Supporting Information

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SI Materials and Methods

Histology. Whole chicken and mice guts were fixed for 1 h in 4% paraformaldehyde (in PBS), permeabilized in 1% Triton (in PBS) for 30 min and incubated over night at 4 °C with primary antibodies in blocking solution (1% DMSO, 0.1% Triton in PBS, 20% FCS). After extensive washes in blocking solution, whole guts were incubated over night at 4 °C, with secondary antibodies in blocking solution. After several washes in 0.1% Triton in PBS, samples were cleared in Glycerol 80% in PBS.

Whole chicken chimeras were cleared using ScaleA2 solution as previously described (48). Samples were imaged using a SP8 or SP5 confocal microscope (Leica). Three-dimensional reconstructions and videos were obtained using the IMARIS imaging software. In situ hybridization and immunochemistry have been described previously (44). Immunofluorescence on cryostat sections was performed as previously described (18). Whole-mount immunofluorescent staining using the 3DISCO method was performed as previously described (31).

Image Generation and Processing. Samples were imaged using a SP8 or SP5 confocal microscope (Leica). Whole-embryo images were obtained by tile-scanning with a 20×-immersion objective and automatic stitching on a Leica SP8 confocal. Three-dimensional reconstructions and videos were obtained using the IMARIS imaging software. Note that the rendering process in IMARIS sometimes leads to the appearance of horizontal or vertical splices in the final whole-embryo images, which are processing artifacts. GFP/TH overlap panels in Fig. S6 are binary masks generated using the ImageJ software. Each .lif confocal file was first converted into .tiff, then run through a customized Macro involving the following processing steps: (*i*) background subtraction of the whole image; (*ii*) "despeckling"; (*iii*) "autothresholding"; (*iv*) binary mask generation (selection of the area of interest exclusively, i.e. the ganglion area); and (*v*) image calculator: calculates final binary image of the colocalized GFP/TH surfaces.

Antibodies and Probes. Antibodies and probes were as follows:

α-2H3 (NF), mouse, 1:500, Hybridoma Bank (#2H3);

α-bIII Tubulin (Tuj1), mouse, 1:500, Covance (#MMS-435P);

α-Phox2b, rabbit, 1:500 (20);

α-Phox2b, guinea pig, 1:500 (48);

α-Sox10, goat, 1:250, Santa Cruz (#SC-17342);

α-βgal, rabbit, 1:400, Cappel (#55976);

α-HuC/D mouse, 1:200, Invitrogen (MABN153);

α-GFP, chicken, 1:500, Aveslab (#GFP-1020);

α-HNK-1, mouse, 1:50, Hybridoma Bank (#3H5);

α-TH, rabbit, 1:800, Merck (#AB152);

α-rabbit Cy3, 1:500, Jackson Immunoresearch Laboratories (#711–165-152);

α-rabbit A488, 1:500, Jackson Immunoresearch Laboratories (#711–545-152);

α-goat Cy3, 1:500, Jackson Immunoresearch Laboratories (#705–166-147);

α-goat A647, 1:500, Jackson Immunoresearch Laboratories (#705–606-147);

α-mouse Cy3, 1:500, Jackson Immunoresearch Laboratories (#715–165-150);

α-mouse A488, 1:500, Invitrogen (#A-21202);

α-guinea pig Cy3, 1:500, Invitrogen (#A-11073);

α-chicken A488, 1:500, Jackson Immunoresearch Laboratories (#103–545-155).

Immunohistochemical reactions were processed with the Vectastain Elite ABC kits (PK-6101 and PK-6012; Vector Laboratories) as indicated by the manufacturer and color development was performed using DAB.

The probes for Cadherin19 and PLP-1 (cDNA from Source Bioscience, clones #IRCKp5014H0217Q and #IRAVp968G0365D, respectively) were synthesized following the distributor's information. The other probe used was *Peripherin* (obtained from M. M. Portier, Collège de France, Paris).

Transgenic Mouse Lines. Mouse lines used were as follows:

Phox2b::Cre(50): BAC transgenic line expressing Cre under the control of the Phox2b promoter.

$Phox2b^{LacZ/+}$ (17): Knockin line expressing the reporter gene *LacZ* from the second exon of the *Phox2b* locus, which is disrupted and lead to a null phenotype in $Phox2b^{LacZ/LacZ}$ embryos.

$Ret^{fl\text{-}CFP/+}$ (51): Knockin line comprising floxed human *Ret9* cDNA and CFP reporter in the first exon of the mouse *Ret* locus. Activation of Cre recombinase results in the removal of floxed *Ret9*, generating a CFP-knockin (*Ret*-null) allele.

Wnt1::Cre(52): Transgenic line expressing Cre under the control of the 3′ enhancer of Wnt1.

Pgk::Cre (53): Transgenic line expressing Cre in the germ line.

$Phox2a^{ASIC2a}$ (*P2aASIC*) (18): Knockin line expressing the toxic G430 mutant of the ASIC2a cation channel upon Cre recombination under the control of *Phox2a* promoter.

$ErbB3^{f}$ ($ErbB3^{Lox/Lox}$) (54): Activation of Cre recombinase results in the removal of floxed *ERBB3*, generating a *ErbB3*-null allele, which lacks the same coding sequences as the previously described ErbB3Δallele (55).

$Nrg1^{tm3Cbm}$ ($Nrg1^{Lox/Lox}$) (56): A floxed allele of the *Nrg-1* gene containing loxP sites flanking exons 7 (containing sequence alterations), 8 and 9 was generated. Activation of Cre recombinase results in the removal of floxed *Nrg1*, generating a *Nrg-1* null allele.

$Neuregulin^{\Delta EGF\text{-}lacZ}$ ($Nrg^{LacZ/+}$) (57, 58): Knock in line expressing the reporter gene *LacZ* from the exon of the *Neuregulin1* locus.

All animal studies were done in accordance with the guidelines issued by the French Ministry of Agriculture and have been approved by the Direction Départementale des Services Vétérinaires de Paris.

Statistical Analyses. For esophageal neuronal counting, transverse sections of whole E13.5 embryos where immunostained for Phox2b and one section of three was selected. The total number of $Phox2b^+$ cells in the esophageal walls was recorded using FIJI Cell Counter.

For postgastric neuronal countings, whole E13.5 guts were dissected and immunostained for Phox2b. The surface occupied by Phox2b$^+$ enteric neurons was measured in three regions up to the cecum (at equal distances from the stomach for both wild-type and mutant embryos). First, stacked confocal images were transformed into Binary images, with a threshold restricted to the Phox2b$^+$ cell surfaces. The total surface was then measured automatically by the FIJI Measuring tool applied to a specific perimeter of the total image (this perimeter was created to avoid areas of the image empty of gut tissue). Then the relation between total surface area/perimeter per region was recorded.

The similarity of variances between each group of data were tested using the F test. Statistical analysis was performed using unpaired two-tailed t test. Results are expressed as mean ±SEM %/wild-type.

Fig. S1. Presence of enteric precursors associated with vagal nerve fibers in *Ret* and *Phox2b* mutants. (*A*) Lateral views of whole-mount E10.5 embryos of the indicated genotypes stained with the indicated markers. A yellow circle indicates the position of the stomach primordium. (*B*) Close-ups of the esophageal region stained with Sox10. (*C*) Close-ups on the esophageal region stained with Neurofilament. In *Wnt1::Cre;Ret$^{CFP/CFP}$* and *Phox2b$^{LacZ/LacZ}$* mice Sox10$^+$ (thus neural crest-derived) cells are mostly confined to the fibers of the vagus nerve, unlike in the wild-type. (*D*, *Upper*) Transverse sections through the gut at the positions indicated in *A*, stained for the indicated markers. (*Lower*) Higher magnifications of groups of cells costained for Sox10 and DAPI and either the reporter Ret-CFP or Phox2b-LacZ. NCC are absent from the postgastric ENS in both *Wnt1::Cre;Ret$^{CFP/CFP}$* and *Phox2b$^{LacZ/LacZ}$* mutants (*D*, sections *d′* and *f′*). C1: first cervical spinal nerve. DRG, dorsal root ganglia; Oe, esophagus; X, vagus nerve; XII, hypoglossal nerve. (Scale bars: *A*–*C*, 500 μm; *D* 50 μm.)

Fig. S2. Vagal-associated enteric precursors share their molecular signature with Schwann cell precursors. (*A*–*C*) Sagittal sections through the foregut of an E11.5 wild-type embryo, stained with the indicated antibodies and probes. Phox2b$^+$ enteric precursors alongside the esophagus express the Schwann cell precursor markers *PLP-1* (*B*) and *Cadh19* (*C*). *Insets* show higher magnifications of double-positive enteric precursors scattered in the esophageal wall. Oe, esophagus; St, stomach. (Scale bars, 100 μm.)

Fig. S3. Axonal Nrg1 is required for the migration of parasympathetic precursors along cranial nerves. (*A* and *B*) Lateral views of the head of whole-mount embryos at E11.5 of the indicated genotypes, immunostained with the indicated markers, showing the facial nerve (VII), Jacobson's nerve (JN), the corda tympani (CT), and the greater petrosal nerve (GSPN), covered with $Sox10^{+}$ neural crest-derived cells (some of them coexpressing Phox2b) in the wild-type (*A*), but naked in the *Nrg1* mutant (white arrowheads in *B*). (*C* and *D*) Parasagittal sections at E13.5 showing parasympathetic ganglia associated with these nerves in the wild-type embryo (*C*)—the otic ganglion (Og), the submandibular ganglion (Smg), and the Sphenopalatine ganglion (Spg)—but absent in the *Nrg1* mutant (asterisks in *D*). Gg, geniculate ganglion; V, trigeminal ganglion. (Scale bars: *A* and *B*, 200 μm; *C* and *D*, 100 μm.)

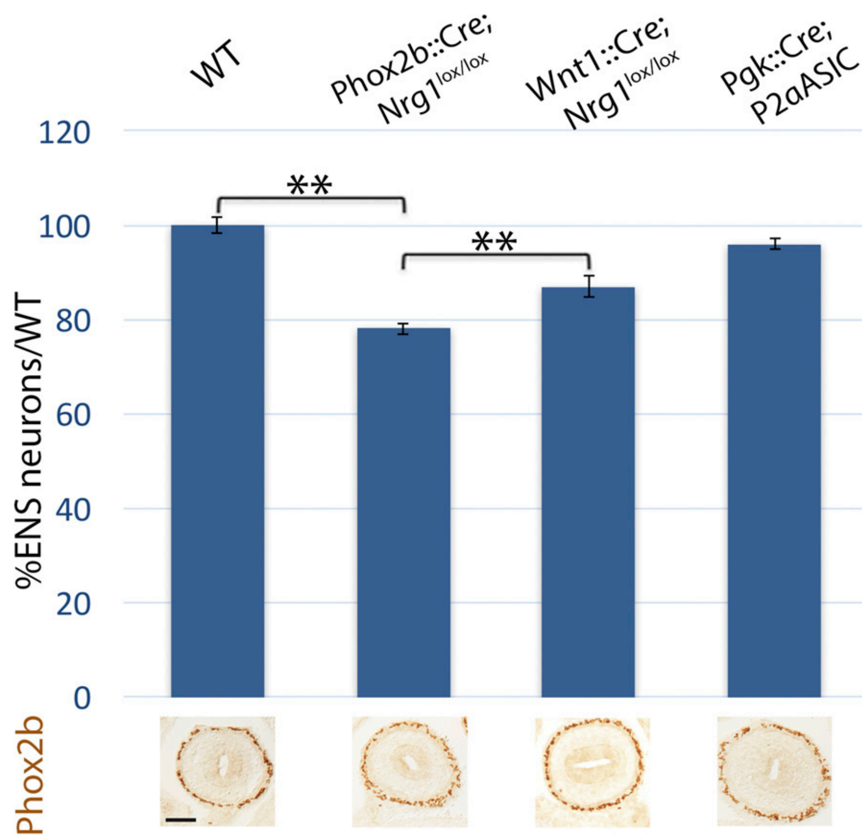
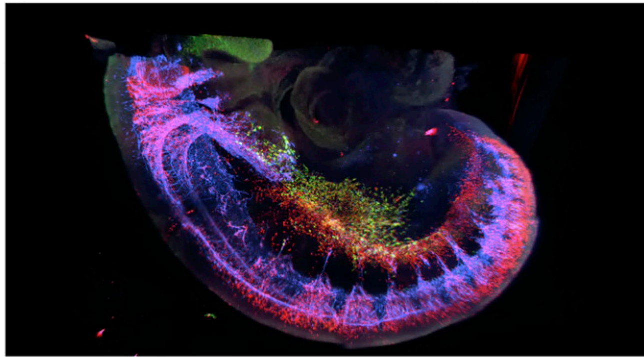


Fig. S4. Consequences on the postgastric ENS of the deletion of the vagus nerve or abrogation of its expression of *Nrg1*. (*Upper*) Quantitative analysis (see *Methods*) of the surface occupied by Phox2b^{+} ENS neurons in the indicated mutant backgrounds. *Phox2b::Cre;Nrg1$^{lox/lox}$* embryos have a mild atrophy of the postgastric ENS (79 ± 1.1%/wild-type, $P = 0.0047$; $n = 9$; and 90 ± 2%/*Wnt1::CreNrg1$^{lox/lox}$*, $P = 0.0040$; $n = 5$), due to loss of *Nrg1* expression by the vagus nerve, and not by enteric precursors, as shown by the lack of phenotype in *Wnt1::Cre;Nrg1$^{lox/lox}$* (87 ± 2.3%/wild-type, $P = 0.237$; $n = 5$). *Pgk::Cre;P2a^{ASIC}* have no phenotype (93 ± 1.2%/wild-type, $P = 0.283$; $n = 4$), presumably because genetic deletion of the nerve is less efficient or occurs later than abrogation of its expression of *Nrg1* (and in line with a weaker phenotype in the esophagus) (Fig. 1*B*). (*Lower*) Representative transverse sections through the postgastric ENS of the indicated mutant backgrounds, immunostained for Phox2b. (Scale bar: 50 μm.) Error bars indicate SEM; $^{**}P < 0.005$.

Fig. S5. Esophageal neuronal precursors do not express *Nrg1*. (*A*) Transverse sections through the esophagus of E11.5 *Nrg1*$^{LacZ/+}$ embryos, immunostained for Sox10 (which labels all enteric precursors at this stage), NF (neurofilament, which labels fibers of the vagus nerve), and β-galactosidase (which labels cells that express the *Nrg1* reporter LacZ). Vagus nerve fibers are LacZ^{+}, whereas enteric precursors are LacZ^{-}. (*Insets*) Close up (3× zoom of a cell in the lower magnification image) of a representative enteric precursor. (*B*) Transverse section through an E13.5 embryo at the level of a dorsal root ganglion (*Left*), the esophagus (*Center*), and an intestinal loop (*Right*). Dorsal root ganglionic cells serve as a LacZ^{+} control, Sox10^{+}/Phox2b^{+} esophageal and enteric precursors are LacZ^{-}. (Scale bars: *A*, 50 μm; *B*, 100μm.)

Fig. S6. The superior cervical ganglion receives contribution of the neural crest from level S3 to S10. Sections through the rostral pole of cervical sympathetic chain of chicken embryos at E5.5, after they received an isotopic graft of the neural tube from a GFP-transgenic donor at stage 10, at the indicated levels, stained with the indicated antibodies. Two sections from two embryos are shown for each type of graft. GFP/TH overlap panels show higher-magnification (*Insets*) of a GFP^{+}/TH^{+} neuron or group of neurons (indicated by white arrowheads, *Upper*, corresponding to yellow arrowheads, *Lower*). S1–2 grafts do not contribute any cell to the SCG, S3 only glia, S4 glia, and an occasional neuron, S5–S10 many neurons and glia. (Scale bar, 50 μm.)

Fig. S7. Atrophy of the sympathetic chain in *Wnt1Cre;ErbB3*$^{lox/lox}$ mutants. Whole-mount views of E10 embryos of the indicated genotypes, immunostained with the indicated antibodies. White arrowhead: sympathetic chain (SC); red arrowhead: cervical contingent of sympatho-enteric cells; X, vagus nerve. (Scale bar, 500 μm.)



Movie S1. Two distinct streams of cells migrate to the esophagus in an E10 mouse embryo. Three-dimensional reconstruction of an E10 *Wnt1::Cre ;Ret*$^{CFP/+}$ embryo. The vagus nerve (NF^{+}, blue) projects dorsolaterally from the hindbrain to the foregut accompanied by neural crest cells (Sox10^{+}, red). Ahead of the vagus nerve and medially to it, a swarm of neural crest cells emerge from the incipient sympathetic chain toward the foregut. Enteric precursors at the level of the future esophagus express the reporter CFP (green) from the *Ret* promoter. The images were captured with a 20× objective on a SP8 confocal microscope.

Movie S1