Neural Crest Cell Origin and Signals for Intrinsic Neurogenesis in the Mammalian Respiratory Tract

Aliete Langsdorf[1], Kelsi Radzikinas[1], Amanda Kroten[2], Sanjay Jain[2], and Xingbin Ai[1]

[1]Pulmonary Center, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts; and [2]Renal Division, Department of Medicine, Pathology, and Immunology, HOPE Center for Neurological Disorders, Washington University School of Medicine, St. Louis, Missouri

Our study investigates the innervation of the respiratory tract during mouse embryonic development, with a focus on the identification of cell origin and essential developmental signals for the resident, or intrinsic, neurons. Using lineage tracing, we show that these intrinsic neurons are exclusively derived from neural crest cells, and cluster to form ganglia that reside in the dorsal trachea and medial bronchi with diminishing frequency. Comparisons of intrinsic neurogenesis between wild-type, glial cell–derived neurotrophic factor (GDNF)$^{-/-}$, neurturin$^{-/-}$, and tyrosine kinase receptor Ret$^{-/-}$ embryos, in combination with lung organ cultures, identified that Ret signaling, redundantly activated by GDNF family members, is required for intrinsic neurogenesis in the trachea and primary bronchi. In contrast, Ret deficiency exerts no effect on the innervation of the rest of the respiratory tract, suggesting that innervation by neurons whose cell bodies are located outside of the lung (so-called extrinsic neurons) is independent of Ret signaling. Furthermore, although the trachea, the esophagus, and their intrinsic neurons share foregut endoderm and a neural crest cell origin, respectively, the signals required for their intrinsic neurogenesis are divergent. Together, our results not only establish the neural crest lineage of intrinsic neurons in the respiratory tract, but also identify regional differences in the abundance and developmental signals of intrinsic neurons along the respiratory tract and in the esophagus.

Keywords: neural crest cell; respiratory tract; intrinsic neuron; extrinsic innervation; GDNF

The mammalian respiratory tract consists of the trachea and lungs, and is innervated by a combination of intrinsic neurons, whose cell bodies reside within the tissue, and extrinsic neurons, whose cell bodies are located elsewhere (1). Extrinsic innervation allows bronchopulmonary communication with the central nervous system, and therefore has been extensively investigated. Compared with extrinsic innervation, the intrinsic neurons of the mammalian respiratory tract are less well-studied. Most previous studies on the intrinsic neurons involved immunohistochemical characterizations of resident ganglia in the embryonic and neonatal respiratory tracts (2–6). However, the developmental mechanisms and functions of these intrinsic neurons are unknown. Greater insight into the innervation of the respiratory tract is of interest because it serves vital life functions such as sensation, breathing, and inflammation (7–9).

The mammalian respiratory tract arises from the ventral foregut endoderm (10). In mouse embryos, two lung buds are induced from the ventral–lateral foregut at E9.5. Along with lung-bud formation, the trachea arises from the ventral foregut and separates from the esophagus, which forms dorsally. During the initiation of respiratory-tract formation, neural crest cells generated from the vagal portion of the neural tube migrate along the rostral–caudal axis into the developing foregut (11, 12). Some vagal neural crest cells were proposed to migrate into the ventral foregut and give rise to intrinsic ganglia in the respiratory tract (13), whereas others give rise to the majority of intrinsic neurons and glia in the gastrointestinal tract (14–16).

Although intrinsic neurons of the gastrointestinal tract are known to be generated from neural crest progenitors (14–16), the cell origin of intrinsic neurons in the mammalian respiratory tract remains to be established definitively by lineage tracing. Results from previous studies in avian and mammalian embryos support a neural crest origin of intrinsic neurons in the respiratory tract. For example, intrinsic neurons in both murine and human embryonic lungs express p75 (4, 6), a neural crest marker that can also be expressed by other cell types. In addition, the engraftment of quail neural crest cells into chicken embryos proves that neural crest cells give rise to intrinsic neurons in avian lungs (17). However, none of these studies address whether neural crest cells are the only source of intrinsic neurons in the mammalian respiratory tract, and if not, whether placodes also contribute to these intrinsic neurons (18).

The signals required for mammalian respiratory neurogenesis have not been identified. The gastrointestinal tract, however, is known to rely on glial cell–derived neurotrophic factor (GDNF) for the development of intrinsic neurons (19–22). GDNF and its family members, neurturin (NRTN), artemin, and persephin, bind to GDNF family coreceptors 1, 2, 3, and 4 (GFRα1–4), respectively, although NRTN and artemin also bind to GDNF receptor GFRα1 with low affinity (23, 24). The ligand and GFR complex subsequently recruits the common tyrosine kinase receptor Ret for signaling (23, 24). Whether GDNF is also essential for the formation of intrinsic neurons in the respiratory tract is unknown, although previous studies showed that the addition of exogenous GDNF to embryonic lung explant cultures induced the migration and neurite outgrowth of intrinsic neurons (25).

Here, we performed lineage tracing using a neural crest–specific Wnt1-Cre line crossed with two R26R reporter lines, to prove definitively the neural crest cell origin of intrinsic neurons in the murine respiratory tract. In addition, we investigated the roles of GDNF family members in the intrinsic neurogenesis of the respiratory tract, using explant cultures and mouse models deficient in GDNF, NRTN, and Ret. Further, we identified differences in the developmental mechanisms of intrinsic neurogenesis between the respiratory tract and the esophagus.

MATERIALS AND METHODS

Mice

Wnt1-Cre transgenic mice, i.e., Gt(ROSA)26Sor (R26R^{lacZ}) and Gt(ROSA)26Sor$^{tm1(eYFP)}$ (R26R^{eYFP}) mice, were purchased from Jackson Laboratories (Bar Harbor, Maine). After crossing, Wnt1-Cre;R26R^{lacZ} and Wnt1-Cre;R26R^{eYFP} reporter lines were generated for neural crest cell lineage tracing (26). GDNF$^{+/-}$ and NRTN$^{-/-}$ mice were described previously (21, 27). Ret-EGFP (enhanced green fluorescent protein) mice were derived from mice carrying a floxed

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Correspondence and requests for reprints should be addressed to Xingbin Ai, Ph.D., Pulmonary Center, Department of Medicine, Boston University School of Medicine, Boston, MA 02118. E-mail: aix@bu.edu

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allele of the Ret gene (28), and contain a functionally null allele of Ret resulting from a knock-in of EGFP cDNA. Timed-pregnant CD1 females were purchased from Charles River (Wilmington, MA). All animal studies were approved by the Institutional Animal Care and Use Committee of the Boston University Medical Campus.

Immunohistochemistry

Dissected embryonic trachea and lungs were fixed in 4% paraformaldehyde/phosphate buffer for 2–5 hours at 4°C, followed by three washes with PBS for 30 minutes each. For immunostaining of the whole respiratory tract, trachea and lungs were treated for 2 hours with a detergent mix (0.5% sodium deoxycholate, 1% Nonidet P-40, and 1% SDS in PBS) to allow permeablization. The whole tissue was then incubated with antibody dilution buffer (10% goat serum, 0.1% Tween-20, and 2% BSA in PBS) to block nonspecific staining, followed by the application of primary antibodies in antibody dilution buffer overnight at 4°C. After extensive washes with PBS, antigen–antibody complexes were detected using biotinylated secondary antibodies and 3,3′-diaminobenzidine (DAB) substrate, following the manufacturer's protocol (Vector, Burlingame, CA). The number of neurons in five ganglia of E13.5 trachea was counted under the microscope, and the average neural number was calculated. Four embryos were examined for each genotype.

For immunostaining of tissue sections, fixed trachea and lungs were cryoprotected overnight in 30% sucrose/PBS at 4°C. After embedding in OCT, 10-μm serial sections were collected on the slide with 100 μm between two adjacent sections. Sections were rinsed with PBS, blocked with antibody dilution buffer, and incubated with primary antibodies. For immunostaining with primary antibodies generated in goat, a gelatin buffer (3% gelatin and 0.1% Tween-20 in PBS) was used as antibody dilution buffer. Antigen–antibody complexes were detected by either DAB substrate or fluorescence-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA). Primary antibodies purchased from R&D Systems (Minneapolis, MN) included mouse anti-neurotubulin (TuJ1; 1:200), goat anti-GFRα1 (1:1,000), and goat anti-GFRα2 (1:200). Primary antibodies from Sigma (St. Louis, MO) included rabbit anti-smooth muscle actin (1:500) and rabbit anti-calcitonin gene-related peptide (1:1,000). Rabbit polyclonal GFP antibody was purchased from Abcam (1:500) (Cambridge, MA). Antigen–antibody complexes were detected using Alexa 488 and 546 conjugated secondary antibodies (Molecular Probes). Nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI) to identify neural cell bodies. After staining, sections were examined with a fluorescence microscope (model DMR; Leica). Images were taken using a digital camera (model DC300F; Leica, Bannockburn, IL). The number of neurons from three serial tracheal sections was counted and added to represent the size and frequency of ganglia along the trachea. A minimum of four embryos was examined for each genotype.

Organ Cultures of the Respiratory Tract

Organ cultures were performed as described previously (29). The respiratory tract, including the trachea and lungs, was dissected from E10.5 and E11.5 CD1 mouse embryos and placed on the membrane of Transwell inserts (Corning, Carlsbad, CA, Lowell, MA). Cultures were maintained in an air–liquid interface for 3 days, with the medium changed every 24 hours. BGJb medium (Invitrogen) contains 25 mg/L ascorbic acid, 1% FBS, and 1% penicillin/streptomycin. To treat cultured lungs with growth factors, NRTN, nerve growth factor (NGF), or fibroblast growth factor 2 (FGF2) (50 ng/ml; R&D Systems) was added to the medium (22, 30). Cultures were either immunostained as a whole, or sectioned followed by immunostaining with TuJ1 antibody. TuJ1-immunoreactive areas on sections were quantified by Image J (NIH, National Institute of Health) and normalized to the size of the section as percentages of innervation.

Detection of β-Galactosidase Activity

Tissues of neural crest cell origin were lineage-labeled by a LacZ reporter in Wnt1-Cre;R26R^{lacZ} embryos. To detect β-galactosidase (gal) activity, embryos were fixed for 2 hours in ice-cold 2% paraformaldehyde/PBS followed by four 30-minute washes with PBS. After washing, embryos were incubated in staining solution (2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.01% Nonidet P-40, 0.1% sodium deoxycholate, and 0.1% X-gal in PBS) at 37°C in the dark. Color development was checked frequently until staining appears dark blue. After staining, embryos were washed in PBS, postfixed with 4% paraformaldehyde/PBS for 30 minutes, and processed for frozen sectioning.

In Situ Analysis of GDNF and NRTN mRNA Expression

A digoxigenin-labeled riboprobe of GDNF was synthesized from a plasmid containing mouse GDNF cDNA sequences (nucleotides 121–2068 of GeneBank Accession Number NM_010275). A digoxigenin-labeled NRTN riboprobe was synthesized from a PCR fragment amplified from genomic DNA, using primers containing the T3 promoter sequence. These primers amplify a 235-base pair (bp) segment of the NRTN coding sequence (GeneBank Accession Number U78109) (31). The primer sequences include: forward, 5′ CCA GCG GAG GCG CGT GCG CAG AGA 3′; and reverse, 5′ GAT CAT TAA CCC TCA CTA AAG GGG AGT TCC CAC ACT TTA TGT GAA 3′. *In situ* hybridization on 10-μm embryonic sections was performed as described previously (32).

RESULTS

Intrinsic Neurons in the Respiratory Tract Have Discrete Anatomic Locations

To identify intrinsic neurons, the respiratory tract was dissected from E13.5, E14.5, and E18.5 mouse embryos and analyzed for neural crest and neural markers, including the expression of GFP in Ret-EGFP embryos and immunohistochemistry for neurotubulin (Figure 1). As described previously, GFP in Ret-EGFP mice is expressed under the control of the Ret promoter, and identifies neural crest cells in the gastrointestinal tract (33). At E13.5, GFP$^+$ cells are found to cluster and align longitudinally on the dorsal side of the trachea (Figure 1A). A majority of them also express the neural marker neurotubulin, as labeled by the TuJ1 antibody (Figure 1B), confirming them as neurons. The GFP$^+$TuJ1$^-$ cells are mostly likely uncommitted neural-crest progenitors or glial progenitors (33). The dorsal alignment of the cell bodies of these intrinsic neurons in the trachea persists at E14.5, E18.5, and postnatally (Figures 1C−1E, and data not shown), and is distinct from the circular distribution of intrinsic neurons in the esophagus (Figure 1C). Sagittal sections of the trachea show cell bodies of the intrinsic neurons located dorsally in association with the smooth muscle that connects the cartilage rings (Figure 1F). The processes of these intrinsic neurons extend into the smooth muscle (Figure 1F, *inset*), supporting the concept that they control the contractility of tracheal smooth muscle (34).

The bronchi and lungs also contain TuJ1$^+$ intrinsic neurons, with the number of neurons diminishing along the proximal–distal axis (Figure 1G). A small number of intrinsic neurons are found along the secondary and tertiary bronchi (Figure 1H), with very few neurons in more distal region. Despite the paucity of intrinsic neurons in the distal lung, TuJ1$^+$ neural fibers are readily identifiable in the proximity of airway smooth muscle and neuroendocrine cells (Figures 1I and 1J) (34–37).

Intrinsic Neurons in the Respiratory Tract Originate from Neural Crest Cells

The expression of neural crest markers p75 and Ret by the intrinsic neurons in the mammalian respiratory tract suggests that these neurons originate from neural crest cells (Figure 1A) (4, 6). To prove definitively their neural crest cell origin, we lineage-labeled neural crest cells *in vivo* using Wnt1-Cre; R26R^{1acZ} embryos, in which neural crest cell–derived cells express β-gal (26). X-gal staining of the respiratory tract of E16.5 Wnt1-Cre;R26R embryos revealed β-gal$^+$ cells in the

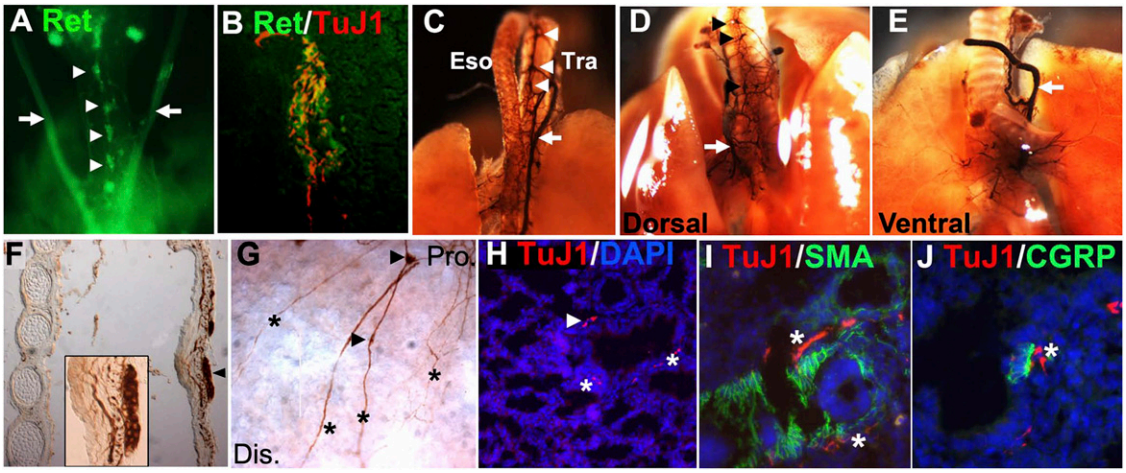

Figure 1. Intrinsic neurons of the developing respiratory tract exhibit discrete anatomic distribution. The trachea and lungs were dissected from E13.5 (*A* and *B*), E14.5 (*C*), and E18.5 (*D–J*) embryos. Intrinsic neurons in the trachea and lung lobes were identified by green fluorescent protein (GFP) expression under the control of the Ret promoter in Ret-EGFP embryos (*A* and *B*) and by TuJ1 immunolabeling of the whole lung and lung sections (*B–J*). *Arrowheads* indicate neural cell bodies. *Arrows* indicate the vagus nerve. *Asterisks* indicate neural fibers. (*A*) Clusters of GFP$^+$ cells are aligned longitudinally in the trachea of E13.5 Ret-EGFP embryos. (*B*) A majority of GFP$^+$ cells are confirmed as neurons by TuJ1 immunolabeling and appear *yellow* after overlay. (*C*) At E14.5, clusters of TuJ1$^+$ neurons are located exclusively on the dorsal side of the trachea facing the esophagus, in contrast to the circular distribution of intrinsic neurons of the esophagus. (*D* and *E*) The asymmetric distribution of intrinsic neurons in the dorsal trachea persists at E18.5. (*F*) Sagittal sections of E18.5 trachea show that intrinsic neurons are absent from the cartilage. Cell bodies of intrinsic neurons cluster on the surface of the tracheal smooth muscle and extend their neurites into the smooth muscle, as shown by enlarged picture in *inset*. (*G* and *H*) Clusters of intrinsic neuronal cell bodies are also found along the bronchi. Only a few neural cell bodies are located distal to the secondary or tertiary bronchi. TuJ1$^+$ neural fibers are identified at more distal regions of the lung than are the cell bodies of intrinsic neurons. (*I*) Double immunostaining with the TuJ1 antibody and an antibody against smooth muscle actin (SMA) identifies TuJ1$^+$ neural fibers in proximity to the airway smooth muscle. (*J*) TuJ1$^+$ neural fibers innervate neuroendocrine bodies that were immunolabeled by an antibody against calcitonin gene–related peptide (CGRP). Eso, esophagus; Tra, trachea; Pro., promixal; Dis., distal.

trachea and the lungs that were distributed in a pattern similar to that of TuJ1$^+$ intrinsic neurons (Figures 2C−2F, compared with Figures 1A−1G). A majority of these β-gal$^+$ cells were confirmed as neurons by TuJ1 antibody staining (Figures 2G and 2H). Therefore, the respiratory tract contains intrinsic neurons that are of neural crest cell origin, similar to the gastrointestinal tract.

In addition to neural crest cells, the placodes also generate neurons (18). To distinguish neural crest from placode contributions to intrinsic neurons in the respiratory tract, we analyzed both Wnt1-Cre;R26R^{lacZ} and Wnt1-Cre;R26R^{eYFP} embryos, in which only neural crest–derived cells are lineage-labeled, by colocalizing TuJ1 immunoreactivity with reporter expression. If neural crest constitutes the only cell of origin of intrinsic neurons in the respiratory tract, all TuJ1$^+$ neurons would be labeled. Alternately, if the placodes contribute to intrinsic neurons, some TuJ1$^+$ neurons would not be labeled. We found that all neurons in the respiratory tract of Wnt1-Cre;R26R embryos are β-gal$^+$ (Figures 2G and 2H), demonstrating that neural crest cells are the only source of intrinsic neurons. This finding was confirmed in Wnt1-Cre;R26R^{eYFP} embryos. Serial sections of the respiratory tract of E16.5 Wnt1-Cre;R26R^{eYFP} embryos were double-stained with an enhanced yellow fluorescent protein (eYFP) antibody and TuJ1 antibody with nuclei labeled by DAPI, to identify cell bodies of intrinsic neurons (Figures 2I−2K). All intrinsic neurons in the trachea, bronchi, and lungs were found to be eYFP$^+$, establishing an exclusive neural crest origin (Figures 2I−2K).

Neural Crest Cells Contribute to Intrinsic Neurons of the Respiratory Tract in a Dynamic and Spatially Regulated Manner

To analyze the spatiotemporal dynamics of neural crest cell migration and intrinsic neurogenesis in the respiratory tract *in vivo*, we characterized the migration of neural crest cells, which were labeled by GFP driven by the Ret promoter in Ret-EGFP embryos, into the trachea, bronchi, and lung buds at E10.5 and E11.5, and we also characterized the generation of TuJ1$^+$ neurons at E12.5 and E14.5 (Figure 3). At E10.5, Ret-GFP$^+$ neural crest cells were identified in the mesenchyme surrounding the esophagus along the dorsal-to-caudal axis (Figure 3A). A few neural crest cells also arrived in the proximity of the bronchi and caudal trachea, where the trachea is separated from the esophagus (Figures 3A2 and 3A3). However, no neural crest cells were present in the lung buds or rostral trachea, where the trachea remains attached to the esophagus (Figure 3A1, and data not shown). At E11.5, abundant neural crest cells were identified in the dorsal trachea and medial sides of the major bronchi, where airway smooth muscle cells form (Figures 3B1 and B2). In addition, a few neural crest cells were detected in the medial and proximal lungs (Figure 3B3). Neural crest cells differentiate into neurons after they migrate into the respiratory tract. A restricted distribution of TuJ1$^+$ intrinsic neurons in the ventral trachea, major bronchi, and proximal lungs was detected at E12.5 (Figure 3C), E14.5 (Figure 3D), and later during development (Figure 1). The migration of neural crest cells into the lungs likely ceases after E14.5, because the location of the intrinsic neurons along the proximal-to-distal axis of E14.5 lungs is similar to that observed at E18.5 (Figure 1).

Intrinsic Neurons in the Trachea and Esophagus Differentially Express GDNF Family Members and Receptors

Before our study of the signals that control intrinsic neurogenesis of the respiratory tract, we analyzed the expression of GDNF, NRTN, and their high-affinity receptors in E12.5 embryos by *in situ* hybridization and immunohistochemistry, respectively (Figure 4). GDNF and NRTN are two GDNF

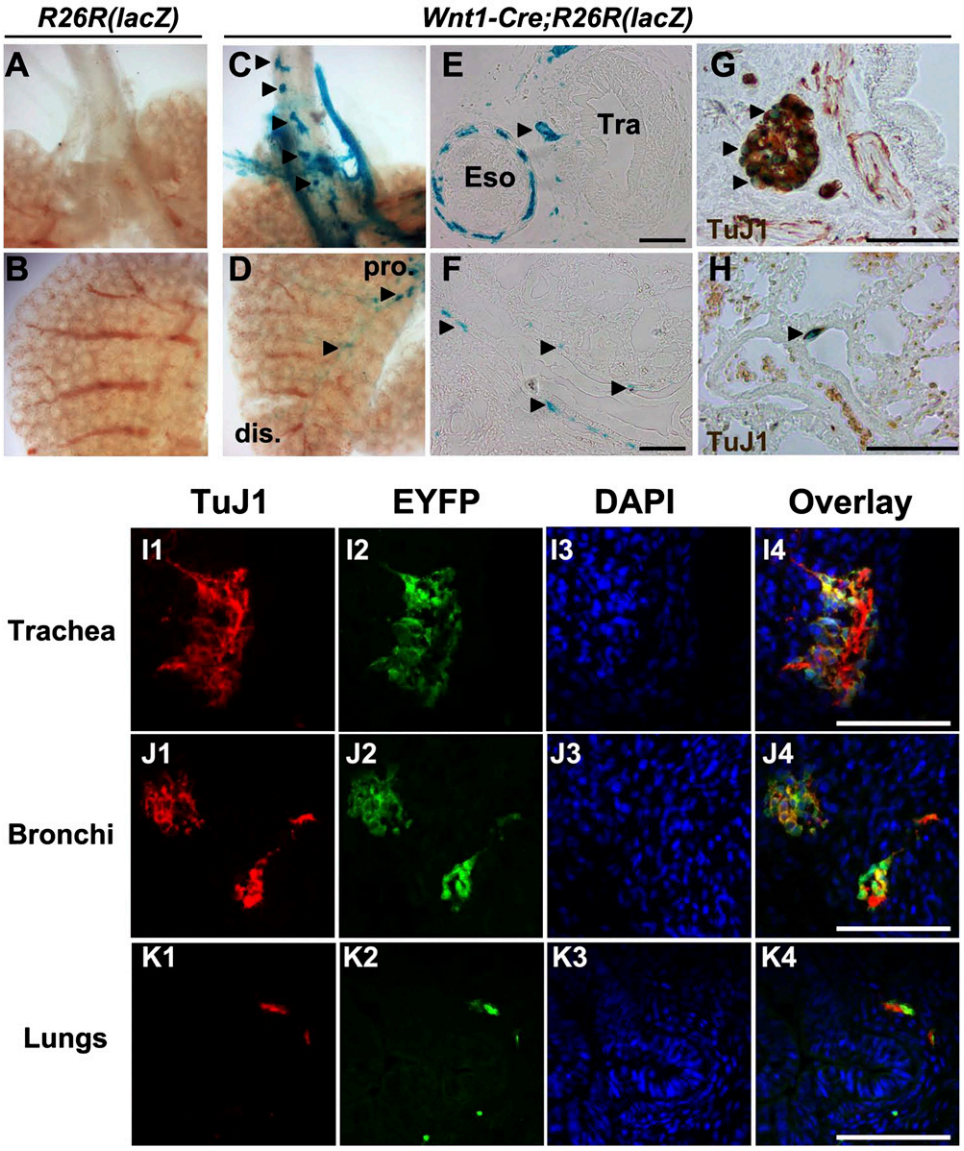

Figure 2. Intrinsic neurons of the respiratory tract are derived exclusively from neural crest progenitors. Neural crest cell linage tracing was performed *in vivo* using E16.5 Wnt1-Cre;Rosa26^{lacZ} embryos (*A–H*) and Wnt1-Cre;Rosa26^{eYFP} embryos (*I–K*). (*A* and *B*) Staining of β-gal activity in E14.5 Rosa26^{lacZ} control embryos identified no β-gal$^+$ cells in the trachea and lung. (*C* and *F*) Clusters of β-gal$^+$ cells are identified in the dorsal trachea and lung. (*G* and *H*) These β-gal$^+$ cells are largely neurons, as demonstrated by TuJ1 immunohistochemistry. Note all TuJ1$^+$ intrinsic neurons express β-galactosidase (β-gal$^+$). (*I–K*) All TuJ1$^+$ intrinsic neurons in the trachea, bronchi, and lungs are colocalized with EYFP after double immunolabeling of E16.5 Wnt1-Cre;Rosa26^{eYFP} embryonic sections. Cell bodies of intrinsic neurons are distinguished from axons by the presence of DAPI nuclear labeling. DAPI, 4′,6-diamidino-2-phenylindole; EYFP, enhanced yellow fluorescent protein; Eso, esophagus; Tra, trachea. *Scale bars*, 100 μm.

family members with essential roles in gastrointestinal innervation, and both are expressed in the esophagus (Figure 4A) (19–22, 27, 31, 38). In contrast, the trachea does not express GDNF mRNA (Figure 4Aa). Furthermore, GDNF mRNA is detected at lower levels in the major bronchi than in the esophagus (Figure 4Ab). However, both the trachea and bronchi express NRTN mRNA at levels comparable to those in the esophagus (Figures 4Ac and 4Ad).

We chose E12.5 Ret-EGFP embryos to analyze the expression of GFRα1 and GFRα2, which are high-affinity receptors for GDNF and NRTN, respectively, using Ret-GFP$^+$ intrinsic neural progenitors in the respiratory tract (Figures 1A and 1B). Immunohistochemistry was performed using specific antibodies against GFRα1 and GFRα2 (39). GDNF family receptor immunoreactivity was colocalized with GFP. GFRα1 is detected in GFP$^+$ neural crest progenitors in the esophagus and the major bronchi, as well as in other cell types, such as smooth muscle (Figures 4Ba−4Bd). In contrast, GFRα2 is expressed by GFP$^+$ neural progenitors in the trachea, but not in the esophagus (Figures 4Be−4Bh). Together, GDNF family ligands and receptors are differentially expressed by intrinsic neurons in the respiratory tract and esophagus, suggesting that intrinsic neurogenesis in these two tissues is regulated by different GDNF family members.

Intrinsic Neurogenesis in the Trachea and Bronchi Requires Ret Signaling

Ret is the tyrosine kinase receptor of all GDNF family members (23). To test whether Ret signaling is required for intrinsic neurogenesis in the respiratory tract, TuJ1 immunostaining was performed in wild-type and Ret$^{-/-}$ embryos at E18.5, and the numbers of TuJ1$^+$ neurons were counted. Compared with the results in wild-type embryos, the disruption of Ret function reduces the number of intrinsic neurons by ∼ 80% in both the trachea and primary bronchi, as shown by whole-mount TuJ1 labeling (Figures 5F and 5I) and quantification on serial cross sections (Figure 3N). The ∼ 20% residual intrinsic neurons may be maintained through the neural cell adhesion molecule receptor (40). Ret-deficiency also eliminates intrinsic neurons in the esophagus (Figures 5A, 5C, and 5M), establishing that Ret is essential for neurogenesis in both the respiratory tract and esophagus.

We further investigated which GDNF family members are required for intrinsic neurogenesis in the respiratory tract. GDNF was previously shown to affect intrinsic neurons in lung organ cultures (25). However, GDNF is not expressed by the trachea (Figure 4A), suggesting that GDNF is not required for

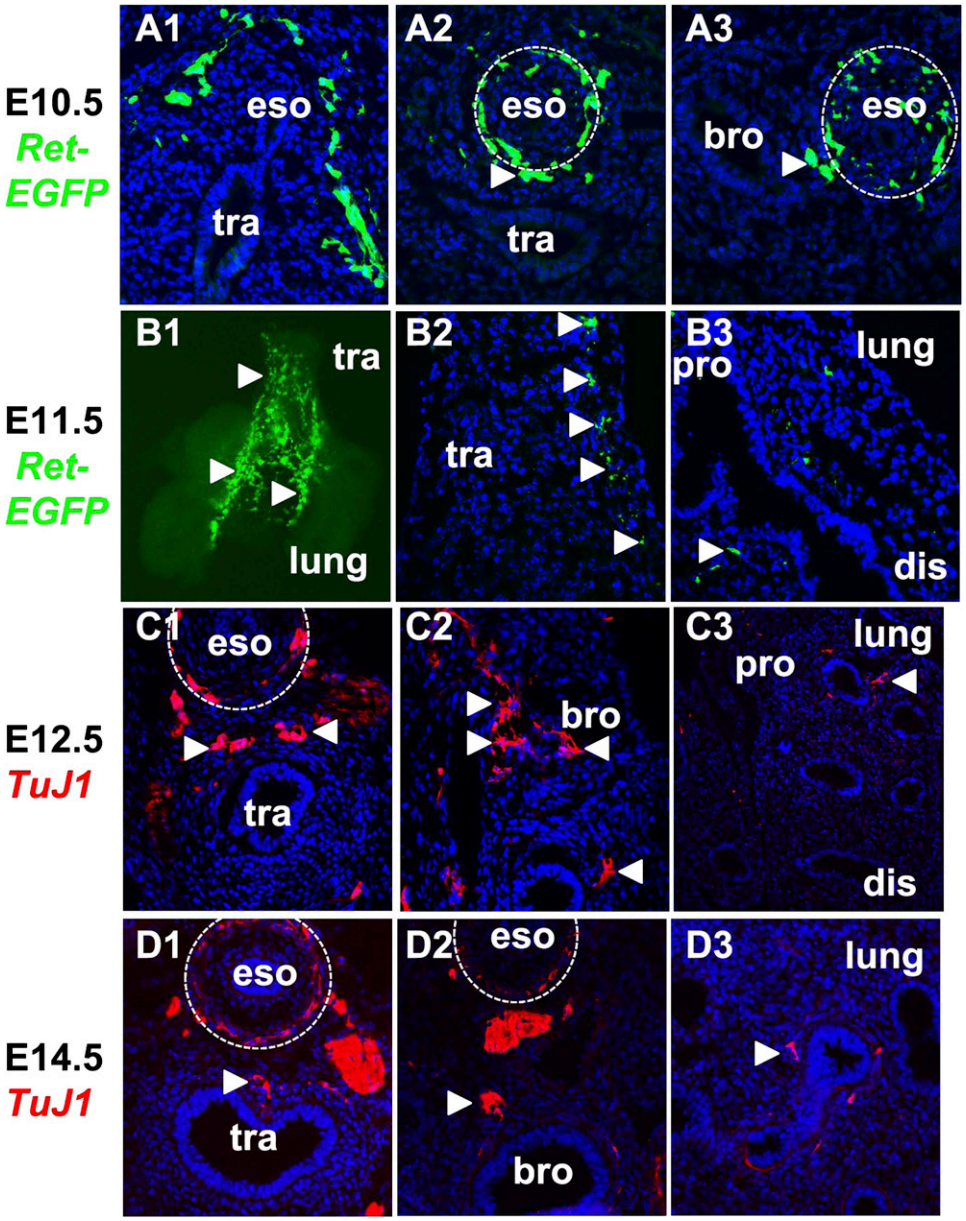

Figure 3. Temporal and spatial characterization of intrinsic neurogenesis in the embryonic respiratory tract. Subparts 1-3 show 3 images. Intrinsic neural progenitors at E10.5 and E11.5 were identified by enhanced green fluorescent protein (EGFP) in Ret-EGFP embryos (*A* and *B*), and intrinsic neurons at E12.5 and E14.5 were immunolabeled by TuJ1 (*C* and *D*). *Arrowheads* indicate cell bodies of intrinsic neural progenitors and neurons. eso, esophagus; tra, trachea; pro, promixal; dis, distal.

neurogenesis in the respiratory tract. To prove our hypothesis, we compared intrinsic neurons between wild-type and GDNF$^{-/-}$ embryos. Indeed, GDNF deficiency exerted no effect on the number and location of intrinsic neurons in the trachea and primary bronchi (Figures 5D, 5E, 5G, 5H, and 5N), in contrast to its essential roles during esophageal neurogenesis (Figures 5A, 5B, and 5M). Therefore, distinct GDNF family members are required for intrinsic neurogenesis in the respiratory tract and esophagus.

Although intrinsic neurogenesis is disrupted in the Ret$^{-/-}$ respiratory tract, comparable densities of TuJ1^{+} neural fibers were evident in the proximity of airway smooth muscle in regions distal to the primary bronchi in wild-type, GDNF$^{-/-}$, and Ret$^{-/-}$ embryos (Figures 5J–L and 5O), suggesting that a majority of innervation in the lungs derives from extrinsic neurons. This finding is also consistent with our observation that the lung is largely devoid of intrinsic neurons (Figures 1G and 1H). Further, this finding indicates that signals controlling the extrinsic innervation of the lungs are different from those controlling intrinsic innervation.

We then tested whether NRTN is required for intrinsic neurogenesis in the respiratory tract. Whole-mount TuJ1 immunostaining was performed with dissected trachea from E13.5 wild-type and NRTN$^{-/-}$ embryos (Figure 6). The number of intrinsic neurons within individual ganglia was quantified. Although NRTN is expressed in the trachea and bronchi (Figures 4Ac and 4Ad), intrinsic neurons were comparable between wild-type and NRTN$^{-/-}$ embryos, demonstrating that NRTN deficiency alone is not sufficient to disrupt intrinsic respiratory neurogenesis (Figure 6).

NRTN Regulates Intrinsic Neurogenesis of the Respiratory Tract in Culture

To gain insights into the functions of GDNF family members during intrinsic respiratory neurogenesis, explant cultures were established from E10.5 and E11.5 embryos after neural crest cells migrated into the respiratory tract (Figure 2) (29). Explants were treated with NRTN, NGF, and FGF2 at a concentration of 50 ng/ml (22, 30). After 3 days in culture (+ 3), the explants were immunolabeled with TuJ1 antibody. NRTN, but none of

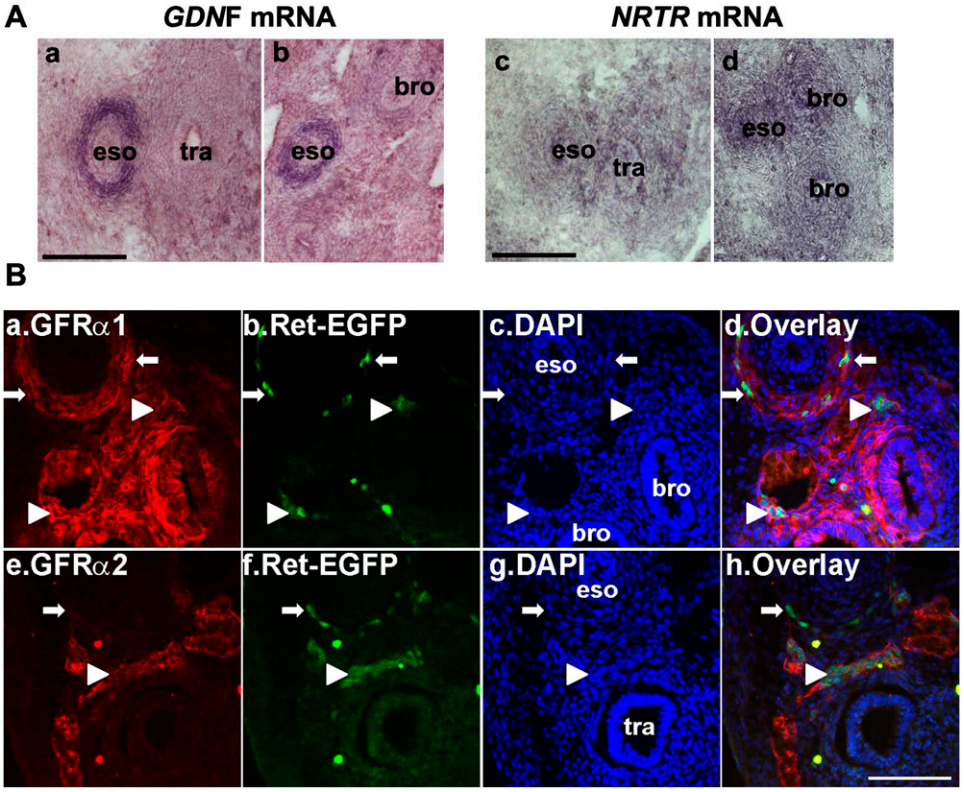

Figure 4. The esophagus, the respiratory tract, and their intrinsic neurons differentially express GDNF family members and receptors at E12.5. (*A*) GDNF and NRTN mRNA expression was analyzed by *in situ* hybridization. GDNF mRNA was detected in the esophagus, but not in the trachea, whereas NRTN mRNA was detected in both tissues. (*B*) The expression of GFRα1 and GFRα2 was assayed by immunostaining. Sections were collected from E12.5 Ret-EGFP embryos in which both Ret expression and intrinsic neurons were labeled by GFP. *Arrows* indicate neurons in the esophagus. *Arrowheads* indicate neurons in the trachea and bronchi. GFRα1 is expressed by intrinsic neurons in both the esophagus and trachea (*a–d*), whereas GFRα2 is detected in intrinsic neurons in the trachea, but not in the esophagus (*e–h*). *Scale bars*, 100 μm. eso, esophagus; tra, trachea; bro, bronchi.

the other factors, affected the intrinsic neurogenesis of the respiratory explant cultures (Figure 7). In E10.5 + 3 cultures, NRTN dramatically increased the number of intrinsic neurons and induced their migration into more distal lung, compared with BSA control, NGF-treated, and FGF2-treated cultures, in which the intrinsic neurons clustered in the trachea and primary bronchi, and only a very low number of neurons was found in the lungs (Figure 7A). In contrast, NRTN had little effect on the migration of intrinsic neurons into the distal lung in E11.5 + 3 cultures (Figure 7A). Instead, NRTN increased TuJ1 immunoreactivity, as compared between control and NRTN-treated explants by whole-mount immunostaining (Figure 7A) and immunostaining of sections collected at four different levels of explants (Figure 7B). Quantification of the TuJ1-immunoreactive area on sections showed a $\sim$50% increase by NRTN, which is likely caused by the NRTN-induced neurite outgrowth of intrinsic neurons. Therefore, NRTN regulates intrinsic neurogenesis of the respiratory tract and exhibits differential effects on the migration, proliferation, and neurite outgrowth of intrinsic neurons at E10.5 and E11.5.

DISCUSSION

Our study investigated the cell origin and developmental signals of neurogenesis in the developing mammalian respiratory tract. We show that intrinsic neurons are distributed asymmetrically in the trachea and primary bronchi, and that the number of neurons decreases along the proximal–distal axis of the pulmonary tree. Such a discrete anatomic distribution of intrinsic neurons likely correlates with the localization of target tissues of innervation, such as smooth muscle cells in the trachea and airways, and neuroendocrine cells, which are more abundant in the proximal lung (34–36). However, according to a recent study, vagal denervation disrupts a large majority of nerve fibers that innervate neuroendocrine cells, suggesting that intrinsic neurons contribute minimally to neuroendocrine innervation (37).

We showed that Ret signaling is required for the formation of intrinsic neurons in the trachea and major bronchi (Figure 5), although the GDNF family ligands that signal through Ret remain to be identified. Our *in situ* analysis showed that the trachea expresses NRTN, but not GDNF (Figure 4). However, NRTN deficiency alone has no effect on the number of intrinsic neurons. Therefore, NRTN, artemin, and persephin likely have redundant roles as chemoattractants for the migration of neural crest cells into the ventral foregut and along the branches of the respiratory tree, and induce the proliferation and differentiation of neural crest progenitors to form resident neurons and glial cells. NRTN consistently induced the migration, proliferation, and neurite outgrowth of intrinsic neurons in our embryonic respiratory explant cultures (Figure 7).

Interestingly, NRTN induces the migration and proliferation of neurons into the distal lung in E10.5 cultures, whereas it mostly promotes neurite outgrowth in E11.5 cultures. The failure of NRTN to induce neural migration into the distal lung in E11.5 cultures is unlikely to be caused by a lack of motile nature of neural crest cells, because they are migratory at this stage, as shown by our characterization of migratory kinetics (Figure 3) and by previous studies (12, 33). In addition, neurons at E12 were induced to migrate and extend their neurites outside of the primary bronchi toward GDNF-impregnated agarose beads (25). Thus, neural crest cells and neuronal progenitors in E11.5 respiratory explants possess a migratory capacity in response to GDNF and NRTN. However, maturation of the lung may lead to the expression of neural repellants to prevent neural migration and neurite outgrowth into the distal lung. For example, Semaphorin 3A, which is enriched in the distal lung and whose mRNA expression peaks at E12.5 (the earliest time point examined) (41), may prevent the migration of neural crest cells into the lung. In addition, Sonic Hedgehog

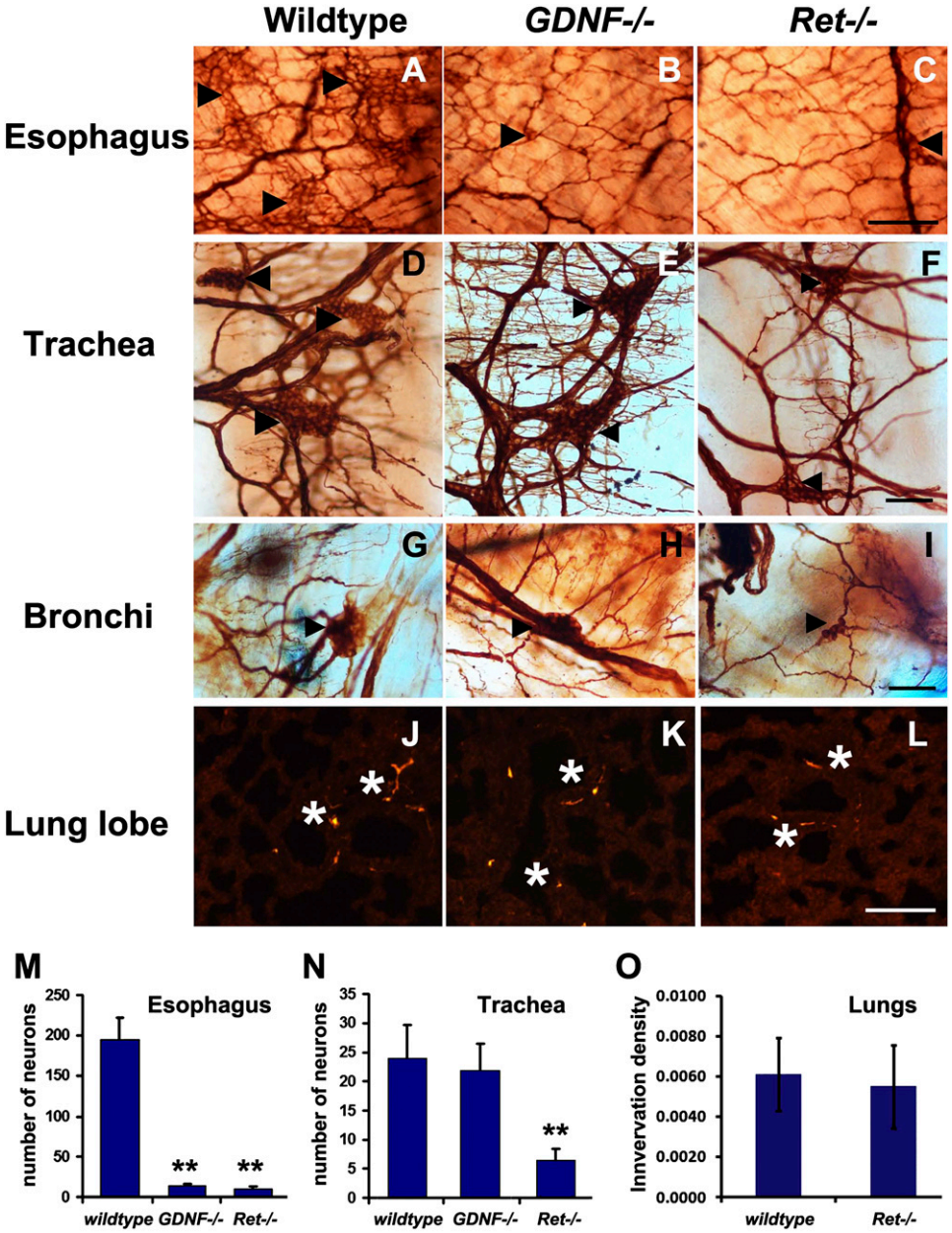

Figure 5. Formation of intrinsic neurons in the respiratory tract requires Ret signaling. Immunostaining using TuJ1 antibody identified neural cell bodies and nerves in the esophagus and lung of E18.5 embryos. *Arrowheads* indicate cell bodies of neurons. (*A–C*) Esophageal neurons are diminished in GDNF$^{-/-}$ and Ret$^{-/-}$ embryos. (*D–I*) Comparable numbers of intrinsic neurons were evident in the trachea of GDNF$^{-/-}$ embryos and controls, whereas the number in the trachea and bronchi was diminished in Ret$^{-/-}$ embryos. (*J–L*) Neural fibers that innervate the distal lung are largely unaffected in GDNF$^{-/-}$ and Ret$^{-/-}$ embryos. Neural fibers are marked by *asterisks*. (*M*) The number of intrinsic neurons on one side of a 1-mm longitudinal segment of the esophagus was counted in wild-type, GDNF$^{-/-}$, and Ret$^{-/-}$ embryos. (*N*) The number of neurons in three serial sections of the trachea was counted after immunostaining with TuJ1 antibody. Data shown in *M* and *N* represent the averages and standard deviations from a minimum of four embryos. (*O*) The innervation density of wild-type and Ret$^{-/-}$ lungs was measured and compared. Data represent the averages and standard deviations from a minimum of nine images from three embryos. *Scale bars*, 50 μm. $^{**}P <$ 0.01.

was shown to counteract the activity of GDNF on neural crest cells in the developing gut (42), and may have a similar inhibitory effect on NRTN-induced neural migration in the respiratory tract. Together, a combination of localized chemoattractants and repellants whose expression is dynamically regulated controls intrinsic neurogenesis in the developing respiratory tract.

The comparison of intrinsic neurogenesis between the trachea and esophagus has identified both similarities and differences. The intrinsic neurons in both organs share a neural crest cell origin and depend on Ret signaling (22, 43). However, the distribution of intrinsic neurons within these two organs is different, likely dictated by structural and cell-type divergence. In addition, essential GDNF family ligands for intrinsic neurogenesis are different in the trachea and esophagus. In contrast to the esophagus, in which intrinsic neurons are dependent on GDNF, the formation of intrinsic neurons in the trachea does not require GDNF. This difference results from differential GDNF family receptor expression by intrinsic neurons as well as the differential expression of GDNF family members. We

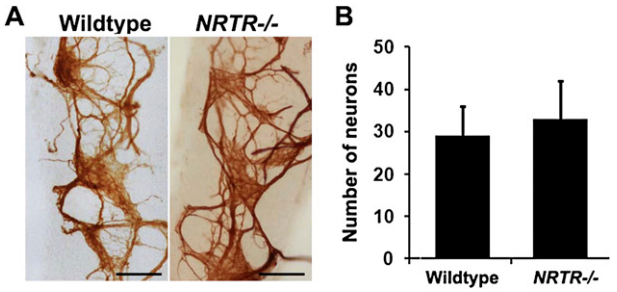

Figure 6. NRTN deficiency alone has no effect on intrinsic neurons in the trachea. Whole-mount TuJ1 immunolabeling was performed with dissected trachea from E13.5 embryos. (*A*) NRTN$^{-/-}$ embryos exhibit a number and size of neural ganglia along the dorsal side of the trachea comparable to those of wild-type embryos. (*B*) The number of TuJ1^{+} neurons in each ganglion of wild-type and NRTN$^{-/-}$ trachea was counted. Data represent the average and standard deviation from four embryos. *Scale bars*, 100 μm.

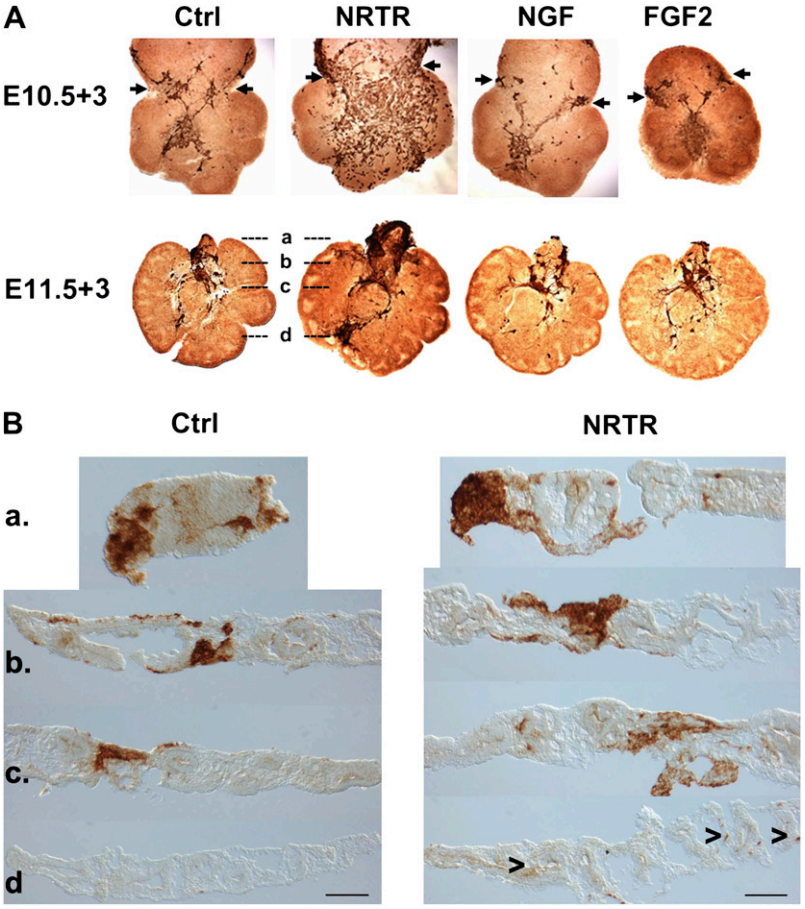

Figure 7. NRTN affects intrinsic neurons in explant cultures. E10.5 and E11.5 lung organ cultures were treated with BSA control, NRTN, NGF, and FGF2 at 50 ng/ml for 3 days (+3) before immunostaining with TuJ1 antibody to label neurons. (*A*) NRTN increased both the migration and number of intrinsic neurons in E10.5 explant cultures, compared with control and other signals. *Arrows* mark the connection between the trachea and the lungs in E10.5 + 3 day cultures. (*B*) In contrast, in E11.5 + 3 explants, NRTN had little effect on the migration of intrinsic neurons, but elicited neurite outgrowth, as shown by an increase of TuJ1 immunoreactivity by ~50% on cross sections at four levels of treated explants *(a–d)*. *Arrowheads* indicate neurites in the distal lung. A minimum of 15 explants from three individual experiments was examined.

show that tracheal neurons express both GFRα1 and GFRα2, whereas neurons in the esophagus only express GFRα1 (43). GFRα1 can bind to GDNF, NRTN, and artemin (23, 24). Therefore, the elimination of NRTN and artemin would likely be required to disrupt intrinsic neurogenesis in the respiratory tract. In contrast, GDNF deficiency alone is able to diminish intrinsic neurons in the esophagus. This indicates that endogenous NRTN is not sufficient to compensate for the loss of GDNF in the esophagus, because esophageal intrinsic neurons do not express GFRα2, the high-affinity NRTN receptor. However, exogenous neurturin was shown to induce the migration and neurite outgrowth of intrinsic neurons in esophageal explants (22). This effect is likely mediated by the low-affinity binding of neurturin to GFRα1 when applied at high concentrations in culture.

Disruption of intrinsic neurons in the respiratory tract in $Ret^{-/-}$ embryos has no effect on extrinsic innervations (Figure 5), indicating that extrinsic innervation is controlled by different signals than is intrinsic innervation. Candidate signals for extrinsic innervation include neurotrophin family members (44, 45). Mice deficient in neurotrophin family members brain-derived neurotrophic factor and neurotrophin 4 have a diminished number of neurons in the nodose ganglia that extend their axons to form the vagus nerve (46). These neurotrophins, including NGF, had no effect on intrinsic neurons in our explant cultures (Figure 7A, and data not shown), further establishing that distinct mechanisms control the extrinsic and intrinsic neurogenesis of the respiratory tract.

Our study establishes the neural crest cell origin, and identifies essential Ret signaling, in the development of intrinsic neurons of the respiratory tract. The functions of these intrinsic neurons and their communication with extrinsic neurons in development, homeostasis, and pulmonary disease remain to be characterized. Future studies will require the identification of molecular markers that distinguish intrinsic from extrinsic neurons, along with analyses of the physiologic changes that result from the uncoupling of intrinsic and extrinsic innervations (47).

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References

1. Loosli CG, Hung KS. Development of pulmonary innervation. In: Lenfant C, Hodson WA, editors. Development of the lung. Volume 6. New York: Dekker; 1977. pp. 269–306.
2. Sparrow MP, Weichselbaum M, McCray PB. Development of the innervation and airway smooth muscle in human fetal lung. *Am J Respir Cell Mol Biol* 1999;20:550–560.
3. Weichselbaum M, Sparrow MP. A confocal microscopic study of the formation of ganglia in the airways of fetal pig lung. *Am J Respir Cell Mol Biol* 1999;21:607–620.
4. Tollet J, Everett AW, Sparrow MP. Spatial and temporal distribution of nerves, ganglia, and smooth muscle during the early pseudoglandular stage of fetal mouse lung development. *Dev Dyn* 2001; 221:48–60.
5. Vaccaro R, Salvi EP, Renda T. Early development of chick embryo respiratory nervous system: an immunohistochemical study. *Anat Embryol (Berl)* 2006;211:345–354.
6. Burns AJ, Thapar N, Barlow AJ. Development of neural crest–derived intrinsic innervation of the human lung. *Am J Respir Cell Mol Biol* 2008;38:269–275.

7. Belvisi MG. Overview of the innervation of the lung. *Curr Opin Pharmacol* 2002;2:211–215.
8. Carr MJ, Undem BJ. Bronchopulmonary afferent nerves. *Respirology* 2003;8:291–301.
9. Widdicombe J. Lung afferent activity: implications for respiratory sensation. *Respir Physiol Neurobiol* 2009;167:2–8.
10. Cardoso WV, Lü J. Regulation of early lung morphogenesis: questions, facts and controversies. *Development* 2006;133:1611–1624.
11. Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E. Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. *Development* 2002;129:5151–5160.
12. Tollet J, Everett AW, Sparrow MP. Migration of neural crest cells into and within the developing lung. *Int J Dev Neurosci* 2002;19:707.
13. Dey R, Hung KS. Development of innervation in the lung. In: McDonald J, editor. Lung growth and development. Volume 100. New York: Dekker; 1997. pp. 244–265.
14. Yntema CL, Hammond WS. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. *J Comp Neurol* 1954;101:515–541.
15. Le Douarin NM, Teillet MA. The migration of neural crest cells to the wall of the digestive tract in avian embryo. *J Embryol Exp Morphol* 1973;30:31–48.
16. Burns AJ, Le Douarin NM. The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. *Development* 1998;125: 4335–4347.
17. Burns AJ, Delalande J. Neural crest cell origin for intrinsic ganglia of the developing chicken lung. *Dev Biol* 2005;277:63–79.
18. Baker CV, Bronner-Fraser M. Vertebrate cranial placodes I. Embryonic induction. *Dev Biol* 2001;232:1–61.
19. Moore MW, Klein RD, Fariñas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A. Renal and neuronal abnormalities in mice lacking GDNF. *Nature* 1996;382:76–79.
20. Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, *et al.* Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* 1996;382:73–76.
21. Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, Barbacid M. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* 1996;382:70–73.
22. Yan H, Bergner AJ, Enomoto H, Milbrandt J, Newgreen DF, Young HM. Neural cells in the esophagus respond to glial cell line–derived neurotrophic factor and neurturin, and are RET-dependent. *Dev Biol* 2004;272:118–133.
23. Baloh RH, Enomoto H, Johnson EM Jr, Milbrandt J. The GDNF family ligands and receptors—implications for neural development. *Curr Opin Neurobiol* 2000;10:103–110.
24. Enomoto H. Regulation of neural development by glial cell line–derived neurotrophic factor family ligands. *Anat Sci Int* 2005;80:42–52.
25. Tollet J, Everett AW, Sparrow MP. Development of neural tissue and airway smooth muscle in fetal mouse lung explants. A role for glial-derived neurotrophic factor in lung innervation. *Am J Respir Cell Mol Biol* 2002;26:420–429.
26. Danielian PS, Echelard Y, Vassileva G, McMahon AP. A 5.5-kb enhancer is both necessary and sufficient for regulation of Wnt-1 transcription in vivo. *Dev Biol* 1998;192:300–309.
27. Heuckeroth RO, Lampe PA, Johnson EM, Milbrandt J. Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. *Dev Biol* 1998;200:116–129.
28. Jain S, Golden JP, Wozniak D, Pehek E, Johnson EM Jr, Milbrandt J. RET signaling is dispensable for maintenance of midbrain dopaminergic neurons in adult mice. *J Neurosci* 2006;26:11230–11238.
29. Park WY, Miranda B, Lebeche D, Hashimoto G, Cardoso WV. FGF-10 is a chemotactic factor for distal epithelial buds during lung development. *Dev Biol* 1998;201:125–134.
30. Ai X, Kitazawa T, Do AT, Kusche-Gullberg M, Labosky PA, Emerson CP Jr. Sulf1 and Sulf2 regulate heparan sulfate–dependent GDNF signaling for esophageal innervation. *Development* 2007;134:3327–3338.
31. Golden JP, DeMaro JA, Osborne PA, Milbrandt J, Johnson EM Jr. Expression of neurturin, GDNF, and GDNF family–receptor mRNA in the developing and mature mouse. *Exp Neurol* 1999;158:504–528.
32. Shepherd IT, Raper J. Collapsin-1/semaphorin D is a repellent for chick ganglion of Remak axons. *Dev Biol* 1999;212:42–53.
33. Young HM, Bergner AJ, Anderson RB, Enomoto H, Milbrandt J, Newgreen DF, Whitington PM. Dynamics of neural crest–derived cell migration in the embryonic mouse gut. *Dev Biol* 2004;270:455–473.
34. Sparrow MP, Lamb JP. Ontogeny of airway smooth muscle: structure, innervation, myogenesis and function in the fetal lung. *Respir Physiol Neurobiol* 2003;137:361–372.
35. Adriaensen D, Timmermans JP, Brouns I, Berthoud HR, Neuhuber WL, Scheuermann DW. Pulmonary intraepithelial vagal nodose afferent nerve terminals are confined to neuroepithelial bodies: an anterograde tracing and confocal microscopy study in adult rats. *Cell Tissue Res* 1998;293:395–405.
36. Pan J, Yeger H, Cutz E. Innervation of pulmonary neuroendocrine cells and neuroepithelial bodies in developing rabbit lung. *J Histochem Cytochem* 2004;52:379–389.
37. Brouns I, Oztay F, Pintelon I, Proost ID, Lembrechts R, Timmermans JP, Adriaensen D. Neurochemical pattern of the complex innervations of neuroepithelial bodies in mouse lungs. *Histochem Cell Biol* 2009;131:55–74.
38. Gianino S, Grider JR, Cresswell J, Enomoto H, Heuckeroth RO. GDNF availability determines enteric neuron number by controlling precursor proliferation. *Development* 2003;130:2187–2198.
39. Leitner ML, Wang LH, Osborne PA, Golden JP, Milbrandt J, Johnson EM Jr. Expression and function of GDNF family ligands and receptors in the carotid body. *Exp Neurol* 2005;191:S68–S79.
40. Paratcha G, Ledda F, Ibáñez CF. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell* 2003;113:867–879.
41. Kagoshima M, Ito T. Diverse gene expression and function of semaphorins in developing lung: positive and negative regulatory roles of semaphorins in lung branching morphogenesis. *Genes Cells* 2001;6:559–571.
42. Fu M, Lui VC, Sham MH, Pachnis V, Tam PK. Sonic Hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut. *J Cell Biol* 2004;166:673–684.
43. Young HM, Hearn CJ, Farlie PG, Canty AJ, Thomas PQ, Newgreen DF. GDNF is a chemoattractant for enteric neural cells. *Dev Biol* 2001;229:503–516.
44. Hoyle GW, Graham RM, Finkelstein JB, Nguyen KP, Gozal D, Friedman M. Hyperinnervation of the airways in transgenic mice overexpressing nerve growth factor. *Am J Respir Cell Mol Biol* 1998; 18:149–157.
45. García-Suárez O, Pérez-Pinera P, Laurà R, Germana A, Esteban I, Cabo R, Silos-Santiago I, Cobo JL, Vega JA. TrkB is necessary for the normal development of the lung. *Respir Physiol Neurobiol* 2009;167:281–291.
46. Erickson JT, Conover JC, Borday V, Champagnat J, Barbacid M, Yancopoulos G, Katz DM. Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. *J Neurosci* 1996;16:5361–5371.
47. Proost ID, Pintelon I, Wilkinson WJ, Goethals S, Brouns I, Nassauw LV, Riccardi D, Timmermans JP, Kemp PJ, Adriaensen D. Purinergic signaling in the pulmonary neuroepithelial body microenvironment unraveled by live cell imaging. *FASEB J* 2009;23:1153–1160.