Frizzled3 Is Required for Neurogenesis and Target Innervation during Sympathetic Nervous System Development

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The sympathetic nervous system has served as an amenable model system to investigate molecular mechanisms underlying developmental processes in the nervous system. While much attention has been focused on neurotrophic factors controlling survival and connectivity of postmitotic sympathetic neurons, relatively little is known about signaling mechanisms regulating development of sympathetic neuroblasts. Here, we report that Frizzled3 (Fz3), a member of the Wnt receptor family, is essential for maintenance of dividing sympathetic neuroblasts. In Fz3−/− mice, sympathetic neuroblasts exhibit decreased proliferation and premature cell cycle exit. Fz3−/− sympathetic neuroblasts also undergo enhanced apoptosis, which could not be rescued by eliminating the proapoptotic factor, Bax. These deficits result in reduced generation of sympathetic neurons and pronounced decreases in the size of sympathetic chain ganglia. Furthermore, the axons of sympathetic neurons that persist in Fz3−/− ganglia are able to extend out of sympathetic ganglia toward distal targets, but fail to fully innervate final peripheral targets. The cell cycle exit, but not target innervation, defects in Fz3−/− mice are phenocopied in mice with conditional ablation of β-catenin, a component of canonical Wnt signaling, in sympathetic precursors. Sympathetic ganglia and innervation of target tissues appeared normal in mice lacking a core planar cell polarity (PCP) component, Vangl2. Together, our results suggest distinct roles for Fz3 during sympathetic neuron development; Fz3 acts at early developmental stages to maintain a pool of dividing sympathetic neuroblasts. In contrast, Fz3 functions at later stages to promote innervation of final peripheral targets by postmitotic sympathetic neurons.

Introduction

The sympathetic nervous system, a branch of the autonomic nervous system, is critical for tissue homeostasis. Postganglionic sympathetic neurons innervate a variety of peripheral targets to regulate several physiological processes including blood glucose levels, cardiac output and body temperature. Development of the sympathetic nervous system is coordinated by a combination of intrinsic transcriptional determinants, and extrinsic signals derived from neighboring vasculature and peripheral targets. Sympathetic neuron development is controlled by the receptor tyrosine kinases, ErbB2/3 (Britsch et al., 1998) and Ret (Enomoto et al., 2001), as well as the guidance receptors, neuropilin1 (Katz et al., 2002), Vangl2, and Plexin A3/A4 (Waimey et al., 2008). While much attention has been focused on neurotrophic factors controlling survival and connectivity of postmitotic sympathetic neurons, relatively little is known about signaling mechanisms regulating development of sympathetic neuroblasts. Here, we report that Frizzled3 (Fz3), a member of the Wnt receptor family, is essential for maintenance of dividing sympathetic neuroblasts. In Fz3−/− mice, sympathetic neuroblasts exhibit decreased proliferation and premature cell cycle exit. Fz3−/− sympathetic neuroblasts also undergo enhanced apoptosis, which could not be rescued by eliminating the proapoptotic factor, Bax. These deficits result in reduced generation of sympathetic neurons and pronounced decreases in the size of sympathetic chain ganglia. Furthermore, the axons of sympathetic neurons that persist in Fz3−/− ganglia are able to extend out of sympathetic ganglia toward distal targets, but fail to fully innervate final peripheral targets. The cell cycle exit, but not target innervation, defects in Fz3−/− mice are phenocopied in mice with conditional ablation of β-catenin, a component of canonical Wnt signaling, in sympathetic precursors. Sympathetic ganglia and innervation of target tissues appeared normal in mice lacking a core planar cell polarity (PCP) component, Vangl2. Together, our results suggest distinct roles for Fz3 during sympathetic neuron development; Fz3 acts at early developmental stages to maintain a pool of dividing sympathetic neuroblasts, while Fz3 functions at later stages to promote innervation of final peripheral targets by postmitotic sympathetic neurons.

Acquisition of noradrenergic features including expression of dopamine β-hydroxylase (DβH) and tyrosine hydroxylase (TH) occurs early in development, and is dependent on transcriptional regulators Mash1, Phox2a/2b, Hand2 and Gata3, as well as diffusible signals such as bone morphogenetic proteins (BMPs) secreted by the dorsal aorta (Goridis and Rohrer, 2002; Howard, 2005). Upon completion of neurogenesis, postmitotic sympathetic neurons are influenced by several extrinsic cues including hepatocyte growth factor, artemin, neurotrophin-3, and nerve growth factor (NGF), to coordinate axonal outgrowth, extension along the vasculature, final target innervation and survival (Glebova and Ginty, 2005). It has been postulated that the majority of the final complement of sympathetic neurons is generated by intensive proliferation of immature noradrenergic sympathetic neuroblasts over a period lasting several days [embryonic day 12.5 (E12.5)–E17.5 in rodents] (Hendry, 1977; Rothman et al., 1978; Rubin, 1985; Fagan et al., 1996). Sympathetic neuroblasts differ from their peripheral sensory and parasympathetic neuronal counterparts and CNS precursors in that they express markers of mature neurons including catecholamine synthesizing enzymes, β-III-tubulin and neurofilament proteins and even bear neuritic processes, but still undergo massive proliferation (Rothman et al., 1978; Rohrer and Thoenen, 1987; DiCicco-Bloom et al., 1990). While several molecular players have been shown to promote proliferation of cultured sympathetic neuroblasts in vitro (DiCicco-Bloom and Black, 1988; DiCicco-Bloom et al., 1990, 2000; Pincus et al., 1990), the signals that...
control proliferation of noradrenergic sympathetic neuroblasts in vivo remains unknown.

Wnt signaling through seven-pass transmembrane Frizzled receptors has been implicated in several developmental events in the nervous system including cell fate specification, cell proliferation, neuronal survival and connectivity (Ciani and Salinas, 2005). During peripheral nervous system (PNS) development, sensory dorsal root ganglia exhibit marked reductions in size in mice lacking both Wnt1/Wnt3a (Ikeya et al., 1997) or with conditional deletion of the cytoplasmic Wnt effector, β-catenin, in premigratory neural crest cells (Hari et al., 2002). However, primordial sympathetic ganglia, which are also derived from neural crest precursors, appear normal in these mutant mice. Currently, 19 Wnt and 10 Frizzled genes have been identified in mammals. Despite the widespread expression of Wnts and Frizzleds in the nervous system and their involvement in almost every aspect of neuronal development in multiple neuronal populations, there is limited information so far about the contribution of Wnt/Frizzled signaling to sympathetic nervous system development. Recently, we defined a role for Wnt5a expressed in sympathetic neurons in mediating axon branching and innervation of final targets during late stages of sympathetic neuron development (Bodmer et al., 2009). Here, we report that Frizzled3 (Fz3) is essential for the normal generation of postganglionic sympathetic neurons. Our analysis of Fz3-deficient mice revealed fewer S-phase proliferating neuroblasts, premature cell cycle exit and enhanced apoptosis in early-stage superior cervical ganglia (SCGs), compared with wild-type littermates. Fz3+/− embryos also showed marked reductions and in some cases, complete absence of sympathetic innervation of several peripheral targets. The neurogenesis, but not innervation, defects in Fz3−/− embryos are also observed in mice lacking β-catenin specifically in the sympathetic neuron lineage. Analysis of homozygous Looptail (Lp/Lp) mice mutant for Vangl2 (Kibar et al., 2001; Montcouquiol et al., 2003), which encodes for a core PCP gene, did not reveal any sympathetic nervous system deficits similar to that observed in Fz3−/− mice. These results identify a critical role for a Fz3/β-catenin signaling pathway in neurogenesis of sympathetic neurons by maintaining a pool of dividing sympathetic precursors. Additionally, our results indicate that Fz3, but not β-catenin, regulates innervation of final peripheral targets by postmitotic sympathetic neurons.

Materials and Methods

Animals. Heterozygous Fz3+/− mice were mated to obtain Fz3−/− and wild-type littermates at various embryonic stages and at postnatal day 0.5 (P0.5). As reported previously, Fz3−/− mice die within a few hours of birth (Wang et al., 2002). To generate conditional mutants of β-catenin, floxed β-catenin (β-Ctnfl/fl) mice (Jackson Laboratory) were crossed to TH-Cre mice. Intercrosses between Fz3+/−/Bax+/− double heterozygotes were set up to obtain Fz3−/−/Bax−/− mice and relevant controls. Bax−/− mice were obtained from Jackson Laboratory. All procedures relating to animal care and treatment conformed to institutional and National Institutes of Health guidelines.

In situ hybridization. In situ hybridization was performed using digoxigenin labeled probes spanning the junction of exons 6 and 7 of Frizzled3. E14.5, E16.5, and P0.5 embryos were fresh frozen in OCT (Tissue-Tek) and serially sectioned (16 μm) using a cryostat. Sections were postfixed in 4% paraformaldehyde (PFA), washed in PBS, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine with 0.9% NaCl. After hybridization with the labeled RNA probe (2 μg/ml) at 68°C overnight, sections were washed with 0.2× SSC buffer at 65°C, blocked with TBS containing 1% normal goat serum and then incubated with alkaline phosphatase-labeled anti-DIG antibody (1:5000; Roche) overnight at 4°C. The alkaline phosphatase reaction was visualized with nitroblue tetrazolium/5-bromo-4-chloro-indolyl phosphate, rinsed in PBS, fixed in 4% PFA and mounted in AquaMount (EMD Chemicals).

Immunohistochemical analysis. Embryos at various developmental ages were fixed in 4% PFA at 4°C for 3–4 h, cryoprotected in 30% sucrose in PBS, frozen in OCT and serially sectioned (10 μm). For immunofluorescence, sections were washed in PBS, permeabilized in PBS containing 1% Triton X-100, and blocked using 5% goat serum in PBS + 0.5% Triton X-100. For diamobenzidine (DAB) immunohistochemistry, endogenous peroxidase activity was quenched in 1% H2O2, before blocking. Sections were incubated in the following primary antibodies overnight: mouse anti-TH (1:200, Sigma-Aldrich), rabbit anti-TH (1:200, Millipore), rat anti-Ki67 (1:100, Dako), rabbit anti-Phox2b (1:200, a kind gift from Jean-François Brunet, Institut de Biologie de l’École Normale Supérieure, Paris, France) and rabbit anti-cleaved caspase3 (1:200, Cell Signaling Technology). Nuclear antigens, Phox2b and Ki67, were subjected to antigen retrieval by boiling sections for 15 min in 1M sodium citrate in 0.5% Tween 20. Following PBS washes, sections were incubated in the appropriate secondary antibodies, diluted 1:200 (anti-mouse Alexa Fluor 488 IgGyl, anti-rabbit Alexa Fluor 488 or Alexa Fluor 546 or anti-rat Alexa Fluor 594 (Invitrogen) for immunofluorescence and anti-rabbit-HRP (GE Healthcare) for DAB immunohistochemistry. Sections were then washed in PBS and mounted in VectaShield (Vector Laboratories) containing 100 μg/ml 4′,6′-diamidino-2-phenylindole dihydrochloride. For colorimetric readout, following secondary antibody incubation, immunoreactivity was detected with diamobenzidine (Sigma-Aldrich), sections were dehydrated in a graded series of ethanol (50%, 70% and 100%) and mounted in Permount (Fisher Scientific). Whole-mount TH immunostaining to visualize sympathetic chains and axons was performed on E12.5, E16.5, and P0.5 mouse embryos as described previously (Bodmer et al., 2009).

Neuronal cell counts. E13.5, E14.5, E16.5, and P0.5 mice for neuronal counts were prepared as described previously by Bodmer et al. (2009). Briefly, mouse torsos were fixed in PBS containing 4% PFA, and then cryoprotected overnight in 30% sucrose-PBS. Superior cervical ganglion (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. In some cases, sections were also stained for TH, and immunopositive cells were counted in every fifth section.

Analyses of proliferation. E13.5 pregnant Fz3−/− dams were injected intraperitoneally with Click-IT EDU (125 μg/ml in PBS, Invitrogen), embryos harvested 2 h later and prepared for immunofluorescence. EDU is a thymidine analog, which like the commonly used bromodeoxyuridine, is incorporated into dividing cells during S-phase but can be easily detected without heat or acid treatment and is thus more compatible for use in fluorescence immunohistochemical analyses (Cappella et al., 2008). Tissue sections (10 μm) were immunolabeled using Click-IT EDU (125 μg/ml), and the Click-IT EDU reaction was performed according to the manufacturer’s protocol to visualize the EDU label. The total number of cells that incorporated EDU in each section were counted and summed for an entire SCG. To assess cell cycle exit, Fz3−/− dams at gestational age E12.5 were injected with Click-IT EDU and harvested 24 h later at E13.5. Following antigen retrieval, sections were immunolabeled with rat anti-Ki67. Cell cycle exit was calculated by determining the percentage of cells positive for EDU but negative for Ki67 among the total number of EDU-labeled cells. To assess cell death of dividing precursors, Fz3−/− dams at gestational age E13.5 were injected with Click-IT EDU and embryos were harvested 2 h later. Visualization of the Click-IT EDU was performed following detection of cell death using terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining (Roche) according to the manufacturer’s protocol. Apoptosis of dividing precursors was calculated by determining the percentage of cells double positive for TUNEL and EDU among the total number of TUNEL-positive cells.

Neuronal cultures and quantification of neurite length and axon branching. Quantification of neurite outgrowth in SCG explants, as well as neurite length and axon branching in dissociated neuronal cultures was performed as previously described (Bodmer et al., 2009). For explants, SCGs dissected from E18.5 wild-type and Fz3−/− embryos were cultured in collagen gel in media containing NGF (25 ng/ml) for 48 h and then...
immunostained with βIII-tubulin (Sigma-Aldrich). Neurite outgrowth in SCG explants was quantified by measuring the area covered by the axons of each explant relative to the area occupied by the cell bodies. Low-density cultures were established from E18.5 wild-type and Fz3<sup>−/−</sup> embryos and cultured on polylysine-coated coverslips for 24 h. Neurons were labeled with β-III tubulin and/or phalloidin to visualize neuronal morphologies. Neurons were imaged using a Zeiss Axiovision microscope with a AxioCam HRC digital camera and analyzed with Axiovision software. Length (micrometers) of the longest neurite as well as the total number of branch points was quantified for each neuron.

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

**Results**

**Sympathetic chain ganglia exhibit hypoplasia in Fz3<sup>−/−</sup> mice**

To determine the role of Fz3 in sympathetic nervous system development, we examined the paravertebral sympathetic chains in Fz3<sup>−/−</sup> mouse embryos using whole-mount tyrosine hydroxylase immunostaining. At E12.5, a stage when the sympathetic chains are still uniform columns of actively proliferating precursors, no morphological differences were apparent between Fz3<sup>−/−</sup> and wild-type embryos (Fig. 1A–D). Neuronal precursors in mutant embryos start extending axons, similar to that observed in wild-type embryos (Fig. 1C, D, arrows). The normal formation of the sympathetic chains and TH expression in Fz3 mutant embryos suggested that dorsoventral migration of neural crest-derived sympathetic precursors and expression of sympathetic lineage markers were unaffected by the absence of Fz3. Normal expression of noradrenergic markers in Fz3<sup>−/−</sup> sympathetic ganglia was also confirmed by immunostaining for Phox2b (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), a homeodomain transcription factor necessary for the induction and maintenance of lineage-specific markers including TH and DBH in sympathetic neurons (Pattyn et al., 1999). At E16.5, when sympathetic axons begin innervating peripheral targets, the chain ganglia appear as discrete, segmented condensations in both wild-type and mutant embryos (Fig. 1E–H). However, a closer inspection revealed that in Fz3<sup>−/−</sup> embryos, sympathetic chain...
ganglia were slightly diminished in size; axonal bundles connecting the ganglia appeared thinner (Fig. 1G,H, arrowheads) while some level of disorganization was seen in projections emanating from the ganglia toward the periphery (Fig. 1G,H, arrows). At P0.5, a stage when sympathetic axons have reached or are actively innervating target tissues, Fz3−/− sympathetic chains were markedly atrophic with smaller ganglia throughout the rostrocaudal extent of the chain and sparser axonal projections (Fig. 1I–L).

To better characterize the defects in sympathetic chain ganglia in Fz3−/− mice, we focused our analyses on the superior cervical ganglia (SCG), the most rostral ganglia in the sympathetic chain. We used in situ hybridization to assess Fz3 expression in the developing SCG. Fz3 expression was observed in the SCG at all the developmental stages examined (E14.5, Fig. 2A,B; E16.5, Fig. 2C,D; and P0.5, Fig. 2E,F). Prominent expression of Fz3 at E14.5, a stage when the SCG is comprised primarily of proliferating sympathetic neuroblasts (Fagan et al., 1996; Francis et al., 1999) suggests that Fz3 is expressed in cells of neuronal origin. We performed TH immunohistochemistry to visualize the SCG and Nissl staining to quantify cells of neuronal origin. We performed in situ hybridization to assess Fz3 expression in the developing SCG. Fz3 expression was observed in the SCG at all the developmental stages examined (E14.5, Fig. 2A,B; E16.5, Fig. 2C,D; and P0.5, Fig. 2E,F). Prominent expression of Fz3 at E14.5, a stage when the SCG is comprised primarily of proliferating sympathetic neuroblasts (Fagan et al., 1996; Francis et al., 1999) suggests that Fz3 is expressed in cells of neuronal origin. We performed TH immunohistochemistry to visualize the SCG and Nissl staining to quantify cell numbers in wild-type (Fig. 2G,I,K,M) and Fz3−/− embryos (Fig. 2H,J,L,M) at various developmental stages. We initiated our analysis at E13.5 when sympathetic ganglia have just coalesced. The wild-type SCG increases in size from E13.5 to E16.5, a time period of robust neurogenesis in the SCG (Fagan et al., 1996; Francis et al., 1999). There is a concomitant increase in SCG cell number from E13.5 (13,714.5 ± 551.4) to E16.5 (20,823.52 ± 1075.9) (Fig. 2M). However, in Fz3−/− mice, significant reductions in SCG cell numbers compared with wild-type embryos were observed at E13.5 (29.8% decrease) and E14.5 (39.15% decrease) (Fig. 2M). By E16.5, when neurogenesis is being completed in the murine SCG (Francis et al., 1999), the Fz3−/− SCG was dramatically reduced in size (Fig. 2J), possessing 68.6% fewer cells than in wild-type embryos (Fig. 2M). The cell loss observed in Fz3−/− SCGs was maximal at E16.5, and no further depletions in cell number were observed between E16.5 and P0.5 (Fig. 2M), when SCG axons are actively innervating target regions and depending on target-derived neurotrophic support (Rubin, 1985; Fagan et al., 1996).

A similar decrease in cell numbers was also evident in the stellate ganglion, a second large ganglion immediately caudal to...
the SCG in the sympathetic chain in Fz3−/− embryos (supplementary Fig. 2, available at www.jneurosci.org as supplemental material). Together, these results reveal that Fz3 is dispensable for early events in sympathetic chain formation including migration and noradrenergic specification, but, is essential for regulating a developmental increase in sympathetic ganglia size.

**Fz3 is necessary for proliferation of sympathetic neuroblasts**

Since the reduction in cell numbers in Fz3−/− SCG is evident during a period of neurogenesis, we assessed cellular proliferation in wild-type and Fz3−/− SCGs using 5-ethyl-2′-deoxyuridine (EdU) incorporation. The SCG was identified by colabeling tissue sections with an antibody against TH (Fig. 3A–E). To determine the cause of reduced proliferating cells in the Fz3−/− SCG, we pulse-labeled sympathetic neuroblasts with EdU at E12.5 for 24 h, and then stained the tissue with Ki67 (Fig. 3F, G). Ki67 labels cells in all phases of the cell cycle except G0. Thus, EdU+/Ki67− cells represent those that have exited the cell cycle during the 24 h time interval. Our analysis revealed that significantly more sympathetic neuroblasts exited the cell cycle over the 24 h interval in the Fz3−/− SCG (Fig. 3H). In the wild-type SCG, 32% of EdU-positive cells exited the cell cycle compared with 43% in Fz3−/− SCG. Together, these results suggest that, during sympathetic nervous system development, Fz3 is necessary to maintain sympathetic neuroblasts in the cell cycle.

**Fz3−/− sympathetic neuroblasts undergo enhanced apoptosis**

Given the dramatic reduction in SCG size in Fz3−/− embryos, we considered whether, in addition to decreased neurogenesis, cell death could also contribute to the significant loss in sympathetic neuron numbers. Thus, we used activated caspase-3 immunostaining to assess apoptotic cell death in wild-type and Fz3−/− embryos (Fig. 4A–D). The number of caspase3-immunoreactive cells per wild-type SCG averaged 38 ± 5 and 49 ± 3 at E13.5 and E14.5, respectively (Fig. 4E). These results point to some degree of naturally occurring cell death in the nascent SCG before target innervation. However, the Fz3−/− embryos showed an increase in apoptotic profiles in the SCG with 65 ± 10 and 83 ± 9 caspase3-positive cells at E13.5 and E14.5, respectively (Fig. 4E). As noted previously, the majority of cells in the SCG at E13.5–E14.5 are proliferating neuroblasts, which we independently confirmed with Ki67 and TH immunostaining (data not shown). These results suggest that enhanced apoptosis in addition to a reduction in the precursor pool as a result of premature cell cycle exit, accounts for the overall decrease in sympathetic ganglia size and cell number in Fz3−/− embryos. We also analyzed apoptosis in wild-type and the Fz3−/− SCGs at E16.5, an intermediate period overlapping the end phase of neurogenesis and the onset of target innervation, and at P0.5, when sympathetic axons are actively competing for limiting amounts of target-derived neurotrophic factors. At E16.5, we observed a twofold increase in apoptotic profiles in the Fz3−/− SCG, compared with wild-type controls (caspase3-positive cells: 397 ± 36.9 in Fz3−/− SCGs versus 191 ± 8.3 in wild-type SCGs) (Fig. 4E). However, at P0.5, we found no significant differences in levels of apoptosis between Fz3−/− and wild-type mice (caspase3-positive cells: 202.3 ± 92.23 in Fz3−/− SCGs versus 430 ± 49.56 in wild-type SCGs) (Fig. 4E). These results suggest that significant cell loss in Fz3−/− sympathetic ganglia occurs before periods of neurotrophin dependence in vivo.

To better define the identity of the dying cells in Fz3−/− sympathetic ganglia, we made use of mice lacking the proapoptotic factor, Bax. Bax is a proapoptotic member of the Bcl-2 family of proteins, and its genetic ablation results in excess cell numbers in various developing tissues including the nervous system (Deckwerth et al., 1996; White et al., 1998; Patel et al., 2000; Lindsten et al., 2003; Lindsten and Thompson, 2006). In vivo studies have shown that postmitotic sympathetic neurons die in the absence of the target-derived survival factor, NGF, and this cell death can be completely rescued by the concurrent elimination of Bax.
(Middleton and Davies, 2001; Glebova and Ginty, 2004). We reasoned that if the cells dying in Fz3−/− SCGs are mature, postmitotic neurons that depend on target-derived trophic support, then enhanced apoptosis observed in Fz3−/− ganglia should be prevented by genetic ablation of Bax. We found a similar depletion of SCG cell numbers in Fz3−/−;Bax−/− double knock-out embryos as in Fz3−/− embryos (Fig. 4G, I, J). In contrast to the P0.5 SCG, in which removal of Bax alone increases neuronal number by twofold (Middleton and Davies, 2001; Glebova and Ginty, 2004), the E16.5 SCG shows no increase in size or cell number in Bax−/− mice (Fig. 4F, H, J). This indicates that naturally occurring cell death of sympathetic neuroblasts occurs via a mechanism independent of the classical Bax-mediated apoptotic pathway used by postmitotic sympathetic neurons that compete for target-derived NGF (Middleton and Davies, 2001; Glebova and Ginty, 2004). The timing of cell death during a period of robust neurogenesis in the SCG, and the Bax-independent mechanism of cell death provides strong evidence that Fz3 deficiency results in depletion of cycling sympathetic neuroblasts or the cells that have prematurely exited the cell cycle, rather than mature postmitotic sympathetic neurons.

To distinguish whether cycling sympathetic neuroblasts or cells that have inappropriately exited the cell cycle undergo enhanced cell death in Fz3−/− sympathetic ganglia, we assessed the percentage of still-dividing sympathetic neuroblasts that are undergoing apoptosis. E13.5 Fz3−/− and wild-type embryos were pulse-labeled with EdU for 2 h to identify S-phase neuroblasts, and the tissue costained with TUNEL to identify dying cells. Although the percentage of TUNEL-positive cells that are also EdU-positive were found to be higher in Fz3 mutant than wild-type SCGs, 30.43 ± 4.9% and 21.1 ± 4.6%, respectively, these numbers were not statistically different (p = 0.195, Student’s t test, n = 6 embryos for wild-type and Fz3−/− embryos, supplemental Fig. 3, available at www.jneurosci.org as supplemental material). This suggests that death of cycling sympathetic neuroblasts does not significantly contribute to the reduction in SCG cell number observed in the absence of Fz3. Together, with the evidence presented above, we infer that cell loss in Fz3−/− SCGs likely results from apoptosis of cells that have prematurely exited the cell cycle, but have yet to rely on survival cues coming from final targets.

Sympathetic innervation of peripheral targets is reduced or absent in Fz3−/− mice

Previous analyses of Fz3−/− mice revealed profound deficits in axon growth in the developing CNS with major losses in thalamocortical, corticothalamic and nigrostriatal tracts (Wang et al., 2002). Additionally, in the absence of Fz3, commissural axons crossing the midline also show targeting errors in their anterior trajectory toward the brain (Lyuksyutova et al., 2003).

To determine whether the postmitotic sympathetic neurons that persist in Fz3−/− embryos were capable of extending axons and reaching their peripheral targets, we used TH immunohistochemical analyses on tissue sections and whole embryos. At E14.5, TH-positive sympathetic axons extended from the SCG and projected along the neighboring carotid artery in both wild-type and Fz3−/− mice (Fig. 5A, B, arrows). Bundles of TH-positive sympathetic fibers were seen coursing adjacent to the cochlea on trajectory toward more distal targets in Fz3−/− mice (Fig. 5D), similar to that in wild-type embryos (Fig. 5C). However, at E16.5, several peripheral targets in Fz3−/− mice showed a complete loss or severe reductions in sympathetic innervation. Sympathetic innervation is markedly reduced in salivary glands (Fig. 5F) or completely absent in the heart (Fig. 5H) of Fz3−/− mice, compared with that in the wild-type tissues (Fig. 5E, G). The salivary glands receive innervation primarily from SCG sympathetic fibers, while innervation of the heart comes from the stellate ganglia, with some contribution from the SCG and thoracic paravertebral ganglia. In Fz3−/− embryos, we also noted...
greatly reduced sympathetic innervation of the kidneys, which receive innervation from paravertebral ganglia in rodents (compare wild-type in Fig. 5J with mutant in Fig. 5J). Fz3−/− sympathetic axons were observed to extend along the renal arteries (Fig. 5J, arrowheads) but these extensions were very sparse and failed to arborize within the kidney parenchyma. The stomach and gastrointestinal tract, normally innervated by sympathetic fibers originating from the prevertebral celiac and mesenteric ganglia, also showed marked reductions in sympathetic innervation in Fz3−/− mice (stomach, Fig. 5L, N; intestine, Fig. 5P, R), compared with wild-type stomach (Fig. 5K, M) and intestine (Fig. 5O, Q). Sympathetic axons entered the stomach in Fz3−/− embryos (Fig. 5L, arrowheads), but a closer examination revealed very little arborization in the stomach wall (Fig. 5N). For the gastrointestinal tract, Fz3−/− sympathetic axons coursed along the mesenteric vasculature as in wild-type embryos (Fig. 5P, arrows), but failed to enter and innervate the proximal small intestine (Fig. 5R). The attenuated sympathetic innervation of target tissues in E16.5 Fz3−/− embryos cannot be attributed solely to reduced neuronal number. For example, the E16.5 stellate ganglia showed a 56% decrease in cell numbers in Fz3−/− embryos, yet, its target, the heart, showed a complete absence of sympathetic fibers. The more caudal thoracic and lumbar sympathetic chain ganglia showed only modest reductions in size at E16.5 (Fig. 1F, H), while pronounced decreases or absence of innervation were evident in their target organs by this stage. Additionally, in some cases, as in the stomach, kidneys and gastrointestinal tract, sympathetic axons were observed to project along the vasculature right up to the periphery of the target. Together, these results suggest an additional role for Fz3 in regulating sympathetic innervation of peripheral target tissues.

The target-derived neurotrophin, NGF, controls axonal extension and arborization when sympathetic axons have reached their final destinations. The deficits in sympathetic innervation of final peripheral targets observed in Fz3−/− embryos are reminiscent of innervation defects observed in mice lacking NGF (Glebova and Ginty, 2004). To determine whether the diminished innervation in Fz3−/− sympathetic targets reflected an inability of the axons to grow in response to NGF, we assessed NGF-dependent growth in SCG explants and dissociated cultures isolated from E18.5 wild-type and Fz3−/− embryos. SCG explants were cultured in collagen gel for 48 h in the presence of NGF (25 ng/ml). Although the ganglia size in Fz3−/− was severely diminished by E18.5, neurites grew outward in a dense axonal halo, similar to wild-type SCGs (supplemental Fig. 4A, B, available at www.jneurosci.org as supplemental material). There were no significant differences in neurite outgrowth between wild-type and mutant explants (supplemental Fig. 4C, available at www.jneurosci.org as supplemental material) when quantified by measuring the area covered by the axons of each explant relative to the area occupied by the cell bodies. That the Fz3−/− SCG explants showed robust growth and no obvious signs of degeneration in the presence of NGF over the 48 h culture period, also suggests that Fz3 deletion does not affect NGF-dependent survival of sympathetic neurons. Examination of morphologies of isolated sympathetic neurons grown in low-density cultures and immunostained with β-III-tubulin (supplemental Fig. 4D, E, available at www.jneurosci.org as supplemental material) revealed no differences in neurite length (supplemental Fig. 4F, available at www.jneurosci.org as supplemental material) or branching (supplemental Fig. 4G, available at
www.jneurosci.org as supplemental material) between wild-type and Fz3−/− sympathetic neurons. Double immunofluorescence labeling to visualize microtubules and actin filaments did not show any obvious differences in morphology or cytoskeletal architecture in the growth cones of sympathetic neurons isolated from Fz3−/− and wild-type embryos (supplemental Fig. 4 H, I, available at www.jneurosci.org as supplemental material). Together, these results suggest that sympathetic innervation deficits seen in Fz3−/− embryos do not arise from an intrinsic inability to grow and branch in response to NGF, or deficiencies in growth cone morphologies.

During CNS development, Fz3 has been considered to predominantly employ the PCP signaling pathway to mediate axonal growth and targeting (Tissir et al., 2005; Wang and Nathans, 2007; Zhou et al., 2008; Fenstermaker et al., 2010). Additionally, double mutant mice lacking Fz3 and another Frizzled receptor, Frizzled6 show deficits in several PCP-dependent processes including neural tube and eyelid closure, and precise orientation of hair bundles on auditory/vestibular sensory cells (Wang et al., 2006b). Thus, we analyzed sympathetic nervous system development in the looptail mouse, which carries a loss-of-function mutation for a core PCP protein, Van Gogh-like 2 (Vangl2) (Kibar et al., 2001). In contrast to the Fz3−/− embryos, we found no differences in SCG cell number between Vangl2Δ/Δ mutants and wild-type embryos at E16.5 (supplemental Fig. 5A, available at www.jneurosci.org as supplemental material). Whole-mount TH immunostaining also showed normal innervation of target tissues by sympathetic fibers in Vangl2Δ/Δ mice (supplemental Fig. 5B–G). Together, these results indicate that Fz3-mediated regulation of sympathetic nervous system development occurs via a mechanism independent of Vangl2.

Conditional ablation of β-catenin in sympathetic ganglia results in enhanced cell cycle exit and reduced neurogenesis, but does not affect target innervation

To determine the downstream signaling effectors that might mediate the effects of Fz3 in sympathetic ganglia, we then focused our attention on β-catenin, a critical component of the canonical Wnt signaling pathway and cadherin-dependent cell–cell adhesion. Loss-of-function and gain-of-function analyses in mice have established a fundamental role for β-catenin in proliferation and expansion of neuronal progenitors in the cortex, dorsal neural crest precursors and ventral midbrain dopaminergic neurons (Chenn and Walsh, 2002; Megason and McMahon, 2002; Woodhead et al., 2006; Tang et al., 2009, 2010). In perhaps the most noted example, expression of constitutively activated β-catenin in mice results in enlarged brains due to the propensity of cortical precursors to reenter the cell cycle and undergo symmetrical divisions (Chenn and Walsh, 2002). To study the role of β-catenin in sympathetic ganglia, we crossed transgenic TH-Cre mice (Gong et al., 2007) with mice harboring a LoxP-based conditional β-catenin allele (β-cateninlox/lox) (Braull et al., 2001), to ablate β-catenin specifically in cells of the sympathetic lineage. Analyses of the SCGs in TH-Cre;β-cateninlox/lox mice revealed a significant reduction in ganglia size (Fig. 6 A, B) and cell number (Fig. 6C) by E16.5, compared with littermate controls that either lacked the TH-Cre transgene or carried a wild-type β-catenin allele. At E16.5, there were 9145 ± 1977 cells in TH-Cre;β-cateninlox/lox SCG, compared with 15,381 ± 1277 cells in wild-type ganglia. These results indicate a cell-autonomous requirement for β-catenin in TH-expressing sympathetic precursors for early SCG development. The significant reductions in SCG cell number in E16.5 embryos deficient for Fz3 and β-catenin suggested that Fz3 might be acting in part, via β-catenin, to promote neurogenesis. To further test this hypothesis, we monitored cell cycle progression in TH-Cre;β-cateninlox/lox embryos by labeling with EdU for 24 h and coinmunostaining with Ki67, thus analyzing how many progenitors have exited the cell cycle. Indeed, similar to the phenotype seen in E13.5 Fz3−/− embryos, more progenitors exited the cell cycle during the 24 h time window in the TH-Cre;β-cateninlox/lox embryos, compared with wild-type control mice (Fig. 6D). At E13.5, 41% of neuroblasts in TH-Cre;β-cateninlox/lox SCG had exited the cell cycle compared with 29% of wild-type progenitors. Together, these results suggest that, like Fz3, β-catenin signaling in sympathetic ganglia positively regulates neurogenesis by maintaining sympathetic neuroblasts within the cell cycle.

To further investigate whether the loss of β-catenin recapitulates all the sympathetic deficits observed in Fz3−/− embryos, we performed whole-mount TH immunostaining to assess sympathetic innervation of peripheral targets in TH-Cre;β-cateninlox/lox embryos. Remarkably, although the SCG cell number was significantly depleted in TH-Cre;β-cateninlox/lox embryos, the pattern and extent of sympathetic innervation of target tissues in mutant embryos was largely indistinguishable from that in wild-type mice. Unlike Fz3−/− embryos, sympathetic fibers were observed to ramify in the parenchyma of the kidneys (Fig. 6F) and stomach (Fig. 6H, I) and within the proximal part of the small intestine (Fig. 6L, N) in E16.5 TH-Cre;β-cateninlox/lox mice, as in wild-type embryos (kidney, Fig. 6E; stomach, Fig. 6G, H; and intestine, Fig. 6K, M). Thus, although neuronal number is reduced with removal of β-catenin in sympathetic ganglia, axons of remaining neurons can reach their destinations and arborize to fully innervate target tissues. Together, these results suggest that the neurogenesis effect of Fz3 might be transduced via β-catenin signaling within sympathetic ganglia, while β-catenin is dispensable for mediating the Fz3-dependent innervation of sympathetic targets.

Discussion

Sympathetic neuroblasts, often referred to as “young neurons” are the immediate precursors of postmitotic sympathetic neurons, and they undergo multiple rounds of cell division to expand the precursor pool and increase sympathetic ganglia size before exiting the cell cycle. However, the molecular events that regulate expansion of sympathetic neuroblasts remain unknown. Using mice lacking Fz3, we now identify Fz3-mediated signaling as being essential for maintaining the proliferative capacity and survival of sympathetic neuroblasts. Fz3 is expressed in SCG at a stage when it is comprised primarily of proliferating sympathetic neuroblasts. In Fz3−/− mice, sympathetic neuroblasts exit the cell cycle precociously and undergo apoptosis, leading to a depletion of progenitor cells and pronounced reductions in sympathetic ganglia size, as development proceeds. Moreover, conditional inactivation of β-catenin, a cytoplasmic Wnt effector, in cells of the sympathetic lineage resulted in neurogenesis deficits similar to that observed in Fz3−/− mice. Together, these findings provide evidence in support of a Fz3/β-catenin signaling pathway acting within sympathetic neuroblasts to regulate proliferation. Our analyses also uncover a temporally and mechanistically distinct role for Fz3 in promoting innervation of peripheral targets by postmitotic neurons, a function that is independent of β-catenin.

We found no effects of Fz3 deletion on the development of early neural crest cells, migration of neural crest–derived sympathetic progenitors and their acquisition of noradrenergic characteristics. Consistent with the notion that absence of Fz3 does not cause a broad loss of neural crest derivatives, we found no signif-
TH-Cre; \( \beta \text{-catenin}^{\text{flox/flox}} \) (Potzner et al., 2010). Several Sox transcription factors including Sox2 (Van Raay et al., 2005), Sox9 (Blache et al., 2004) and Sox17 (Ye et al., 2009) are downstream mediators of Frizzled signaling and it remains to be determined whether Fz3 and Sox11 are involved in the same pathway that regulates sympathetic neuroblast proliferation.

The similar alterations in cell cycle exit and SCG size in \( Fz3^{-/-} \) mice, and mice with conditional deletion of \( \beta \text{-catenin} \) in the sympathetic lineage suggests that the proliferative effects of Fz3 are likely to be transduced by \( \beta \text{-catenin} \). Previously, deleting \( \beta \text{-catenin} \) in Wnt1-expressing premigratory neural crest cells resulted in reduced sensory ganglia due to a role for \( \beta \text{-catenin} \) in specification of cells in the sensory neuron lineage, but sympathetic ganglia were reported to be normal (Hari et al., 2002). However, in our analyses, removal of \( \beta \text{-catenin} \) in neural crest-derived cells already committed to a sympathetic lineage using \( TH-Cre \) results in normal formation and aggregation of primordial sympathetic ganglia, but pronounced decrease in ganglia size later on due to reduced proliferation of sympathetic neuroblasts. Thus, the timing of \( \beta \text{-catenin} \) removal reveals distinct requirements for \( \beta \text{-catenin} \) during PNS development. \( \beta \text{-Cat} \) could exert its effects by direct or indirect regulation of core components of the cell cycle machinery (Shuttman et al., 1999; Tetsu and McMahon, 1999; Megason and McMahon, 2002; ten Berge et al., 2008). \( \beta \text{-catenin} \) might also influence proliferation of neuroepithelial progenitors through its function as an adhesion protein and regulation of adherens junction integrity and epithelial architecture in the developing cortex (Woodhead et al., 2006; Stocker and Chenn, 2009) and ventral midbrain (Tang et al., 2009). However, in the developing SCG, it remains unclear whether and how cell adhesion defects could underlie the reduced proliferation that we observed in \( \beta \text{-catenin} \) mutant mice.

The loss of Fz3 and presumably \( \beta \text{-catenin} \) in sympathetic ganglia not only causes defects in proliferation, but also affects cell survival. Enhanced apoptosis is seen in early E13.5–E16.5 \( Fz3^{-/-} \) SCG with significant reductions in ganglia size by E16.5 in both \( Fz3^{-/-} \) and \( \beta \text{-catenin} \)-mutant embryos. Several lines of evidence suggest that Fz3 depletion results in the apoptosis of an intermediate sympathetic population that has inappropriately exited the cell cycle, rather than the loss of actively dividing sympathetic precursors or mature postmitotic neurons depending on trophic support from final target tissues. First, we did not detect significant differences in apoptosis of proliferating sympathetic neuroblasts between wild-type and \( Fz3^{-/-} \) embryos. Second, peak levels of apoptosis in the \( Fz3^{-/-} \) SCGs are observed at E16.5, just before the period of active sympathetic innervation of final targets. Concomitantly, reduction in cell numbers in the \( Fz3^{-/-} \)
SCGs is complete by E16.5, and there is no further increase in apoptosis and depletion of cell numbers during the period of target-dependent trophic support of postmitotic sympathetic neurons at P0.5. In contrast, in mice lacking target-derived NGF signaling, excessive cell death in the SCG is first evident at E17.5 and is exacerbated postnatally, with near complete loss of mature sympathetic neurons a week after birth (Fagan et al., 1996; Glebova and Ginty, 2004). Last, the enhanced apoptosis observed in Fz3/H11002 SCGs uses a Bax-independent pathway. The finding that SCG cell death in Fz3/H11002 mice is not prevented by Bax removal contrasts sharply with the protection afforded by Bax elimination in postmitotic sympathetic neurons deprived of NGF (Middleton and Davies, 2001; Glebova and Ginty, 2004). Together, these findings suggest that the precise timing of production of postmitotic neurons is critical during development. Thus, sympathetic neuroblasts that improperly exit the cell cycle in the absence of Fz3 or β-catenin may not be exposed in a timely manner to regulatory signals necessary for proper transition to mature postmitotic neurons, and subsequently undergo cell death.

Previous analyses of Fz3/H11002 mice showed dramatic losses in major axon tracts in the CNS but no phenotypes were observed in the PNS (Wang et al., 2002), presumably because the analyses were performed at early embryonic stages (E12.5) before the formation of sympathetic chain ganglia. We observed a complete absence or marked reduction of sympathetic fibers in many peripheral targets by E16.5 in Fz3/H11002 embryos. Given the demonstrated role of Fz3 in axon guidance in the CNS (Lykusyutova et al., 2003; Wang et al., 2006a), it is possible that failure of Fz3/H11002 sympathetic axons to enter final target areas reflects an axonal guidance function for Fz3. Axonal bundles emanating from the Fz3/H11002 sympathetic ganglia were defasciculated and more disorganized, suggesting that deficits in target innervation could stem, in part, from early navigational errors as soon as axons extend from the ganglia toward the periphery. However, at least for some targets such as the stomach and intestinal tract, Fz3/H11002 sympathetic axons were capable of approaching their final target fields, but failed to enter and fully innervate the entire target fields. Available evidence supports a role for PCP signaling in Fz3/H11002-mediated axonal targeting in the CNS (Bovolenta et al., 2006; Wang and Nathans, 2007; Fenstermäker et al., 2010). Like PCP-dependent processes, axon navigation is a directional cellular behavior that depends on cytoskeletal dynamics. Axonal targeting phenotypes observed in Fz3/H11002 CNS are similar to those in mice lacking another well established PCP gene, Celsr3, encoding for a seven-pass transmembrane cadherin (Tissier et al., 2005; Zhou et al., 2008). We found normal sympathetic innervation of targets in Vangl2H11002 mutant mice, however, it is feasible that Vangl2 might function redundantly with other PCP components such as Celsr3 or Vangl1 in Fz3-dependent targeting of sympathetic fibers. Vangl1 is closely related to Vangl2 (with the proteins sharing 70% similarity) and functions redundantly with Vangl2 to mediate several PCP processes (Torban et al., 2008; Song et al., 2010). Further studies will delineate the contributions of PCP signaling in promoting Fz3-mediated axon targeting in the sympathetic nervous system.

Our work underscores the diversity of functions of a single Frizzled receptor, Fz3, in the developing nervous system. Fz3 function and mechanism of action is strongly context-dependent. In addition to its previously described roles in PCP-dependent processes, axonal outgrowth and guidance in the CNS, we uncover a critical role for Fz3 in sympathetic neurogenesis and target innervation in the PNS in this study.

References
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