An autocrine Wnt5a-Ror signaling loop mediates sympathetic target innervation

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ABSTRACT

During nervous system development, axon branching at nerve terminals is an essential step in the formation of functional connections between neurons and target cells. It is known that target tissues exert control of terminal arborization through secretion of trophic factors. However, whether the in-growing axons themselves produce diffusible cues to instruct target innervation remains unclear. Here, we use conditional mutant mice to show that Wnt5a derived from sympathetic neurons is required for their target innervation in vivo. Conditional deletion of Wnt5a resulted in specific deficits in the extension and arborization of sympathetic fibers in their final target fields, while no defects were observed in the overall tissue patterning, proliferation, migration or differentiation of neuronal progenitors. Using compartmentalized neuronal cultures, we further demonstrate that the Ror receptor tyrosine kinases are required locally in sympathetic axons to mediate Wnt5a-dependent branching. Thus, our study suggests an autocrine Wnt5a-Ror signaling pathway that directs sympathetic axon branching during target innervation.

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Introduction

A major challenge to understanding nervous system development has been to determine how a limited number of signaling pathways can establish a complex nervous system. In the last decade, a remarkable finding in developmental neurobiology is that many classical morphogens that direct cell fate specification and embryonic tissue patterning can be "recycled" to regulate later events in neuronal connectivity (Zou and Lyuksyutova, 2007). Wnts are secreted cysteine-rich glycoproteins with 19 vertebrate members that influence early embryonic patterning through regulation of cell fate decisions, proliferation, cell polarity, movement and apoptosis (Veeman et al., 2003; Angers and Moon, 2009). However, recent evidence, based on studies in neuronal culture systems and the analyses of conventional Wnt knockout mice, have identified surprising roles for Wnts in post-mitotic neurons in axon pathfinding, dendritogenesis and synapse formation (Salinas and Zou, 2008; Budnik and Salinas, 2011). Despite emerging information regarding Wnt functions in vertebrate neural circuit assembly, fundamental questions have remained regarding tissue-specific and temporal roles of Wnt

signaling in the formation and maintenance of neuronal circuits in vivo. Some of these questions regarding the spatial and temporal aspects of Wnt signaling are best addressed by the use of conditional or inducible genetic mouse models.

During nervous system development, many axons undergo reiterative branch formation at their terminals to generate large tree-like arbors that completely cover their final target fields (Gibson and Ma, 2011). This feature of developing axons is a critical step in synapse formation and the establishment of functional neuronal circuits. It has been shown previously that target-derived growth factors instruct terminal arborization (Kennedy and Tessier-Lavigne, 1995). A notable example is found during the innervation of peripheral tissues by post-ganglionic sympathetic neurons. Nerve growth factor (NGF) is a target-derived signal that promotes terminal arborization once sympathetic axons have reached their final destinations (Glebova and Ginty, 2005). During target innervation, spatial and temporal mechanisms exist to ensure that terminal arborization into target fields is only initiated once axons have reached their final destinations (Gibson and Ma, 2011). One such regulatory mechanism would be to have the neurons produce a branching factor upon arrival at target tissues. We previously reported that Wnt5a, a member of the Wnt family of secreted proteins, is robustly expressed in sympathetic neurons during target innervation, and that Wnt5a induces axon branching in cultured sympathetic neurons (Bodmer et al., 2009). In addition, analyses of global Wnt5a knockout mice revealed reduced sympathetic innervation of target tissues (Bodmer et al., 2009). These

findings suggested that Wnt5a produced by sympathetic neurons acts in an autocrine manner to promote axon branching in target tissues.

To directly test the hypothesis that autocrine Wnt5a signaling in sympathetic axons regulates terminal arborization during target innervation *in vivo*, we generated conditional *Wnt5a* mutant mice to allow for selective deletion of Wnt5a in sympathetic neurons. We found that conditional *Wnt5a* ablation using neural crest-specific *Wnt1::Cre* or catecholaminergic neuron-specific *Tyrosine Hydroxylase* (*TH*)::*Cre* mice circumvented the gross tissue patterning abnormalities previously reported in the *Wnt5a*$^{-/-}$ mice (Yamaguchi et al., 1999). The loss of neuronal Wnt5a did not compromise early stages of peripheral nervous system development, but had a specific effect in perturbing sympathetic axon innervation of target fields. We further show that the Ror receptor tyrosine kinases are required locally in sympathetic axons to mediate the branching effects of Wnt5a, using compartmentalized neuronal cultures. Together, these results define an autocrine Wnt5a signaling loop within sympathetic neurons that directs terminal axon arborization during neural circuit assembly.

Materials and methods

Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. *Wnt5a*$^{+/-}$ (stock number: 004758) and *Wnt1::Cre* (stock number: 009107) mice were obtained from Jackson Laboratory. *TH::Cre* mice were a generous gift from Dr. Charles Gerfen (National Institute of Mental Health, Bethesda, MD). *Wnt1::Cre;Wnt5a*$^{+/-}$ males were mated with *Wnt5a*$^{fl/fl}$ females or *Wnt5a*$^{+/-}$ males mated with *Wnt1::Cre;Wnt5a*$^{fl/+}$ females to obtain *Wnt5a*$^{fl/+}$ off-spring. Consistent with previous reports of *TH::Cre*-mediated deletion in female germline cells (Lindeberg et al., 2004), we observed global deletion of *Wnt5a* when *TH::Cre*-carrying females were used to generate off-spring. Thus, *TH::Cre;Wnt5a*$^{fl/+}$ males were mated with *Wnt5a*$^{fl/fl}$ females to obtain *TH::Cre;Wnt5a*$^{fl/fl}$ off-spring.

Generation of floxed Wnt5a mice

Exon 2 of the *Wnt5a* gene on chromosome 14 was selected to be flanked by *loxP* sequences. The *loxP* sequences were inserted into intronic locations (285 bp upstream and 306 bp downstream of exon 2) to avoid splicing disruption. A 3.2 kb genomic sequence upstream and a 3 kb sequence downstream of the *loxP* insertion sites were used as homologous sequences for gene targeting in embryonic stem (ES) cells. The targeting vector also contained a *FRT*-flanked neomycin selection cassette constructed downstream of the *loxP*-flanked exon 2, and upstream of the 3 kb 3′ homologous sequence. 129/S6/SvEv mouse ES cells (MC1 line, Johns Hopkins University transgenic core) were electroporated with the targeting vector, and selected in media containing G418. PCR-based screening was used to identify ES clones with correct homologous recombination. Positive ES cells were injected into blastocysts, which were then implanted in a surrogate mother to generate chimeric mice (JHU transgenic core). Male chimeric mice were mated with C57BL/6 wild-type mice to obtain the heterozygous mice carrying the modified *Wnt5a* allele (*Wnt5a*$^{fl-neo/+}$ mice). *Wnt5a*$^{fl-neo/+}$ mice were then mated with mice expressing germline FLP recombinase to remove the *FRT*-flanked neomycin selection cassette.

In situ hybridization

In situ hybridization was performed as described previously with digoxigenin-labeled probes against *Wnt5a* exon 2 (Bodmer et al., 2009). The Wnt5a *in situ* hybridization probe is 250 bp long and spans part of the 5′ untranslated region (5′ UTR) to the end of exon 2 of *Wnt5a*. Fresh frozen E16.5 mouse tissues were embedded in OCT (Tissue-Tek) and serially sectioned (10 μm). Sections were post-fixed in 4% paraformaldehyde (PFA) in PBS, washed in PBS and acetylated in 0.25% acetic anhydride, 0.1 M triethanolamine and 0.2% HCl. After hybridization with DIG-labeled RNA probe (3 μg/ml) at 64 °C overnight, sections were washed with $0.2 \times$ SSC buffer at 65 °C, blocked with 1% normal goat serum/TBS and incubated with alkaline phosphatase (AP) labeled anti-DIG antibody (1:2000; Roche) overnight. Following TBS washes, the AP substrate, nitroblue tetrazolium/5-bromo-4-chloro-indolyl phosphate, was added. Sections were washed in PBS, fixed in 4% PFA and mounted in AquaMount (EMD Chemicals).

Quantitative real-time PCR (Q-PCR) analyses

Total RNA was prepared from dissected SCGs, salivary glands or heart using RNeasy mini columns (Qiagen). RNA was then reverse transcribed using a RETROscript kit (Ambion). Real-time qPCR was performed using a Maxima SYBR Green/Rox Q-PCR Master Mix (Thermo Scientific), in a 7300 Real time PCR System (Applied Biosystems). *Wnt5a* mRNA levels were measured by using primers targeting exon 2 (*Wnt5a*-F: 5′-CTCGGGTGGCGACTTCCTCTCCG-3′ and *Wnt5a*-R: 5′-CTATAACAACCTGGGCGAAGGAG-3′). GAPDH was used as a control (*GAPDH*-F: 5′-CCTGCACCACCAACTGCTTA-3′ and *GAPDH*-R: 5′-CCACGATGCCAAAGTTGTCA-3′). Each sample was analyzed in triplicate reactions. Fold change in *Wnt5a* transcript levels was calculated using the $2^{(-\Delta\Delta Ct)}$ method, normalizing to GAPDH transcript.

Immunohistochemical analyses

Embryos of various developmental stages were fixed in 4% PFA/PBS for 4 h, cryoprotected in 30% sucrose/PBS, frozen in OCT and serially sectioned (10 μm). For immunofluorescence, sections were permeabilized in 1% Triton X-100/PBS, and blocked in 5% normal goat serum/0.5% Triton X-100/PBS. Sections were incubated with the corresponding primary antibodies overnight: mouse anti-TH (1:200, Sigma-Aldrich), rabbit anti-TH (1:200, Millipore), rabbit anti-Phox2b (1:200, a kind gift from JF Brunet), rabbit anti-cleaved caspase3 (1:200, Cell Signaling Technology), rabbit anti-TrkA (1:200, Millipore) and rabbit anti-brain lipid binding protein (BLBP) (1:200, Abcam). For Phox2b staining, sections were subjected to antigen retrieval by boiling sections for 15 min in 1 M sodium citrate containing 0.5% Tween-20. Following PBS washes, sections were incubated with appropriate secondary antibodies (1:200). Sections were washed in PBS and mounted in VectaShield (Vector Laboratories).

Whole-mount TH immunostaining to visualize sympathetic chains and axons was performed on E16.5 mouse embryos as described previously (Bodmer et al., 2009). Embryos were fixed in 4% PFA overnight, then dehydrated in a graded series (30%, 50% and 80%) of methanol/PBS, and incubated overnight in 20% dimethylsulfoxide (DMSO)/80% methanol solution containing 3% H_2O_2 to quench endogenous peroxidase activity. After rehydration, embryos were blocked in 4% BSA/1% Triton X-100/PBS and incubated with rabbit anti-TH (1:200, Millipore) over 3 days at 4 °C. Embryos were washed in 1% Triton X-100/PBS overnight and secondary antibody, anti-rabbit IgG HRP (1:200, GE Healthcare), was added overnight at 4 °C. Following washes, immunoreactivity

was detected with diaminobenzidine (Sigma-Aldrich), followed by serial dehydration in methanol/PBS and clearing in 2:1 benzyl benzoate/benzyl alcohol (Sigma-Aldrich).

Analyses of sympathetic axon branching in peripheral targets

After whole-mount TH immunostaining, images of peripheral organs were captured at $4\times$ or $5\times$ magnification on a Zeiss Stemi SV6 dissecting scope using an AxioCam HRc camera. Images were saved as JPEG files. All TH-positive axon fibers in each tissue were manually traced using Adobe Photoshop CS3, and the branch points counted using ImageJ. For some organs (thymus, bladder, spleen) all visible TH-positive fibers within the organ were analyzed. For the heart and salivary glands that receive dense sympathetic innervation by E16.5, axon branching was represented as branches per μm^2. More than three different litters were used for all analyses; the number of mutant and wild-type animals used per experiment are indicated in the figure legends.

Analysis of proliferation

E12.5 pregnant dams were injected intraperitoneally with Click-IT EdU (125 μg/ml in PBS, Invitrogen), embryos harvested 24 h later and prepared for immunofluorescence. Tissue sections (10 μm) were immunolabeled for TH to visualize the SCG, and the Click-IT EdU reaction was performed according to the manufacturer's protocol to visualize the EdU label.

Neuronal cell counts

E16.5 and P0.5 mice were prepared for neuronal counts as described in Bodmer et al. (2009). Mouse torsos were fixed for 4 h in PBS containing 4% PFA, and then cryoprotected overnight in 30% sucrose-PBS. SCG sections (10 μm) were stained with a solution containing 0.5% cresyl violet (Nissl). Cells with characteristic neuronal morphology and visible nucleoli were counted in every fifth Nissl stained section.

Axonal extension and branching in compartmentalized cultures

Compartmentalized cultures of sympathetic neurons from P0.5 rat pups were established, and axonal elongation (in micrometers per day) was measured as described previously (Bodmer et al., 2009). Compartmentalized cultures were initially maintained in NGF-containing media for 5–7 days to allow robust axonal growth into axonal compartments. To analyze the effect of Ror activity on Wnt-mediated growth and branching, NGF was withdrawn from the culture media bathing cell bodies and axons and Ror1/2 inhibitory antibodies (120 μg/ml in PBS), (Ho et al., 2012) were added either to the axon compartment or the cell body compartments in the presence of either control- or Wnt5a-conditioned medium. Control conditioned media were obtained from the parental fibroblasts (L cells) used to generate the Wnt5a-expressing cell line. Quantification of axonal growth was performed by photographing axons using a Zeiss Axiovert 200 microscope at 0 and 24 h after treatment and measuring the extension of the axon using the Openlab 4.04 software. To measure axonal branching, neurons were fixed and immunostained for β-III-tubulin (1:500 dilution, Sigma), the axonal compartments were photographed using a Zeiss Axiovert microscope equipped with a Retiga EXi camera, and the total number of axonal branch points counted for all axons per collagen track. For every axon, each point of divergence from the main axonal shaft was counted as a branch point.

Statistical analyses

Statistical comparisons were determined by the Student's t-test for pair comparisons and by one-way analysis of variance (ANOVA) for multiple comparisons. Post-hoc analyses were done using the Tukey–Kramer test. Values and error bars indicate mean ± SEM.

Results

Embryonic mice with conditional deletion of Wnt5a in the developing neural crest appear grossly normal

To generate the conditional Wnt5a allele, we flanked exon 2 of the Wnt5a gene with loxP sites (Fig. 1A and B). Disruption of exon 2 has been shown to lead to a null mutation in the conventional $Wnt5a^{-/-}$ mouse (Yamaguchi et al., 1999). In the absence of Cre-mediated recombination, the $Wnt5a^{fl/fl}$ mice had no apparent mutant phenotype and were morphologically indistinguishable from wild-type C57BL/6 mice, suggesting that the insertion of loxP sites did not perturb the Wnt5a locus. Quantitative real-time PCR analyses on sympathetic ganglia and target tissues i.e. the salivary glands and heart tissue revealed no differences in Wnt5a transcript levels between $Wnt5a^{fl/fl}$ and wild-type animals (Supplementary Fig. 1). In order to delete Wnt5a in sympathetic neurons, we crossed mice homozygous for the floxed Wnt5a allele ($Wnt5a^{fl/fl}$) with mice carrying a Wnt1::Cre transgene and one Wnt5a null allele ($Wnt1::Cre;Wnt5a^{+/-}$) to generate the $Wnt1::Cre;Wnt5a^{fl/-}$ mice. This mating strategy optimizes Cre-mediated deletion efficiency. In all of our analyses, we used littermate control mice that always carried at least one functional copy of the Wnt5a allele ($Wnt5a^{fl/+}$, $Wnt5a^{fl/-}$ or $Wnt1::Cre;Wnt5a^{fl/+}$). Heterozygous Wnt5a mice ($Wnt5a^{+/-}$) are viable, fertile and show no obvious developmental abnormalities (Yamaguchi et al., 1999). Wnt1-driven Cre induces recombination, starting at embryonic day 8.5 (E8.5), in pluripotent neural crest cells (Danielian et al., 1998), which give rise to both neurons and glial cells in the peripheral nervous system (PNS).

In sharp contrast to the conventional $Wnt5a^{-/-}$ embryos which exhibit major embryonic patterning deficits including dwarfism, a shortened anterior–posterior axis, truncated limbs and tails and craniofacial abnormalities, $Wnt1::Cre;Wnt5a^{fl/-}$ mice appeared grossly normal with normal anterior–posterior axis, limbs and tail structures (Fig. 1C), (Yamaguchi et al., 1999). However, craniofacial abnormalities were evident in the conditional mutants similar to that in $Wnt5a^{-/-}$ embryos, suggesting a role for neural crest-derived Wnt5a in proper formation of craniofacial structures (He et al., 2008). $Wnt1::Cre;Wnt5a^{fl/-}$ mice were born in typical Mendelian ratios, but, like the $Wnt5a^{-/-}$ mice, also died within a few hours after birth, likely due to the inability to feed owing to craniofacial defects. In situ hybridization analyses using a probe directed against the targeted exon 2 of the Wnt5a gene showed a pronounced decrease in Wnt5a transcript in the developing superior cervical ganglia (SCG) in E16.5 $Wnt1::Cre;Wnt5a^{fl/-}$ embryos (Fig. 1D), whereas the salivary glands (a sympathetic target tissue) showed detectable levels of Wnt5a (Fig. 1E). Moreover, the Wnt5a transcript was not detected in $Wnt1::Cre;Wnt5a^{fl/-}$ sympathetic ganglia in RT-PCR analyses using primers that target sequences outside of exon 2 (Supplementary Fig. 2), suggesting that the transcript missing exon 2 likely undergoes nonsense-mediated mRNA decay in Cre-expressing tissues. Together, the above results demonstrate the specific reduction of Wnt5a in sympathetic neurons but not in target tissues in $Wnt1::Cre;Wnt5a^{fl/-}$ animals.
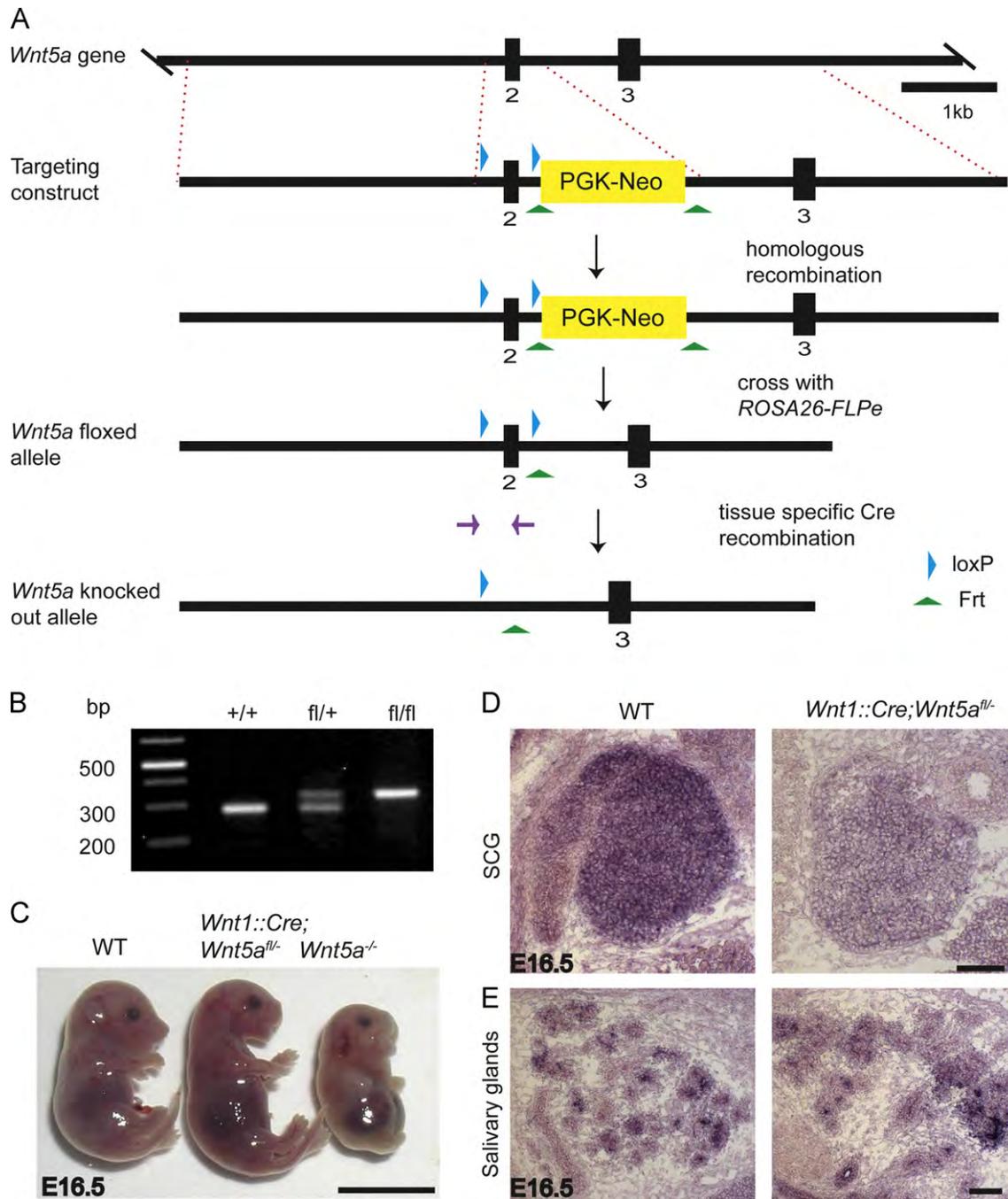
Fig. 1. Generation of conditional *Wnt5a* mutant mice. (A) Strategy for generating the conditional *Wnt5a* allele. Exons 2 and 3 in the *Wnt5a* gene are indicated in each line. (B) Genotyping results from *Wnt5a*$^{+/+}$, *Wnt5a*$^{fl/+}$ and *Wnt5a*$^{fl/fl}$ mice using primers (purple arrows in A) targeting the first *loxP* site. (C) An E16.5 *Wnt1*::*Cre*;*Wnt5a*$^{fl/-}$ embryo shows normal anterior–posterior length, limbs and tail but has craniofacial abnormalities as compared to a wild-type embryo. The craniofacial defects in the *Wnt1*::*Cre*;*Wnt5a*$^{fl/-}$ embryo are similar to that seen in a constitutive *Wnt5a* null embryo obtained from the same litter due to occasional germline expression of Cre. (D and E) *In situ* hybridization using a probe that spans the targeted exon 2 of *Wnt5a* shows decreased *Wnt5a* transcript in E16.5 *Wnt1*::*Cre*;*Wnt5a*$^{fl/-}$ mice in the superior cervical ganglia (SCG) (D) but the Wnt5a signal can be detected in salivary glands (E). Scale bar: 1 cm for C and 100 μm for D and E. For all experiments, 3 embryos for each genotype were analyzed.

Wnt5a is dispensable for early stages of sympathetic nervous system development

Using the *Wnt1*::*Cre*;*Wnt5a*$^{fl/-}$ mice, we asked whether loss of Wnt5a in the developing PNS would affect early stages of sympathetic nervous system development. At E13.5, we observed normal expression of Phox2b, a homeodomain transcription factor necessary for the induction and maintenance of sympathetic lineage-specific markers (Pattyn et al., 1999), in both mutant and control embryos (Fig. 2A). Assessment of cellular

proliferation using a 24-hr pulse of 5-ethynyl-2′-deoxyuridine (EdU) incorporation revealed no major differences between *Wnt1*::*Cre*;*Wnt5a*$^{fl/-}$ and control mice at E13.5 (3,997 ± 336 EdU-positive cells in *Wnt1*::*Cre*;*Wnt5a*$^{fl/-}$ embryos *versus* 3964 ± 365 in control mice); the SCG was identified by co-labeling tissue sections with an antibody against tyrosine hydroxylase (TH) (Fig. 2B and C). To visualize the spatial organization of the entire sympathetic chain and initial projections of sympathetic axons out of ganglia, we employed whole-mount TH immunostaining in *Wnt1*::*Cre*;*Wnt5a*$^{fl/-}$ embryos and control litter-mates. In contrast
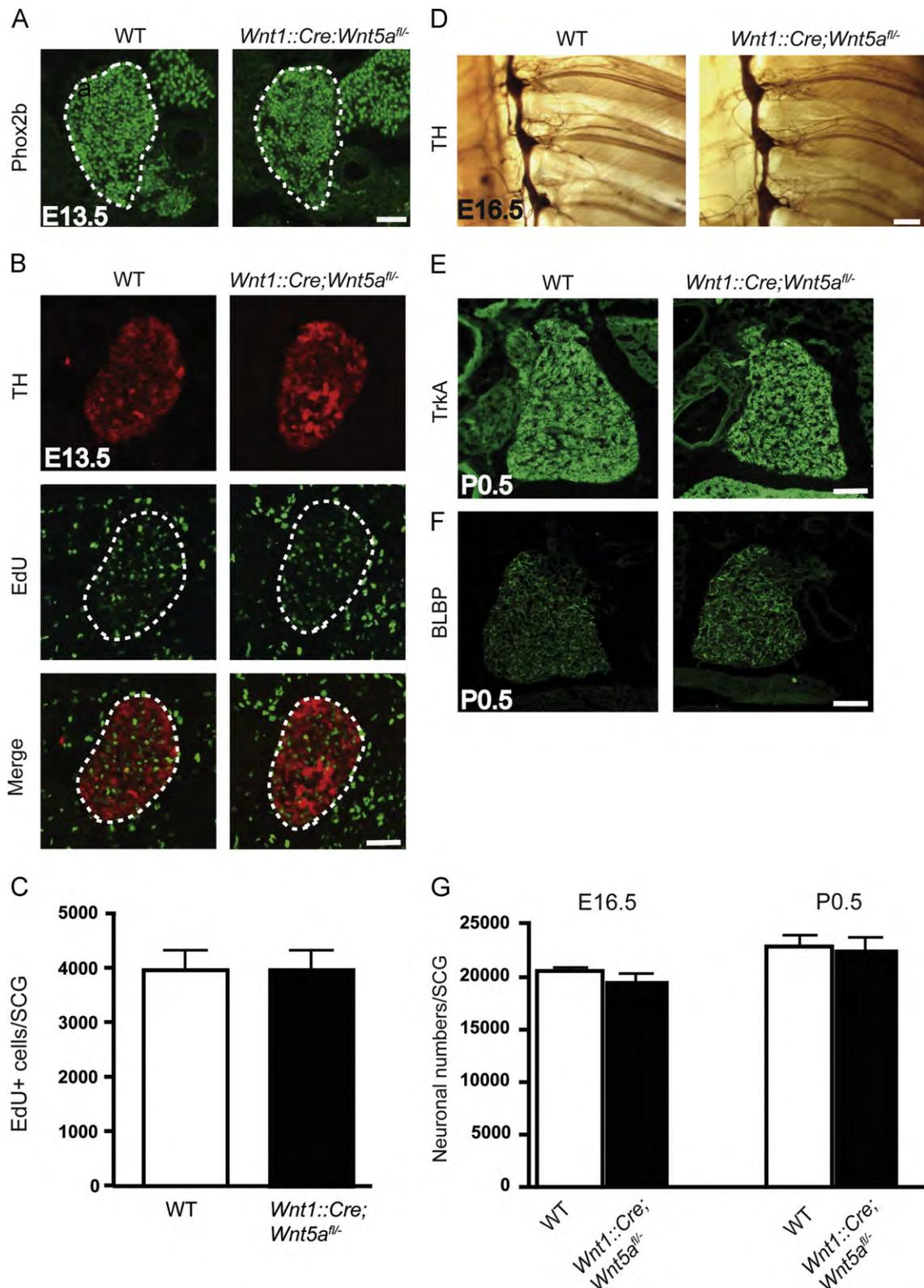
Fig. 2. Neuronal Wnt5a is dispensable for sympathetic neuron differentiation, proliferation and survival. (A) Normal expression of the sympathetic lineage marker, Phox2b, in E13.5 control and *Wnt1::Cre;Wnt5a$^{fl/-}$* SCGs. Scale bar: 50 μm. (B) A 24-hr pulse of EdU reveals similar levels of proliferating cells in *Wnt1::Cre;Wnt5a$^{fl/-}$* and control SCGs at E13.5. Scale bar: 100 μm. Dashed lines outline the SCG. (C) Quantification of the total number of EdU-positive cells per SCG. Values are the mean ± SEM, $n=3$ embryos. (D) The general architecture of the sympathetic chain is normal in E16.5 *Wnt1::Cre;Wnt5a$^{fl/-}$* mice, with ganglia size comparable to that in wild-type controls. Scale bar: 250 μm. (E and F) Immunostaining against TrkA (E) and brain-lipid-binding protein (BLBP) (F) at P0.5 show no changes in the expression of mature neuronal and glial markers upon conditional deletion of *Wnt5a*. Scale bar: 100 μm. (G) Quantification of neuronal numbers show that there is no significant cell loss in the developing SCG at E16.5 and P0.5 in *Wnt1::Cre;Wnt5a$^{fl/-}$* mice compared to controls. Values are the mean ± SEM, $n=3$ animals for each genotype at each developmental stage.

to the aberrant segmentation and organization of the sympathetic chains that we previously reported in $Wnt5a^{-/-}$ mice (Bodmer et al., 2009), no structural deficits were observed in sympathetic chains of E16.5 conditional Wnt5a mutants (Fig. 2D). Sympathetic chain ganglia were intact and showed normal coalescence in $Wnt1::Cre;Wnt5a^{fl/-}$ embryos. In addition, proximal projections out of sympathetic ganglia were seen coursing along the intercostal arteries in $Wnt1::Cre;Wnt5a^{fl/-}$ mice, similar to that in wild-type animals. This is in marked contrast to our previous observations in $Wnt5a^{-/-}$ mice, where axonal projections emanated from mutant ganglia at aberrant locations and appeared to be highly disorganized (Bodmer et al., 2009). Together, these results suggest that the deficits in sympathetic chains and proximal sympathetic projections in $Wnt5a^{-/-}$ mice do not stem from a cell-autonomous requirement for Wnt5a in sympathetic neurons. Using immunostaining against TrkA and brain-lipid-binding protein (BLBP) to mark neuronal and glial differentiation, respectively, we found no effects of conditional Wnt5a deletion on these parameters of sympathetic ganglia maturation (Fig. 2E and F). Quantification of neuronal numbers in the developing SCG confirmed a normal complement of SCG neurons in $Wnt1::Cre;Wnt5a^{fl/-}$ mice at E16.5 ($19,975 \pm 454$ in mutants versus $20,663 \pm 364$ in control mice) and postnatal day 0.5 (P0.5) ($22,968 \pm 927$ in mutants versus $22,973 \pm 1150$ in controls) (Fig. 2G). Together, these results suggest that loss of Wnt5a in the embryonic PNS does not affect neurogenesis, specification and viability in sympathetic ganglia at early developmental stages.

Wnt5a is required for terminal arborization of sympathetic axons

We next employed whole-mount TH immunostaining of several sympathetic targets in $Wnt1::Cre;Wnt5a^{fl/-}$ mice to visualize final target innervation in vivo. At E16.5 when sympathetic axons are just beginning to reach and innervate final target tissues, we observed defects in the extension and branching of sympathetic fibers within the final target fields in several peripheral tissues, including salivary glands (Fig. 3A and B), thymus (Fig. 3C and D), heart (Fig. 3E and F), spleen (Fig. 3G and H) and bladder (Fig. 3I and J). Thus, target organs of both paravertebral sympathetic ganglia (salivary glands, thymus, heart) and prevertebral ganglia (spleen and bladder) are incompletely innervated by sympathetic axons lacking Wnt5a. The innervation deficits in $Wnt1::Cre;Wnt5a^{fl/-}$ embryos are also reminiscent of the phenotypes observed in NGF/Bax double null mice (Glebova and Ginty, 2004), although not as severe as that observed with NGF loss. Notably, in the spleen of $Wnt1::Cre;Wnt5a^{fl/-}$ embryos, sympathetic axons extend along the vasculature right up to the target but fail to grow and arborize within the spleen parenchyma (Fig. 3G), similar to their aberrant behavior reported in NGF-deficient embryos (Glebova and Ginty, 2004). In contrast to marked hypoplasia of sympathetic targets in conventional $Wnt5a^{-/-}$ embryos (Cervantes et al., 2009), we observed no defects in the integrity of peripheral organs in conditional Wnt5a mice. Together, these results suggest that neuronal Wnt5a is dispensable for initial stages of sympathetic axon outgrowth and extension along the intermediate arteries, but is specifically required for terminal arborization into final NGF-expressing target fields.

Although we did not observe a significant depletion of SCG neuronal numbers in P0.5 $Wnt1::Cre;Wnt5a^{fl/-}$ mice (see Fig. 2G), we found a significant increase in apoptotic profiles in mutant ganglia, as assessed by immunostaining for cleaved caspase-3 (Supplementary Fig. 3). Since $Wnt1::Cre;Wnt5a^{fl/-}$ mice die within a few hours after birth, we were unable to determine whether the increased apoptosis manifests as a significant decline in neuronal numbers at later postnatal stages. Nevertheless, these results suggest that enhanced apoptosis in mutant SCGs is

likely a secondary consequence of impaired innervation of NGF-expressing targets.

$TH::Cre;Wnt5a^{fl/fl}$ mice exhibit sympathetic innervation deficits similar to that observed in $Wnt1::Cre;Wnt5a^{fl/-}$ mice

Since the $Wnt1::Cre$ driver also induces recombination in glial cells in the peripheral nervous system (Danielian et al., 1998), the potential loss of Wnt5a in glia and compromised glial integrity could underlie the observed sympathetic innervation deficits. We consider this scenario less likely since we previously observed Wnt5a expression in sympathetic ganglia to be largely neuronal (Bodmer et al., 2009). Moreover, in $Wnt1::Cre;Wnt5a^{fl/-}$ mice, significant deficits in sympathetic innervation were observed by E16.5, a time at which gliogenesis has just been initiated in superior cervical ganglia (Hall and Landis, 1991). However, to directly address the role of Wnt5a in sympathetic neurons, we crossed the floxed Wnt5a mice to a $TH::Cre$ line (Gong et al., 2007). TH is expressed in catecholaminergic cells including sympathetic neurons and is not expressed in sympathetic glia. The $TH::Cre;Wnt5a^{fl/fl}$ mice were morphologically indistinguishable from their wild-type litter-mates and survived to adulthood, unlike the $Wnt1::Cre;Wnt5a^{fl/-}$ mice. It is also interesting to note, that in contrast to the $Wnt1::Cre;Wnt5a^{fl/-}$ mice, $TH::Cre;Wnt5a^{fl/fl}$ mice did not exhibit any gross abnormalities in craniofacial structures.

In situ hybridization analyses revealed attenuated Wnt5a expression in the developing superior cervical ganglia (SCG) but not in the salivary glands in E16.5 $TH::Cre;Wnt5a^{fl/fl}$ embryos compared to $Wnt5a^{fl/fl}$ litter-mate controls (Fig. 4A and B). Moreover, quantitative real-time PCR (Q-PCR) analyses revealed a substantial (60%) depletion of Wnt5a transcript in sympathetic ganglia in $TH::Cre;Wnt5a^{fl/fl}$ embryos compared to $Wnt5a^{fl/fl}$ litter-mate controls (Fig. 4C). However, the Wnt5a levels in the salivary glands were unperturbed (Fig. 4C). Whole-mount TH immunostaining of the salivary glands and heart in E16.5 $TH::Cre;Wnt5a^{fl/fl}$ mice revealed significant defects in sympathetic innervation (Fig. 4D–G), that phenocopied the defects seen in $Wnt1::Cre;Wnt5a^{fl/-}$ mice (Fig. 3A–J). These results suggest that indeed, the sympathetic innervation defects observed in the $Wnt1::Cre;Wnt5a^{fl/-}$ mice arise from a loss of neuronal Wnt5a. Since $TH::Cre;Wnt5a^{fl/fl}$ mice survived after birth, we asked whether impaired innervation of NGF-expressing targets at embryonic stages would result in enhanced apoptosis and a depletion in neuronal viability at postnatal stages. We observed a significant depletion (30% decrease) in SCG neuronal numbers by postnatal day 7 in $TH::Cre;Wnt5a^{fl/fl}$ mice ($19,750 \pm 2119$ in $TH::Cre;Wnt5a^{fl/fl}$ mice versus $28,376 \pm 2268$ in $Wnt5a^{fl/fl}$ mice) (Fig. 4H and I). However, $TH::Cre;Wnt5a^{fl/fl}$ mice had a normal complement of SCG neurons at the day of birth ($24,995 \pm 578$ in $TH::Cre;Wnt5a^{fl/fl}$ mice versus $23,920 \pm 1166$ in $Wnt5a^{fl/fl}$ mice). The observed decrease in neuronal viability in postnatal $TH::Cre;Wnt5a^{fl/fl}$ mice, long after the deficits in target innervation seen at E16.5, indicates that neuronal loss is a secondary consequence of the reduced innervation of peripheral tissues and the failure to gain access to target-derived survival factor, NGF.

Ror receptors are required locally in sympathetic axons to mediate Wnt5a-dependent branching

Using conditional mutant mice, we recently reported that the atypical receptor tyrosine kinases, Ror1 and Ror2, mediate several Wnt5a-dependent embryonic morphogenetic processes including formation of facial structures, limb development and axis elongation (Ho et al., 2012). During sympathetic nervous system development, Ror2 is expressed in sympathetic neurons during the period of target
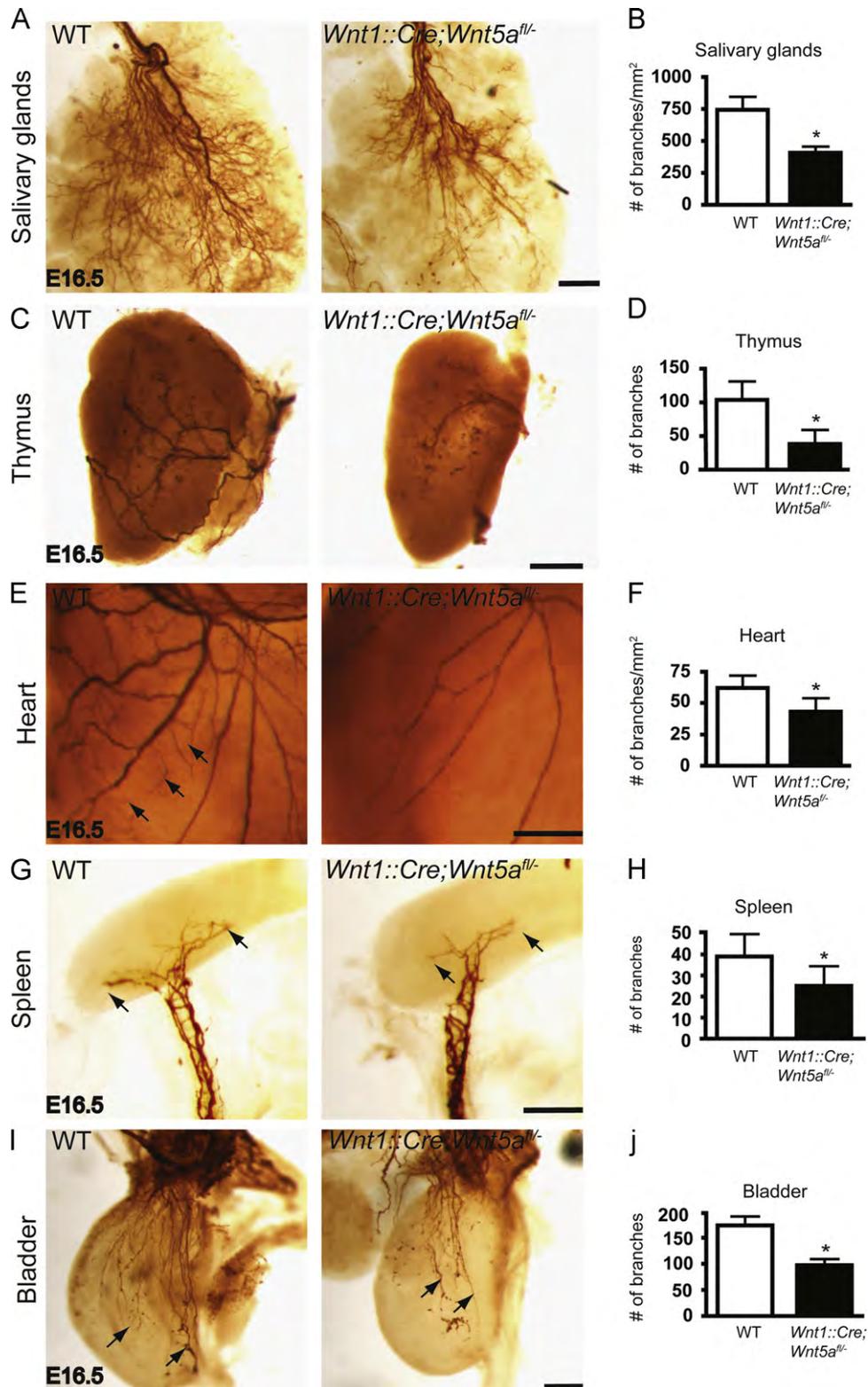
Fig. 3. Wnt5a is required for terminal arborization of sympathetic axons. $Wnt1::Cre;Wnt5a^{fl/-}$ mice show reduced sympathetic arborization in several peripheral targets. Major axon bundles reach the final targets in the mutants, but they fail to extend and branch within final target fields as compared to control litter-mates. (A–J) Whole-mount TH staining of salivary glands (A), thymus (C), heart (E), spleen (G) and bladder (I) in E16.5 WT and $Wnt1::Cre;Wnt5a^{fl/-}$ mice. Quantification of branching in the target tissues are shown in (B, salivary glands), (D, thymus), (F, heart), (H, spleen) and (J, bladder). Scale bar: 200 μm, $n=5$ embryos for each genotype. Values are the mean ± SEM *$p < 0.05$.

innervation. Conditional ablation of *Ror1/2* in peripheral neurons resulted in deficient innervation of several sympathetic targets (Ho et al., 2012) in a manner that closely resembled the phenotypes observed in $Wnt1::Cre;Wnt5a^{fl/-}$ and $TH::Cre;Wnt5a^{fl/fl}$ mice. These findings prompted the hypothesis that Rors are the Wnt5a receptors that promote sympathetic axon branching during target innervation.
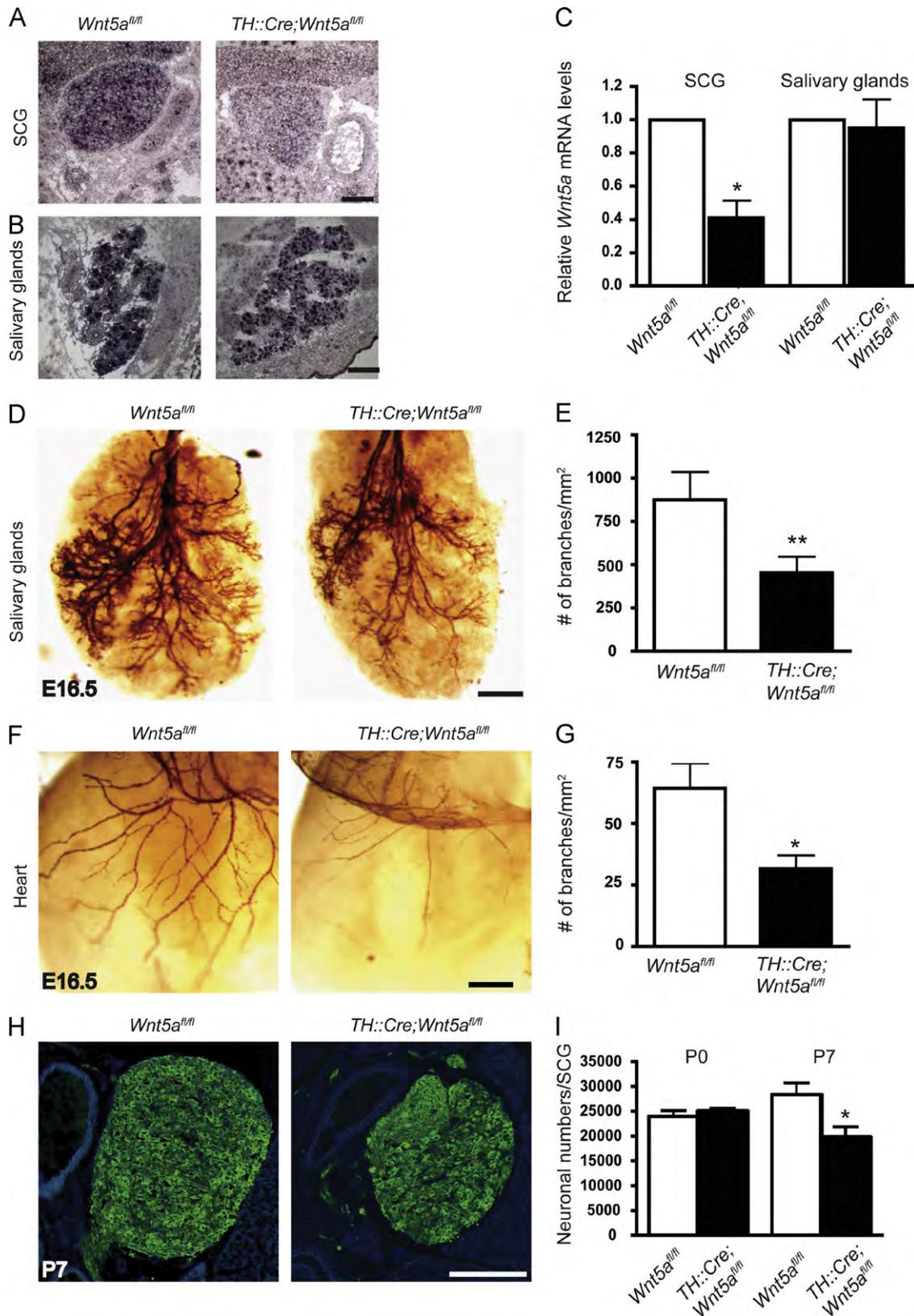
Fig. 4. Sympathetic innervation defects in *TH::Cre;Wnt5a$^{fl/fl}$* embryos. (A and B) *In situ* hybridization shows decreased *Wnt5a* transcript in E16.5 *TH::Cre;Wnt5a$^{fl/fl}$* mice in the superior cervical ganglia (SCG) (A) but the Wnt5a signal can be detected in salivary glands (B). Scale bar: 100 μm. (C) Q-PCR analyses show that Wnt5a transcript levels are significantly reduced in sympathetic ganglia, but not salivary glands in *TH::Cre;Wnt5a$^{fl/fl}$* mice at P0.5. SCGs were harvested from $n=5$ *Wnt5a$^{fl/fl}$* and $n=8$ *TH::Cre;Wnt5a$^{fl/fl}$* mice. Salivary glands were dissected from $n=5$ *Wnt5a$^{fl/fl}$* and $n=9$ *TH::Cre;Wnt5a$^{fl/fl}$* mice. Values are the mean $\pm$ SEM $^*p < 0.05$. (D–G) Whole-mount TH immunostaining of target tissues shows reduced sympathetic innervation of the salivary glands (D) and heart (F) in E16.5 *TH::Cre;Wnt5a$^{fl/fl}$* embryos compared to control *Wnt5a$^{fl/fl}$* mice. Scale bar: 200 μm. Quantification of the branching are indicated in (E, salivary glands) and (G, heart). Salivary glands were harvested from $n=5$ animals and hearts from $n=4$ animals, for each genotype. $^*p < 0.05$, $^{**}p < 0.01$. (H) TH immunostaining of tissue sections show a reduction in superior cervical ganglia (SCG) in *TH::Cre;Wnt5a$^{fl/fl}$* mice compared to control *Wnt5a$^{fl/fl}$* mice at one week after birth (P7), Scale bar: 200 μm. (I) Quantification of neuronal numbers show no change in SCG cell numbers at P0.5 but significant cell loss by P7 in *TH::Cre;Wnt5a$^{fl/fl}$* mice compared to *Wnt5a$^{fl/fl}$* mice. $n=4$ animals for each genotype at P0 and $n=3$ animals at P7. Values are the mean $\pm$ SEM, $^*p < 0.05$.
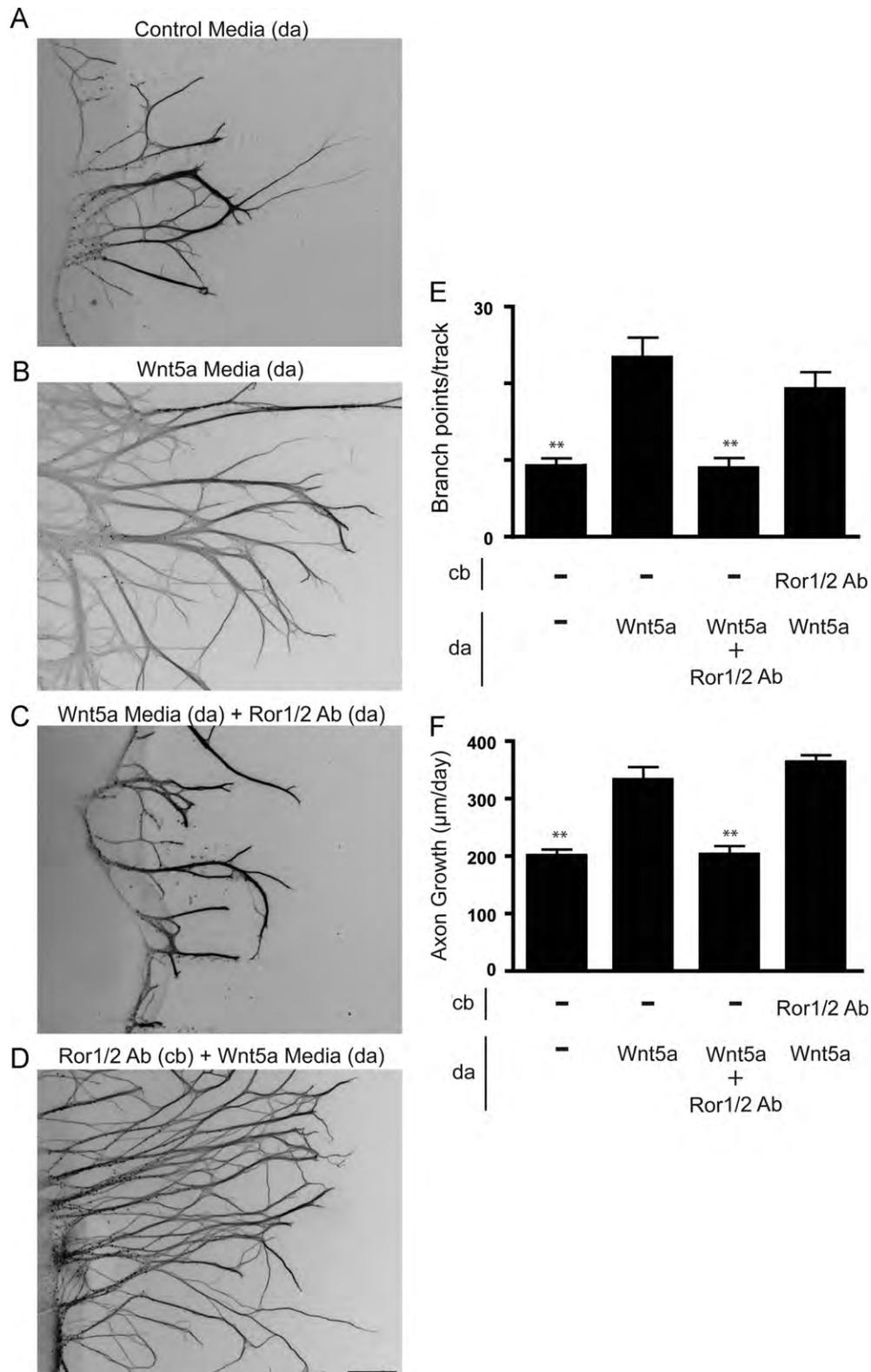
Fig. 5. Wnt5a-dependent branching is mediated by local actions of Ror receptors in sympathetic axons. (A–D) In compartmentalized cultures, Wnt5a-treated axons exhibit robust axon growth and branching (B), which is eliminated by the addition of Ror1/2 neutralizing antibodies to distal axons (da) (C), but not cell bodies (cb) (D). Compartmentalized cultures were initially maintained in NGF-containing media for 5–7 days to allow robust growth into axon compartments. NGF was then withdrawn from the culture media, and Ror1/2 inhibitory antibodies added either to the axon or cell body compartments in the presence of either control- or Wnt5a-conditioned medium for 24 h. The broad-spectrum caspase inhibitor, BAF, was added to the cell body compartments to prevent neuronal apoptosis in the absence of NGF. Axons were stained with β-III-tubulin for visualization. Scale bar: 100 μm. Quantification of Wnt5a-mediated axon branching (E) and growth (F) in compartmentalized cultures over 24 h. $n = 4$ independent experiments, $^{**}p < 0.01$.

To directly test whether Rors act as Wnt5a receptors in mediating branching, we examined the effects of function-blocking antibodies against Ror1/2, (Ho et al., 2012), on Wnt5a-dependent branching

in vitro. Additionally, to identify the sub-cellular location of Ror activity, we assessed Wnt5a-mediated axon branching and growth in compartmentalized sympathetic cultures treated with Ror

neutralizing antibodies added exclusively to distal axon or cell body compartments. Wnt5a-conditioned media added only to sympathetic axon terminals promoted robust axon branching and extension compared to control media (Fig. 5A and B). To monitor the effects of Wnt5a alone, these analyses were performed in the absence of NGF with the addition of the broad-spectrum caspase inhibitor, BAF, to keep the neurons alive in the absence of NGF. The axonal effects of Wnt5a were diminished by the inclusion of Ror neutralizing antibodies to axon terminals (Fig. 5C). Sympathetic axons treated with Wnt5a in the presence of the Ror neutralizing antibodies were shorter and highly fasciculated, with substantially reduced branching at their terminals (Fig. 5C). However, addition of Ror antibodies to the cell body compartments had no effect on Wnt5a-dependent branching and growth (Fig. 5D). Quantification of axon branching and elongation over a period of 24 h showed that Wnt5a-mediated branching and growth responses were abolished with Ror neutralization in sympathetic axons, but not cell bodies (Fig. 5E and F). These results indicate that Ror receptors transduce the effects of Wnt5a on sympathetic axon branching and more importantly, define a local action of Wnt5a-Ror signaling in axons in promoting branching.

Discussion

Of the Wnts, Wnt5a has been intensely investigated in non-canonical Wnt signaling and planar cell polarity-related morphogenetic movements during embryogenesis (Montcouquiol et al., 2006). During nervous system development, emerging evidence implicates Wnt5a as a key regulator of neuronal connectivity events, including axon outgrowth, guidance, and synapse formation (Davis et al., 2008; Li et al., 2009; Varela-Nallar et al., 2010; Blakely et al., 2011; Shafer et al., 2011). However, all these studies were based on morphological assessment of cultured neurons treated in vitro with exogenous Wnt5a, and analyses of global Wnt5a knockout mice lacking Wnt5a at all developmental stages and in all tissues. Little is known about the tissue-specific and temporal requirements for Wnt5a signaling in neural development in vivo. Moreover, the global tissue patterning defects and neonatal lethality of conventional $Wnt5a^{-/-}$ mice (Yamaguchi et al., 1999) have also hindered genetic analyses in neural circuit assembly, maturation and maintenance. Here, we reveal a tissue-specific requirement for Wnt5a in regulating vertebrate neural circuit assembly, using conditional mutant mice. We also provide evidence that Ror receptor activity is required locally in sympathetic nerve terminals to mediate Wnt5a-dependent branching. These findings point to an autocrine Wnt5a-Ror signaling loop acting within vertebrate axons to contribute to circuit formation.

Previous studies have indicated that Wnts produced by neuronal targets act in a paracrine manner to prompt axons to undergo extensive remodeling during target innervation. Wnt7a expressed by cerebellar granule cells induces growth cone enlargement and axonal spreading of presynaptic mossy fibers (Hall et al., 2000). Wnt3 secreted by motoneurons regulates axonal branching and terminal arborization of efferent spinal sensory neurons (Krylova et al., 2002). The only evidence so far to support an autocrine Wnt signaling loop in neuronal development comes from the Drosophila larval neuromuscular junction, where Wingless is secreted by motor neurons and binds to DFz2 receptors localized in these same neurons to regulate presynaptic differentiation during muscle innervation (Packard et al., 2002). Wnt proteins are lipid-modified and highly hydrophobic (Port and Basler, 2010). Thus, secreted Wnts could exert very localized actions on distinct sub-cellular domains in polarized neurons. However, very little is known about the autocrine effects of Wnt signaling in neurons, and in particular, about their sites of action

in morphologically complex neurons. In this study, the combined use of conditional mutant mice and compartmentalized sympathetic neuronal cultures allowed us to define that Wnt5a derived from sympathetic neurons acts locally at sympathetic nerve terminals to direct their terminal axon branching during target innervation.

The localized effects of autocrine Wnt5a-Ror signaling in axon terminals may be a critical regulatory step in ensuring that axon branching is only initiated once the axons have arrived at their appropriate target fields. Wnt5a levels in sympathetic neurons are enhanced by the neurotrophin, NGF, secreted by target tissues (Bodmer et al., 2009), suggesting a regulatory mechanism by which in-growing axons themselves produce diffusible cues in a manner dependent on target interactions to instruct target innervation. Although our study focused on peripheral neurons, it is likely that such interplay between axons and targets would exist in any neuronal cell type, to ensure precise spatial and temporal control of terminal arborization. Currently, the precise mechanisms by which Wnt5a's actions are restricted to the axons, remain unclear. It is possible that Wnt5a synthesized in neuronal soma is packaged, anterogradely transported in vesicles, and primarily released in nerve terminals by mechanisms similar to those demonstrated for some neurotrophins and neurotransmitters (Altar and DiStefano, 1998). Alternatively, differential receptor/effector distribution could dictate the site of Wnt5a actions in neurons. Testing of these possibilities awaits the generation of suitable reagents including effective antibodies to monitor the endogenous sub-cellular distribution of Wnts and Ror proteins.

Wnt5a is also expressed in sympathetic target tissues (Figs. 1E and 4B and C), raising the question of the contribution of neuronal versus target-derived Wnt5a to target innervation. Our results suggest that neuron-derived Wnt5a is required to promote initial stages of branch formation just when sympathetic axons reach their peripheral targets at E16.5. However, the mature pattern of terminal arbors is also dictated by other mechanisms, among which a key process is the guidance of the newly formed axon branches (Gibson and Ma, 2011). Paracrine Wnt5a signaling has been implicated in axon repulsion, a function conserved in Drosophila and mice (Yoshikawa et al., 2003; Liu et al., 2005; Keeble et al., 2006; Li et al., 2009). Thus, a possibility is that Wnt5a derived from neurons and targets might have a dual role in promoting branch formation and branch repulsion, respectively, to establish the final pattern of terminal arborization.

Sympathetic neurons innervate a variety of peripheral targets to regulate many physiological processes including blood glucose levels, cardiac output and body temperature. Dysfunction of the sympathetic nervous system is associated with prevalent diseases including diabetes, congestive heart failure and peripheral neuropathies (Goldstein et al., 2002; Low, 2002; Esler, 2010), which may originate from perturbation of sympathetic target innervation during development. Thus, elucidating an autocrine Wnt5a signaling mechanism in the terminal arborization of sympathetic axons, is a critical step toward understanding the molecular cues that control distinct aspects of sympathetic innervation, and will guide the development of therapeutic strategies to counteract neuronal injury or disease. Finally, the identification of physiological modes of Wnt5a signaling may have implications that extend beyond the sympathetic nervous system. Wnt5a-Ror signaling is a critical regulator of morphogenetic movements, cell polarity and cell shape in multiple developing tissues (Nishita et al., 2010). Constitutive activation of Wnt5a-Ror signaling also contributes to tumor invasion and metastasis (Nishita et al., 2010). Thus, the conditional mutant mice and the autocrine mode of Wnt5a signaling described in this study have broad implications that can guide many future investigations of Wnt5a signaling.

Author contributions

Y.K.R. performed experiments and analyzed data with R.K. S.C. and H.Z. generated Wnt5a conditional mice. H.H. generated the Ror neutralizing antibodies and provided critical input into the project. Y.K.R. and R.K. designed the research and wrote the paper.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.02.013.

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