yellow arrows) surrounding the exit of superior mesenteric artery (dotted circle) is dramatically reduced in size, whereas the aortico-renal ganglion (white arrowhead) is abnormally large in *Ret*$^{-/-}$ mice. (I,J) Sympathetic innervation along the branch of superior mesenteric artery is severely depleted in *Ret*$^{-/-}$ mice. Ad, adrenal gland. Scale bar: 900 μm in A,B; 600 μm in C,D; 550 μm in E-H; 100 μm in I,J.

Impaired axonal outgrowth and migration of sympathetic cells in *Ret*$^{-/-}$ embryos

Our analysis has demonstrated an in vivo requirement for RET signaling throughout the entire peripheral sympathetic nervous system. Newborn *Ret*$^{-/-}$ mice display complex deficits in the sympathetic ganglion that include impaired migration, reduced axonal projection and increased neuronal cell death. Because cell survival often depends on target-derived trophic factors and neuronal migration may require appropriate axonal projection, it is important to determine whether RET plays a primary role in any of these processes. To address this issue, we analyzed sympathetic nervous system development in *Ret*$^{-/-}$ embryos and their wild-type littermates.

Recent genetic evidence has shown that induction of *Ret* gene expression is dependent on Phox2b (Pattyn et al., 1999), which begins to be expressed immediately after the formation of the primary sympathetic chain (Ernsberger et al., 2000). Phox2b is also required for induction and maintenance of Phox2a and Mash1 expression in sympathetic cells, respectively (Pattyn et al., 1999). These transcription factors act in concert to regulate expression of lineage-specific and pan-neuronal markers including TH, intermediate neurofilament (NF160), SCG10 and class III β tubulin (Guillemot et al., 1993; Pattyn et al., 1999; Sommer et al., 1995). Therefore, we examined expression of these transcription factors and neuronal markers in *Ret*$^{-/-}$ sympathetic cells during embryonic days 10.5-11.5 (E10.5-11.5). No difference was observed in the expression of these molecules between *Ret*$^{-/-}$ and wild-type embryos (data not shown). Therefore, RET does not appear to be essential for the commitment of sympathetic lineage or determination of neuronal cell fate.

However, despite the intact expression of these differentiation-associated molecules, *Ret*$^{-/-}$ sympathetic neuronal precursors display severe deficits in axonal projection. For example, at E10.5, when some sympathetic precursors in wild-type embryos begin to extend long processes as revealed by anti-Class III β tubulin antibodies (Fig. 4A), very few cells were observed to extend long processes in *Ret*$^{-/-}$ embryos (Fig. 4D; *n*=3). Impaired axonal projection of sympathetic cells in *Ret*$^{-/-}$ mice became more obvious in the SCG, STG and prevertebral ganglia during the period from E12.5 to E13.5 (Fig. 4B-F, data not shown, *n*=3). The SCG and STG in *Ret*$^{-/-}$ embryos also failed to undergo morphogenetic alteration to form their characteristic ganglion shape (Fig. 4E,F).

Whole-mount TH immunohistochemistry of embryos enabled us to visualize the overall configuration of sympathetic ganglion formation and their fibers during development. This analysis revealed further morphological abnormalities in the entire sympathetic ganglia. At E10.5, the primitive sympathetic chain is recognized from lower cervical to lumbar levels as revealed by whole-mount TH immunohistochemistry. At this period, the rostral part of the developing sympathetic chain was observed to reach the comparable axial level between *Ret*$^{-/-}$ embryos and their wild-type littermates (Fig. 4G,J; *n*=5). By E11.5, the rostral end of the sympathetic chain extends further rostrally into the upper cervical region, forming a long primitive SCG in wild-type embryos. This morphogenetic alteration is speculated to result from rostral migration of sympathetic neuronal precursors from the caudal area, as well as their proliferation (Rubin, 1985). In *Ret*$^{-/-}$ embryos, the SCG primordia were round-shaped and invariably shorter (Fig. 4H,K), suggesting that there are fewer precursors. Indeed, cell count of the SCG primordia at E11.5 revealed approximately 30% decrease of sympathetic cells in *Ret*$^{-/-}$ when compared with wild type (wild type: 4020±535; *n*=3 and *Ret*$^{-/-}$: 2780±600; *n*=2). Deficits in the more caudal region are also

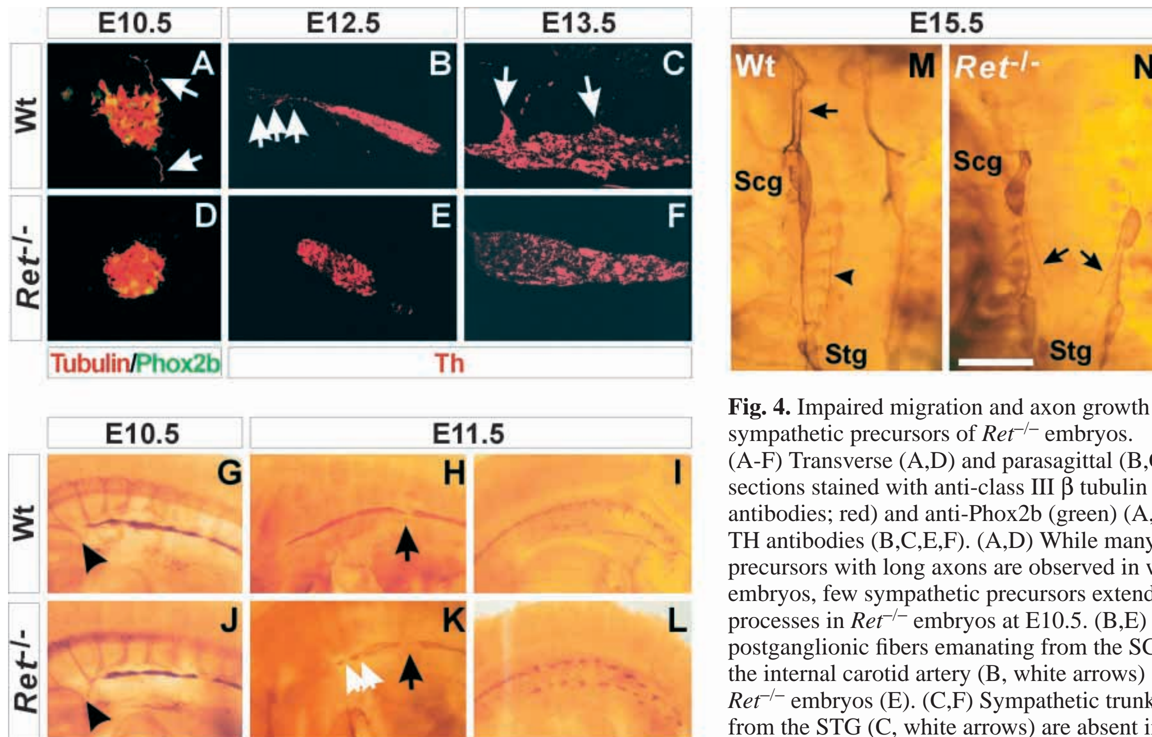

Fig. 4. Impaired migration and axon growth in sympathetic precursors of *Ret*$^{-/-}$ embryos. (A-F) Transverse (A,D) and parasagittal (B,C,E,F) sections stained with anti-class III β tubulin (TUJ1 antibodies; red) and anti-Phox2b (green) (A,D) or anti-TH antibodies (B,C,E,F). (A,D) While many sympathetic precursors with long axons are observed in wild-type embryos, few sympathetic precursors extend long axonal processes in *Ret*$^{-/-}$ embryos at E10.5. (B,E) Tips of postganglionic fibers emanating from the SCG towards the internal carotid artery (B, white arrows) are absent in *Ret*$^{-/-}$ embryos (E). (C,F) Sympathetic trunks exiting from the STG (C, white arrows) are absent in *Ret*$^{-/-}$ embryos (F). (G-L) Pictures of whole-mount TH immunohistochemistry showing rostral and ventral region to the left and bottom, respectively. (G,J) Formation of primary sympathetic chain is comparable between *Ret*$^{-/-}$ and wild-type littermates at E10.5. Black arrowheads depict the developing brachial plexus. (H,K) The SCG primordium is short and displays an exaggerated constriction (K, white arrows) in *Ret*$^{-/-}$ embryos (K) at E11.5. Black arrows indicate C7 vertebral level. (I,L) While sympathetic precursors are evenly distributed in the lumbar chain and prevertebral regions of wild-type embryos (I), these precursors are found as clumps of cells in *Ret*$^{-/-}$ embryos (L). (M,N) Ventral view of whole-mount TH staining of E15.5 embryos, showing rostral region on top. In this preparation, the lower jaw is removed for better visualization of the sympathetic trunks. Major sympathetic trunks of the SCG project rostrally in E15.5 wild-type embryos (M, arrow). In some *Ret*$^{-/-}$ embryos, these trunks are thin and project caudally (N, arrows). Note that the vertebral nerve growing from the STG (M, arrowhead) is not yet formed in *Ret*$^{-/-}$ embryos at this developmental stage. Abbreviations: Scg, the superior cervical ganglion; Stg, the stellate ganglion. Scale bar: 80 μm in A,D; 220 μm in B,C,E,F; 55 μm in G,H,J,K; 70 μm in I,L; 1 mm in M,N.

recognizable at E11.5. For example, in wild-type embryos, sympathetic precursors were evenly distributed along the dorsal aorta and mesenteric arteries (Fig. 4I). By contrast, sympathetic precursors in *Ret*$^{-/-}$ embryos formed cell clumps and failed to distribute properly (Fig. 4L). These deficits, including the smaller SCG and abnormal cell distribution in the more caudal region, were consistently observed in all *Ret*$^{-/-}$ embryos examined (n=7).

Importantly, although the cell number in the SCG primordia is decreased and the axonal projection of the STG and SCG is severely impaired, no increased cell death was detected in both ganglia of *Ret*$^{-/-}$ embryos from E10.5-E13.5, as judged by nuclear morphology or activated caspase 3 immunohistochemistry (not shown). Furthermore, proliferating population in the sympathetic precursors at E11.5 identified by BrdU and Phox2b double-labeling was comparable (approx. 40% of the total precursors were doubly labeled) between *Ret*$^{-/-}$ embryos and their wild-type littermates. While BrdU labeling may not detect subtle differences in cell proliferation, RET deficiency does not appear to have a major impact on proliferation. Thus, we conclude that the reduced cell number in the SCG primordia of *Ret*$^{-/-}$ embryos is primarily caused by a failure in rostral migration of the sympathetic precursors, rather than increased cell death or impaired proliferation of these cells.

Finally, we observed that RET deficiency affects the proper pathfinding of growing sympathetic axons. At E15.5, profuse nerve fibers from the SCG project rostrally along the internal carotid artery to form the internal carotid nerve in wild-type embryos (Fig. 4M, arrow). Although these nerve fibers were recognizable in most *Ret*$^{-/-}$ embryos by E15.5, these nerve bundles were thin. Moreover, in some *Ret*$^{-/-}$ embryos (two out of four examined), these nerve bundles projected caudally, instead of rostrally (Fig. 4N, arrows), indicating that axon projection is mis-routed. Formation of the sympathetic trunk from the STG was not yet observed at this period in *Ret*$^{-/-}$ embryos. Collectively, in sympathetic neuron development, RET is required primarily for migration as well as axonal growth and guidance of sympathetic neuronal precursors.

RET does not directly support the survival of sympathetic neurons

Although the analysis of sympathetic chain at early embryonic stages has revealed the primary requirement of RET for differentiation of sympathetic precursors, not for their survival, it remained unclear why all sympathetic ganglion of *Ret*$^{-/-}$ embryos exhibit dramatic reduction in size at birth. To answer this question, we examined SCG and STG neurons in later development. This analysis revealed retarded differentiation of

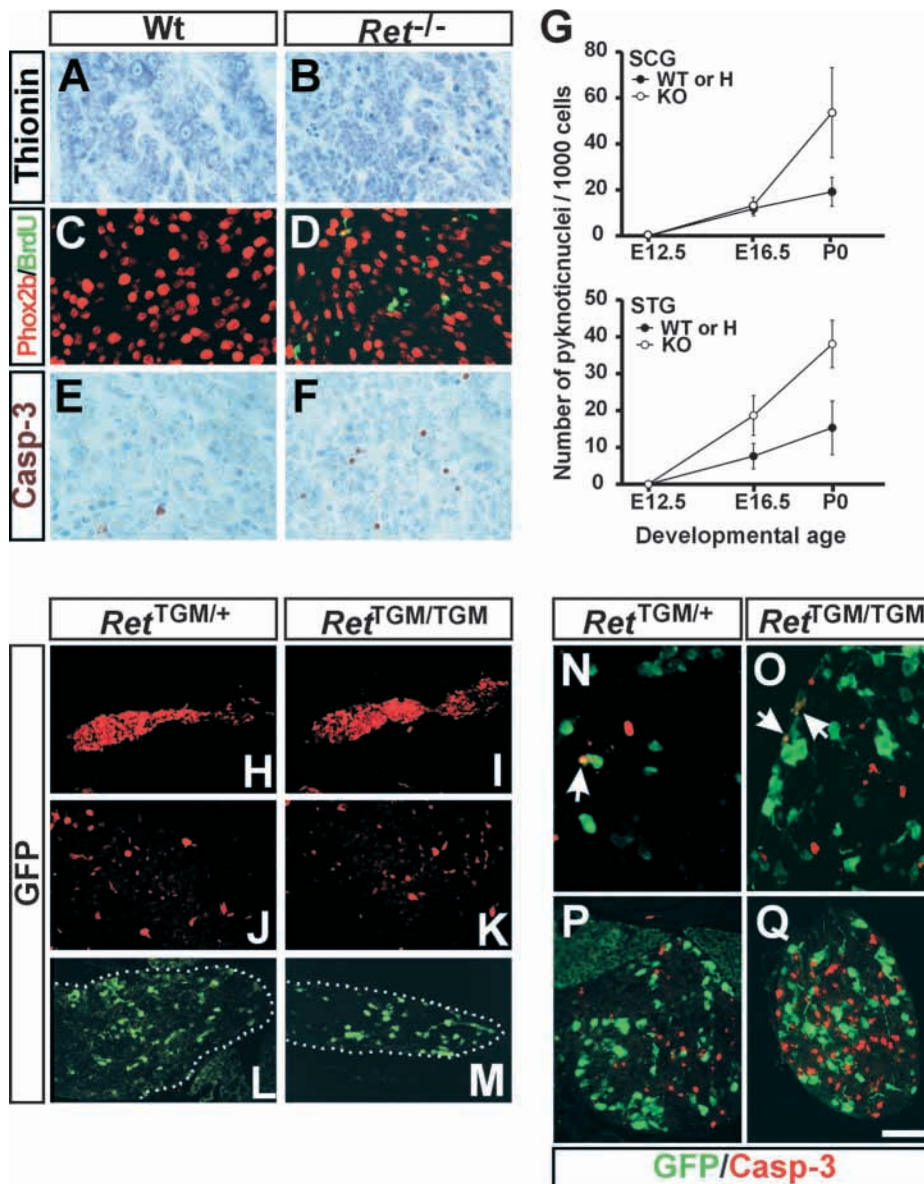

Fig. 5. Cell death of sympathetic neurons increases perinatally in *Ret*$^{-/-}$ mice, owing to lack of non-GFL neurotrophic factors. (A-F) Thionin (A,B), Phox2b and BrdU (C,D) and activated-caspase 3 (E,F) staining of E16.5 STG. The STG of *Ret*$^{-/-}$ embryos is mostly composed of neurons containing little cytoplasm (B), and with mixture of excessive dividing neuronal precursors (D) and dying cells (F). (G) Neuronal cell death is increased during the perinatal period in the SCG and STG of *Ret*$^{-/-}$ animals. Error bars indicate the standard deviation. (H-M) *Ret* gene expression monitored by GFP immunohistochemistry in the SCG of *Ret*$^{\mathrm{TGM/+}}$ and *Ret*$^{\mathrm{TGM/TGM}}$ animals at E11.5 (H,I), E15.5 (J,K) and P0 (L,M). *Ret* expression is markedly downregulated in most developing neurons in the SCG by E15.5. The *Ret*-expressing population in the SCG is comparable in size between *Ret*$^{\mathrm{TGM/+}}$ and *Ret*$^{\mathrm{TGM/TGM}}$ mice at P0. The broken line demarcates the SCG (L,M). (N-Q) GFP (green) and activated caspase 3 staining (red) of the SCG of newborn *Ret*$^{\mathrm{TGM/+}}$ (N, P) and *Ret*$^{\mathrm{TGM/TGM}}$ (O,Q) mice. Arrows (N,O) depict cells that are doubly stained. Despite the apparent increase of caspase 3-positive cells in *Ret*$^{\mathrm{TGM/TGM}}$ mice, GFP and caspase 3-positive populations remain largely distinct (P,Q). Scale bar: 35 μm in A-F, 140 μm in H,I,L,M,P,Q; 80 μm in J,K; 50 μm in N,O; 100 μm in P,Q.

neurons in these ganglia of *Ret*$^{-/-}$ embryos. For example, many sympathetic cells contained little cytoplasm and displayed neuroblast-like morphology at E16.5 (STG shown in Fig. 5A,B). Indeed, Phox2b and BrdU staining showed the presence of proliferating sympathetic neuronal precursors in the SCG and STG of *Ret*$^{-/-}$ embryos, which was not detected in their wild-type or heterozygous counterparts (Fig. 5C,D). In addition, more apoptotic figures were observed in the STG, but not the SCG, of *Ret*$^{-/-}$ embryos at E16.5 (Fig. 5G), an observation that is confirmed by increased numbers of cells that stain for activated caspase 3 (Fig. 5E,F). Moreover, a significant increase in neuronal cell death in *Ret*$^{-/-}$ embryos was observed in both the SCG and STG at P0 (Fig. 5G). Thus, in *Ret*$^{-/-}$ animals, sympathetic neuronal differentiation is severely delayed, and neuronal cell death increases during E16.5-P0.

The increased cell death in sympathetic neurons after E16.5 in *Ret*$^{-/-}$ embryos suggested that RET may be required for survival of sympathetic neurons in later development. To address this issue, we monitored *Ret* expression in sympathetic ganglia at different embryonic stages in *Ret*$^{\mathrm{TGM/+}}$ and *Ret*$^{\mathrm{TGM/TGM}}$ mice using GFP immunohistochemistry. Interestingly, whereas virtually all neuronal precursors expressed *Ret* in the SCG and STG at E11.5 (SCG shown in Fig. 5H,I), most of these cells lose *Ret* expression by E15.5 and *Ret* expression is detected in only a small subpopulation of sympathetic neurons in these ganglia at P0 in *Ret*$^{\mathrm{TGM/+}}$ and *Ret*$^{\mathrm{TGM/TGM}}$ animals (Fig. 5J-M). This downregulation of *Ret* expression was confirmed using RET immunohistochemistry (data not shown). This suggested that RET may be required perinatally for the survival of a specific subpopulation of sympathetic neurons of these ganglia. Surprisingly, however, the proportion of TGM-positive cells in the SCG and STG in was comparable between *Ret*$^{\mathrm{TGM/+}}$ and *Ret*$^{\mathrm{TGM/TGM}}$ mice at P0 (SCG: *Ret*$^{\mathrm{TGM/+}}$ versus *Ret*$^{\mathrm{TGM/TGM}}$ = 5.5% versus 6.6%; STG: *Ret*$^{\mathrm{TGM/+}}$ versus *Ret*$^{\mathrm{TGM/TGM}}$ = 7.8% versus 8.0%; Fig. 5L,M; data not shown). As *Ret* is expressed in only a small subset of sympathetic neurons perinatally, we examined whether

increased cell death is preferentially occurring in this small *Ret*-expressing population. The SCG and STG in newborn $Ret^{TGM/+}$ and $Ret^{TGM/TGM}$ animals were doubly stained with anti-GFP and anti-activated-caspase 3 antibodies to reveal relationship between dying cells and *Ret*-expressing cells. Although some doubly positive cells were identified in ganglia from both $Ret^{TGM/+}$ and $Ret^{TGM/TGM}$ mice (Fig. 5N,O, arrows), dying and TGM-positive populations were largely non-overlapping (Fig. 5P,Q), suggesting that excessive cell death is not occurring preferentially in *Ret*-expressing populations. Together, RET deficiency does not lead to selective elimination of *Ret*-expressing neurons in sympathetic ganglia by P0, despite the fact that excessive cell death occurs in *Ret*-deficient sympathetic ganglia. We conclude that the increased cell death of sympathetic neurons after E16.5 is indirectly related to lack of RET signaling. As innervation failure precedes the increased cell death, inaccessibility to other target-derived growth factors such as NGF may be a likely cause of the accelerated cell death. Supporting this hypothesis, expression of TRKA occurred normally in $Ret^{-/-}$ embryos (data not shown).

Artemin is a vascular-derived factor that promotes and attracts sympathetic axonal growth

While the analysis above has underscored the primary importance of RET signaling for inducing differentiation of early sympathetic precursors, it remained unclear which factors activate RET during the period of early sympathetic differentiation. Previous expression studies have shown that neither GDNF nor NRTN is expressed at the site of sympathetic ganglion formation (Golden et al., 1999). Examination of E11.5 $GDNF^{-/-}$ embryos using whole-mount TH immunohistochemistry revealed no discernible deficits in the sympathetic chain in these animals (data not shown). Furthermore the SCG was found in the normal location in newborn mice lacking both GDNF and NRTN (data not shown). Thus, it is unlikely that GDNF or NRTN is responsible for in vivo activation of RET in early differentiating sympathetic precursors. As migration of the SCG is affected by GFRα3 deficiency (Nishino et al., 1999), and GFRα3 is expressed in the primary sympathetic chain (data not shown), we examined the expression pattern of ARTN, a specific ligand for GFRα3 in rat embryos, by in situ hybridization.

The analysis revealed a strong correlation between the site of *Artn* mRNA expression and sympathetic neuron development. *Artn* mRNA was expressed at high levels in the wall of the dorsal aorta and its dorsal proximity at E12.5 (Fig. 6A, arrow), around the period of primitive sympathetic chain formation in the rat. At E14.5, high levels of *Artn* mRNA were detected in many blood vessels, including the dorsal aorta, celiac, superior mesenteric (Fig. 6B), inferior mesenteric and vertebral arteries (Fig. 6C, data not shown). At E16.5, whereas expression of *Artn* in the dorsal aorta became almost undetectable (Fig. 6D, arrow), *Artn* expression persisted in the small peripheral branches of the celiac and mesenteric arteries (Fig. 6D; arrowheads, 6E,F; arrows). Modest expression of *Artn* was also detected in the SCG and the internal carotid artery (data not shown). *Artn* expression was also observed in the esophagus and the stomach, but not in the digestive tract distal to the stomach (data not shown). Importantly, sympathetic axons grow towards or along these blood vessels during these periods and deficits in sympathetic innervation in $Ret^{-/-}$ mice are found at sites expressing *Artn*.

This expression analysis strongly suggested that loss of ARTN-mediated RET activation leads to impaired migration and axonal growth of sympathetic neurons observed in $Ret^{-/-}$ mice. To test this hypothesis, we analyzed the potential activity of ARTN in promoting and directing neurite outgrowth of sympathetic ganglion neurons. Nascent sympathetic ganglia were dissected from E13.5 mouse embryos and cultured in a collagen gel containing GDNF, NRTN ARTN or NT3, and axon outgrowth was evaluated using β tubulin immunohistochemistry. The effect of GDNF and NT3 was negligible at various concentrations tested (Fig. 7B,G). Although NRTN induced axon outgrowth from these ganglia at low concentrations (20 ng/ml; Fig. 7C), these fibers tended to fasciculate and neurite outgrowth was suppressed at higher concentration of NRTN (100 ng/ml; Fig. 7D). By contrast, ARTN elicited long neurite outgrowth and the density of neurites increased in a dose-dependent manner (Fig. 7E-G). Interestingly the axons induced by ARTN projected radially and displayed more branching than those induced by NRTN. We further tested whether NRTN or ARTN can influence the directionality of growing axons of the sympathetic explant. A factor-impregnated heparin bead (NRTN or ARTN bead) was placed approximately 300-400 μm from the explant in a collagen gel and axon outgrowth was examined after 40 hours of incubation. Although NRTN beads were capable of directing neurite outgrowth toward the bead in some explants, the response was modest and variable (three out of 24 explants, not shown). By contrast, approximately 90% of the explants

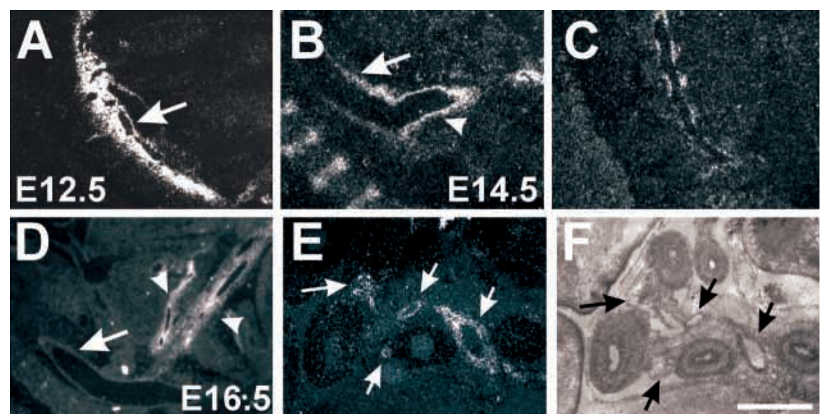

Fig. 6. Expression analysis of *Artn* mRNA in developing rat embryos. Radioactive in situ hybridization for *Artn* mRNA on parasagittal sections of E12.5 (A), 14.5 (B,C) and 16.5 (D-F) rat embryos. High levels of *Artn* mRNA are detected in the dorsal aorta (A,B, white arrows), the superior mesenteric, vertebral and celiac arteries (B,D, arrowheads). At E14.5, the vertebral artery also expresses high levels of *Artn* mRNA (C). Note that *Artn* expression in the dorsal aorta is undetectable at E16.5 (D, arrow). By contrast, persistent high expression is detected in small branches of the mesenteric arteries (arrows in dark- (E) and bright- (F) field photographs) at E16.5. Scale bar: 500 μm in A,C,E,F; 250 μm in B; 1100 μm in D.

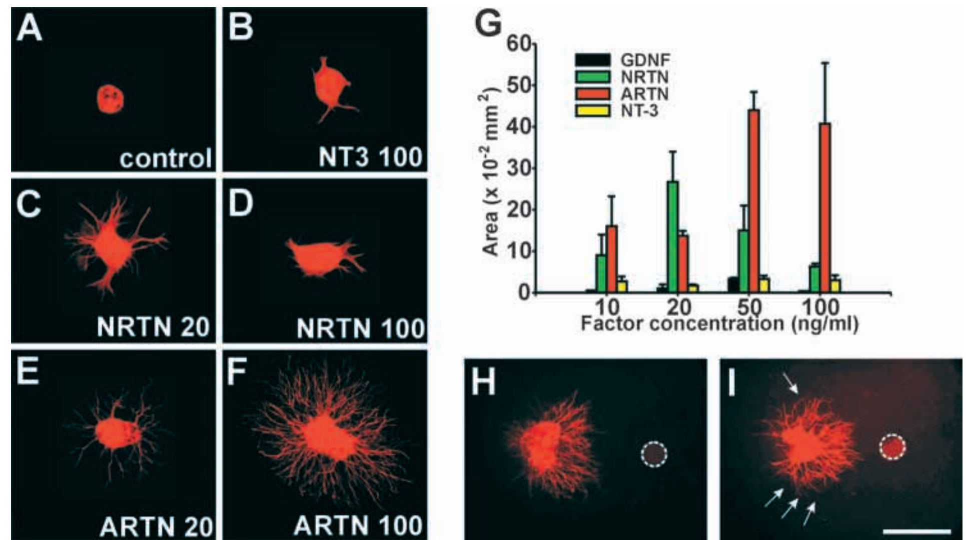

Fig. 7. ARTN promotes neurite outgrowth and attracts growing axons. (A-F) Representative pictures of STG explants stained with anti-β tubulin antibodies. Explants were dissected out from E13.5 mouse embryos and cultured in the presence of NT3 (100 ng/ml (B)), NRTN (20 ng/ml (C), 100 ng/ml (D)) and ARTN (20 ng/ml (E), 100 ng/ml (F)). (A) No factor. (E,F) Moderate and robust neurite outgrowth is induced by lower and higher concentrations of ARTN, respectively. (G) Axon-covering area was measured by use of NIH Image software (three explants for each condition; bars indicate mean standard errors). (H,I) ARTN-impregnated bead (dotted circle) induces robust neurite outgrowth of the sympathetic ganglion explant and directs growing axons toward the bead. Note that axons emanating from the distal side of the explant steer towards the source of ARTN (arrows in I). Scale bar: 350 μm for A-F; 250 μm for H,I.

projected profuse and long axons toward ARTN-impregnated beads (21 out of 24 explants tested, Fig. 7H). Furthermore, in a number of distally located axons, the axonal trajectory is steered toward the ARTN-beads (Fig. 7I, arrows), indicating that ARTN has tropic activity. Thus, the in vivo expression pattern of ARTN, together with its pharmacological activity to promote and direct axonal growth in vitro, strongly suggests that ARTN guides sympathetic axons by activating RET in early sympathetic neuron development.

DISCUSSION

The complexity of the nervous system demands extensive positional and morphological changes in neurons during development, including extension of axons and subsequent proper synapse formation. In vertebrates, peripheral sympathetic ganglia are formed in para- and pre-vertebral regions, and the post-ganglionic axons need to travel across significant distances to reach the final target tissues. For sympathetic neurons, blood vessels serve as intermediate targets during development for the axonal projections that will eventually innervate end organs. The interaction between sympathetic neuronal precursors and blood vessels occurs early in development, when these cells migrate and initiate axonal projection. Our present study has demonstrated that ARTN-mediated RET signaling is essential for this biological process, thereby revealing RET and ARTN as key regulators of sympathetic neuron development.

RET signaling is required by all sympathetic ganglia

It has previously been reported that RET deficiency affects only the SCG, but not other sympathetic ganglia, eliminating all SCG neuronal precursors by E12 in mice (Durbec et al., 1996). As the enteric neurons are completely depleted in the gut distal to the stomach in *Ret*$^{-/-}$ mice, it has been proposed that the SCG and those enteric neurons derive from a commonly shared precursor pool. Our present study has shown that RET deficiency does not eliminate all SCG neurons, but instead leads to formation of the SCG in an aberrant, more caudal location. We speculate that the aberrantly located SCG was misinterpreted as being absent in the previous report. The presence of the SCG in *Ret*$^{-/-}$ mice indicates that precursors for the SCG and those enteric neurons are distinct at least in GFL dependency. Furthermore, our results have shown that RET deficiency influences precursor migration and their axonal projections quite uniformly throughout the entire sympathetic nervous system. Thus, it is reasonable to conclude that SCG precursors are more similar to precursors of the more caudal sympathetic ganglia, rather than to precursors of enteric neurons. This conclusion is consistent with the previous lineage studies showing that enteric and sympathetic precursors are derived from distinct levels of the neural crest (Le Douarin and Teillet, 1973; Le Douarin, 1986).

Biological role of RET signaling bridges neural commitment and neuronal survival in sympathetic neuron development

Present analysis demonstrates that RET signaling is primarily required for axonal growth and migration of sympathetic precursors, but not for their survival or proliferation. Despite the profound migration and innervation deficits in *Ret*$^{-/-}$ embryos, the primary sympathetic chain formation and expression of adrenergic as well as pan-neuronal phenotypes occur essentially normally in these embryos, indicating that RET signaling is not essential for the generation or determination of neuronal identity of sympathetic neurons. This raises the question as to whether RET is regulating migration and axon projection through activating expression of other molecules yet to be identified, or, alternatively, whether RET signaling is directly linked to the cell locomotive machinery (see below).

It is noteworthy that, in *Ret*$^{-/-}$ embryos, the innervation and migration deficits of sympathetic precursors precede the abnormal cell death that occurs both in the SCG and STG during the perinatal period. Paradoxically, this abnormal cell death only becomes evident after *Ret* expression is confined to a small subpopulation of neurons in these ganglia. Indeed our

analysis demonstrated that the *Ret*-expressing population is not selectively dying in the absence of RET. Because of the severe depletion of sympathetic fibers, these sympathetic neurons are likely to be dying because of deprivation of the target-derived survival-promoting factors such as NGF. Collectively, our present study, together with previous in vivo evidence, provides new insight into sympathetic neuron development. Generation of the primitive chain, commitment to sympathetic lineage and expression of catecholaminergic traits requires Neuregulin signaling, BMPs, Mash1 and Phox2 proteins (Britsch et al., 1998; Guillemot et al., 1993; Pattyn et al., 1999; Schneider et al., 1999). Phox2b induces RET expression in sympathetic neuronal precursors (Pattyn et al., 1999). The activation of RET in sympathetic neuron precursors allows these cells to initiate axonal growth and/or migrate to the proper location, steps that are crucial for successive maturation and reaching a source of NGF for survival later in development.

RET and ARTN are required for sympathetic axon guidance

The present study also establishes ARTN as a strong candidate responsible for RET activation in early sympathetic development. Expression of *Artn* is detected in a number of blood vessels including the dorsal aorta, mesenteric, celiac, vertebral and internal carotid arteries during periods of axonal projections by sympathetic neurons. Importantly, sites of *Artn* expression significantly overlap regions where innervation deficits are found in *Ret*$^{-/-}$ mice. Consistent with the in vivo expression pattern, ARTN has a strong neurite outgrowth-promoting activity. Sympathetic neurons from E13.5 mouse embryos extend neurites in response to ARTN in a dose-dependent fashion, and the response is not suppressed even at high concentration of ARTN. This suggests that growing axons are capable of responding continuously to higher levels of ARTN in vivo. In this respect, temporal changes in the sites of *Artn* expression are particularly intriguing. *Artn* mRNA is initially detected in the dorsal aorta. As development proceeds, *Artn* expression in the dorsal aorta is downregulated, whereas expression in the peripheral branches of the mesenteric arteries persists. This movement of *Artn* expression towards the periphery would allow sympathetic growth cones to grow in a directed fashion, towards the site of highest ARTN expression.

Although RET deficiency severely affects the initiation of axonal growth in early embryos, axonal outgrowth is not completely halted. In fact, many sympathetic ganglia begin to project fibers late in development. The presence of abnormal trajectories of sympathetic fibers, which are occasionally seen in these late-projecting sympathetic fibers, confirms the critical requirement of RET signaling in axon guidance of sympathetic neurons (Song and Poo, 2001). We propose that ARTN-mediated RET activation provides a crucial guidance signal required for sympathetic axons to travel along blood vessels. The late onset of axonal projection may be potentiated by intrinsic mechanisms, or, alternatively, may reflect compensatory effects by other neurotrophic factors. One such candidate is NT3, which is expressed in blood vessels to support perinatal survival of sympathetic neurons (Francis et al., 1999). Thus, it would be interesting to examine whether RET and NT3 double mutation results in a more dramatic reduction and a greater abnormality in the trajectory of sympathetic nerves.

Common biological responses are induced by GFL-mediated RET signaling in development of the autonomic nervous system

While recent genetic evidence has established GFL-mediated RET signaling as crucial for proper development of a variety of neuronal populations including enteric, sensory, sympathetic, parasympathetic and motoneurons, the central biological process dependent on RET signaling has been unclear. We have recently conducted detailed analysis on development of parasympathetic neurons in *Gdnf*$^{-/-}$, *Nrtn*$^{-/-}$, *GFRα1*$^{-/-}$ or *Ret*$^{-/-}$ animals and found that proper migration of parasympathetic neuronal precursors as well as target innervation of those neurons require RET signaling (Enomoto et al., 2000). Thus, together with the present study, RET activation by different ligands, in different types of cells, and at different developmental periods leads to two closely related biological responses: enhanced cell motility and axon outgrowth. Similar regulation of cell migration and axonal growth by a single molecule has been demonstrated in a number of axon guidance molecules including netrin, slit and Eph receptors (Chisholm and Tessier-Lavigne, 1999).

The mechanism by which RET controls cell migration and axon growth is unclear. One possibility is that RET activation is directly involved in regulating reorganization of cytoskeletal proteins such as actin or neurofilament. Indeed, multiple lines of evidence have shown that activation of RET signaling is capable of enhancing cell motility, which is associated with rapid actin reorganization (Tang et al., 1998; van Weering and Bos, 1997). It is also noteworthy that RET signaling mediated by GFLs requires recruitment of RET to lipid rafts and interaction with Src family kinases (SFKs) for efficient neurite outgrowth in vitro (Encinas et al., 2001; Tansey et al., 2000). SFKs are enriched in neuronal growth cones (Maness et al., 1988) and have been shown to play a crucial role in guidance and fasciculation of olfactory axons in vivo (Morse et al., 1998). Moreover, recent genetic evidence has demonstrated the critical requirement of the Src substrate, p190 GAP, for proper axonal projection of the anterior commissure and formation of the subcortical axons (Brouns et al., 2001), supporting further the importance of Src signaling in axon guidance and growth. Thus, it will be intriguing to determine the relationship between RET-mediated Src-activation and cytoskeletal protein organization. Finally RET signaling appears to be important not only in physiological but also pathological conditions, as GDNF significantly promotes regeneration of spinal nerves after injury (Ramer et al., 2000). Elucidation of mechanisms that underlie neurite outgrowth-promoting activity of RET will be a crucial step toward potential application of GFLs for treatment of neurodegenerative diseases and nerve or spinal cord injuries.

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