Development/Plasticity/Repair

TGFβ Type II Receptor Signaling Controls Schwann Cell Death and Proliferation in Developing Nerves

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During development, Schwann cell numbers are precisely adjusted to match the number of axons. It is essentially unknown which growth factors or receptors carry out this important control *in vivo*. Here, we tested whether the type II transforming growth factor (TGF) β receptor has a role in this process. We generated a conditional knock-out mouse in which the type II TGFβ receptor is specifically ablated only in Schwann cells. Inactivation of the receptor, evident at least from embryonic day 18, resulted in suppressed Schwann cell death in normally developing and injured nerves. Notably, the mutants also showed a strong reduction in Schwann cell proliferation. Consequently, Schwann cell numbers in wild-type and mutant nerves remained similar. Lack of TGFβ signaling did not appear to affect other processes in which TGFβ had been implicated previously, including myelination and response of adult nerves to injury. This is the first *in vivo* evidence for a growth factor receptor involved in promoting Schwann cell division during development and the first genetic evidence for a receptor that controls normal developmental Schwann cell death.

Key words: **Schwann cell; proliferation; death; TGFβ; neuregulin; CRE recombinase**

Introduction

During the development of the nervous system, mitogens, survival factors, and death signals work in concert to generate appropriate cell numbers. We have previously proposed that TGFβ acts as a Schwann cell death signal in developing nerves, a suggestion based on *in vitro* experiments and injections of TGFβ or TGFβ-blocking antibodies *in vivo* (Skoff et al., 1998; Parkinson et al., 2001). Other observations have indicated a more complex involvement of TGFβ in Schwann cell development, because TGFβ also promotes or inhibits Schwann cell proliferation or inhibits myelin gene expression in various *in vitro* models, depending on culture conditions (Eccleston et al., 1989; Ridley et al., 1989; Mews and Meyer, 1993; Scherer et al., 1993; Morgan et al., 1994; Einheber et al., 1995; Guenard et al., 1995a,b; Stewart et al., 1995; Awatramani et al., 2002). These data indicate that the effects of TGFβ on Schwann cells are strongly context dependent. Its role during nerve development is therefore hard to predict.

The use of mouse mutants to determine the function of TGFβ has been hampered by the broad spectrum of abnormalities and early death of mice in which TGFβ-1, -2, or -3 have been inactivated (Geiser et al., 1993; Kulkarni et al., 1993; Dickson et al., 1995; Kaartinen et al., 1995; Sanford et al., 1997). However, the progeny of TGFβ-1-null mice in a nude background survive into adulthood and show uncompacted myelin (Day et al., 2003).

To determine the function of TGFβ in Schwann cells, we used mice in which TGFβ type II receptors (TβRIIs) have been selectively inactivated only in Schwann cells. TGFβ signaling is initiated by binding and bringing together type I and type II receptor serine/threonine kinases on the cell surface (Massague, 1998; Massague et al., 2000; ten Dijke and Hill, 2004). After TGFβ binding, type II receptor phosphorylates the type I receptor kinase domain, which then propagates the signal through phosphorylation of Smad proteins (Shi and Massague, 2003; Izzo and Attisano, 2004).

We report here the generation of a conditional knock-out mouse in which the TGFβ receptor type II is specifically ablated only in Schwann cells. To do so, we crossed mice carrying a floxed type II receptor (Cazac and Roes, 2000) with P0 CRE mice, in which the CRE recombinase protein is expressed under the control of the myelin P_0 protein promoter (Feltri et al., 1999). We find that mice lacking TGFβ signaling to Schwann cells have a reduced rate of Schwann cell death in embryonic and perinatal nerves. Likewise, injury-induced Schwann cell death in neonates is suppressed. Notably, these mice also show a strong reduction in Schwann cell proliferation.

The intriguing fact that the type II TGFβ receptor is involved in regulating these two distinct developmental programs indicates that TGFβ is a key part of the mechanism that ensures correct Schwann cell numbers in developing nerves. It also points to the signaling complexity in perinatal nerves, raising the question of how the dual action of TGFβ is controlled and integrated with other signals.

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Materials and Methods

Generation of P0 CRE/TβRII$^{f/f}$ mice. Mice homozygous for the TβRII$^{f/f}$ locus (Cazac and Roes, 2000) were crossed with P0 CRE mice (Feltri et al., 1999) to obtain P0 CRE/TβRII$^{f/+}$ mice, which were then back-crossed with TβRII$^{f/f}$. The resulting P0 CRE/TβRII$^{f/f}$ mutants, which delete TβRII efficiently in Schwann cells, were used as a test group. TβRII$^{f/f}$, TβRII$^{f/+}$, and P0 CRE/TβRII$^{f/+}$ littermates were used as controls.

Southern blot. The efficiency of recombination was established on Southern blots performed on genomic DNA extracted from tails (control) and purified Schwann cells from postnatal day 5 (P5) P0 CRE/TβRII$^{f/f}$ and P0 CRE/TβRII$^{f/+}$ mice. DNA was digested with the restriction enzyme *Nco*I, separated by 1% agarose gel electrophoresis, and transferred to a nylon membrane (Duralon-UV; Stratagene, La Jolla, CA). TβRII exon II probe (Cazac and Roes, 2000) was radiolabeled using Prime-a-Gene Labeling System (Promega, Southampton, UK) following the manufacturer's instructions. Deletion efficiency of the TβRII$^{f/f}$ loci was assessed with ImageJ.

Peripheral nerve injury experiments. All of the experiments with animals were performed following United Kingdom Home Office guidelines. P1 or adult transgenic mice were anesthetized with halothane, and the right sciatic nerve was exposed at midthigh level and either crushed (3 times, 10 s each) or cut (in which case the distal stump was diverted to limit the possibility of religation). Resulting wounds were sutured with 3/0 or 8/0 black polyamide monofilament, Mersilk (Johnson & Johnson, New Brunswick, NJ). At the relevant day after injury, the animals were killed and both the distal stump of the right, injured sciatic nerve and the contralateral control nerve were excised, frozen, and immediately processed for mRNA or protein extraction or embedded in OCT compound (Agar Scientific, Stansted, UK) for immunohistochemistry.

Electron microscopy. Mutant and control mice of various ages were killed with a method appropriate for the age of the animal. Electron microscopy (EM) analysis of sciatic nerve sections was essentially performed as described previously (Wrabetz et al., 2000). Ultrathin sections were taken using an Ultracut E ultramicrotome (Leica, Nussloch, Germany). EM sections were viewed in a Jeol 1010 electron microscope (Jeol, Peabody, MA), and images were captured on x-ray film (Ilford, Ilford, UK). Films were printed using a DeVere enlarger (KHB Photographix, Mississauga, Ontario, Canada) on Ilford multigrade paper using Ilford multigrade gel filters.

Schwann cell cultures. Cultures of Schwann cells from embryonic day 18 (E18) and P1, P3, and P5 sciatic nerves were prepared essentially as described previously (Jessen et al., 1994; Parkinson et al., 2001) and purified by negative immunopanning on dishes coated with Thy1.1 antibodies (Dong et al., 1997).

For survival assay and test of TGFβ-induced apoptosis, freshly purified Schwann cells were plated on poly-D-lysine-coated coverslips. TGFβ-induced apoptosis was tested as described previously (Parkinson et al., 2001). For the serum deprivation assay, Schwann cells from P3 sciatic nerves from mutant and control mice were plated at high density (2000 cells per 15 μl per coverslip) on poly-D-lysine coverslips. Cells were cultured in DMEM with 10% fetal calf serum (FCS) for 16 h. At this point, half of the coverslips were fixed in 4% paraformaldehyde (PF) for 10 min, washed for 20 min in PBS, and immunolabeled with L1 or S100 to estimate purity. They were also stained with Hoechst nuclear dye. The rest of the coverslips had their medium changed to DMEM alone and were left for at least 72 h, after which they were fixed and immunolabeled. The number of surviving cells is indicated as survival percentage, that is, the number of living cells present at the end of the experiment as a percentage of the living cells after 16 h.

Western blotting. Extracts from frozen tissues were prepared and blotted as described previously (Parkinson et al., 2004). Primary antibody dilutions were as follows: anti-mouse P_0 (1:1000; gift from J. Archelos, Karl-Franzens University, Graz, Austria), anti-rabbit periaxin (1:10,000; gift from P. Brophy, Edinburgh University, Edinburgh, UK), anti-rat L1 (1:100; gift from R. Martini, Würzburg University, Würzburg, Germany), and anti-mouse β-tubulin (1:5000; Sigma-Aldrich, Gillingham, UK). Blots were then incubated with horseradish-conjugated secondary antibody and developed with ECL reagent (Amersham Biosciences, Essex, UK).

RNA isolation and reverse transcription-PCR. RNA isolation, reverse transcriptase reaction, and reverse transcription (RT)-PCR experiments were performed as described previously (D'Antonio et al., 2006). Primers for periaxin were as described by Parkinson et al. (2003). Primers for P_0 were as follows: P_0 sense, 5′-GTCCAGTGAATGGGTCTCAG-3′; P_0 antisense (AS), 5′-GCTCCCAACAACACCCCATA-3′. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: GAPDH sense, 5′-ACCACAGTCCATGCCATCAC-3′, GAPDH AS, 5′-TCCACCACCCTGTTGCTGTA-3′.

Immunofluorescence. After fixation in 4% PF in PBS for 10 min, cultured cells were blocked and permeabilized in antibody-diluting solution (ADS) (PBS containing 10% FCS, 0.1 M lysine, and 0.02% sodium azide) containing 0.2% Triton X-100 for 30 min to 1 h. Antibodies for immunolabeling were diluted in ADS as follows: rabbit anti-P_0, 1:500 (Morgan et al., 1994); rabbit anti-S100 (1:2000; DakoCytomation, Ely, UK); rabbit anti-periaxin, 1:8000; mouse anti-Smad2 (1:500; BD Biosciences, Bedford, MA); and mouse anti-Smad4 (1:400; Santa Cruz Biotechnologies, Santa Cruz, CA). The signal was detected with secondary antibody conjugated to either FITC or tetramethylrhodamine isothiocyanate, diluted in ADS.

Mouse sciatic nerves of different ages were embedded in OCT compound and fresh frozen in liquid N_2. Transverse sections (6–8 μm) were dried for 45 min, fixed in 4% PF, and incubated with primary antibody. Slides were mounted in Citifluor (Citifluor, London, UK) and examined with a fluorescence microscope (Eclipse E800; Nikon, Kingston upon Thames, UK). Images were captured with a digital camera (DMX1200; Nikon) and ACT-1 acquisition software (Nikon). UMAX PowerLook II (UMAX Technologies, Dallas, TX) was used to digitalize the images.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assay. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay was performed as described previously (Grinspan et al., 1996; Feltri et al., 2002) with some minor modifications. Briefly, 6–8 μm cryostat sections of fresh-frozen nerves from E18, P2, and adult mice were fixed by submersion in 4% PF for 10 min at room temperature and labeled for S100 as described by Parkinson et al. (2001) (data not shown). The sections were then washed in PBS and preincubated in terminal transferase buffer for 15 min at room temperature. Terminal transferase and biotinylated-D-UTP (Roche Diagnostics, Mannheim, Germany) were added to the sections in a TUNEL reaction mixture, as recommended in the manufacturer's protocol, and incubated at 37°C for 60 min. Nonspecific binding sites were blocked using PBS with 10% FCS for at least 60 min at room temperature. The sections were then incubated with streptavidin conjugated to Cy3 (cyanine 3), at a concentration of 1:50 in ADS with 0.1% Triton X-100 for 30 min at room temperature. Nuclei were then counterstained with Hoechst dye. For quantification, only Hoechst-positive nuclei associated with nerves were counted, and the fraction of TUNEL-positive nuclei was determined. At least 10,000 nuclei per animal at each time point were examined.

Proliferation assay. The ratio of cells undergoing proliferation *in vivo* was measured with phospho-histone-3 (PH3) immunolabeling. Cryostat sections (6–8 μm thick) were cut from fresh-frozen sciatic nerves at different ages, mounted on Superfrost slides, and allowed to dry for 45 min to 1 h at room temperature. Sections were fixed for 10 min with 4% PF and, after washings, were blocked using ADS for 1 h. Anti-rabbit PH3 antibody (Upstate, Charlottesville, VA), diluted 1:5000 in ADS, was applied either for 1 h at room temperature or overnight at 4°C. Nuclei were labeled with Hoechst dye. At least 10,000 nuclei per animal were examined.

The bromodeoxyuridine (BrdU) *in vitro* incorporation assay was performed essentially as described by Stewart et al. (1993). Briefly, Schwann cells from P3 wild-type and mutant mice were isolated, purified, and plated (2000 cells per 15 μl per coverslip) on poly-L-lysine–laminin-coated coverslips. Cells were cultured in defined medium (DM) (Jessen et al., 1994) plus insulin (1 μM) plus 0.5% FCS for 16 h, after which the medium was changed to DM plus insulin plus neuregulin-1 (R&D Sys-

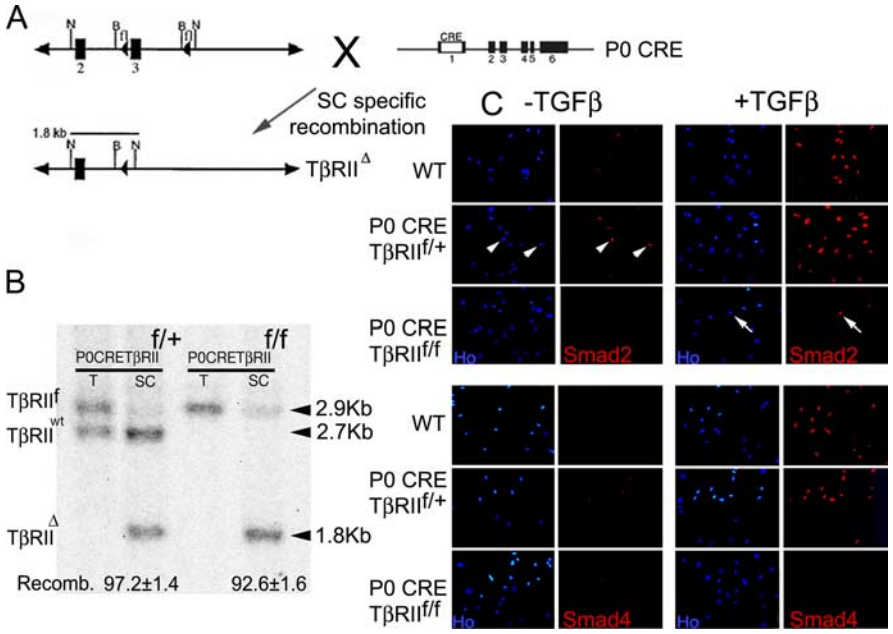

Figure 1. Inactivation of the TβRII locus and assessment of the efficiency of deletion. ***A***, Schwann cell (SC)-specific inactivation of the TβRII locus, obtained by crossing TGFβRII $^{f/f}$ mice with P0 CRE mice. The length of the recombined restriction fragment in Southern blot (1.8 kb) is indicated. ***B***, Southern blot of *Nco*I-digested DNA from tail (T) or purified Schwann cells from P0 CRE/TβRII $^{f/+}$ or P0 CRE/TβRII $^{f/f}$ P5 mice ($n = 5$). Efficiency of deletion, normalized for cell purity (~90%, established by S100 staining) (data not shown), is indicated. Recomb., Percentage of recombination. ***C***, Nuclear localization of Smad2 and Smad4 was essentially absent in P0 CRE/TβRII $^{f/f}$ mice even after TGFβ-1 treatment. Very occasionally, nuclear staining was detectable in the mutant mice (arrows), indicating that the recombination, although highly successful, is probably not complete. In some cases in the control wild type or P0 CRE/TβRII $^{f/+}$, nuclear localization is detectable even in the absence of TGFβ-1 (arrowheads). This is possibly attributable to the action of endogenous TGFβ. Ho, Hoechst nuclear dye; WT, wild type.

tems, Minneapolis, MN) (0, 1, 2, 5, and 10 ng) for 10 h. When TGFβ receptor inhibitor 4-(5-benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1*H*-imidazol-2-yl)-benzamide hydrate (SB431542; Tocris Biosciences, Bristol, UK) was included, it was added at a concentration of 10 μM, 30 min before the addition of neuregulin-1. BrdU staining was then performed as described previously (Stewart et al., 1993). To evaluate the cooperation between TGFβ and neuregulin-1 in promoting proliferation, sciatic nerve and brachial plexi of P3 wild-type mice were dissected and purified for 3 d in DMEM plus 5% horse serum containing 10 μM cytosine arabinoside. The cells were then plated on laminin-coated coverslips at a density of 5000 cells per 15 μl drop in DM plus 0.5% horse serum plus insulin (1 μM) and left overnight to flatten down. The next day, the medium was changed to DM plus insulin (1 μM) (no serum), and neuregulin-1 (1, 2, and 5 ng) and TGFβ (5 ng) were added. After 24 h, BrdU was added and the immunolabeling was performed as described previously (Stewart et al., 1993).

Cell counts. Sciatic nerves were dissected from P15 P0 CRE/TβRII $^{f/f}$ or wild-type mice, carefully desheathed (to minimize the contribution of perineurial cells) and embedded in OCT compound. Sections (10 μm) were cut and the nuclei were labeled with Hoechst dye. Equal areas from every section were selected and, using ImageJ, the threshold image program was used to highlight nuclei. The areas of individual nuclei were calculated using the particle analysis program, and the areas of the nuclei were summed. On the assumption that the average nuclear size is the same in the two genotypes, the sum of the area is proportional to the number of cells. Seven animals per genotype were used, with wild-type and mutant animals being littermates from four different litters. Ten to fifteen sections of each nerve were analyzed, the nuclear area per genotype was calculated and averaged, and the SEM was derived.

Statistical analysis. The statistical significance of differences between the experimental groups was analyzed using a two-tailed Student's *t* test.

Results

Generation of the mice and calculation of the efficiency of recombination

To disrupt the TβRII gene specifically in Schwann cells, TβRII $^{f/f}$ mice (Cazac and Roes, 2000) were crossed with P0 CRE mice (Feltri et al., 1999), which induce efficient Schwann cell lineage-specific deletion of loxP-flanked target sequences (Feltri et al., 2002; Saito et al., 2003) (Fig. 1*A*). In the TβRII $^{f/f}$ mouse, loxP sites flank exon 3 of the TβRII gene, which encodes for the membrane-proximal extracellular part of the protein (Cazac and Roes, 2000). If exon 3 is ablated, the resulting protein is truncated and translation terminates before the extracellular domain. This should result in the complete inactivation of the type II receptor.

To determine the recombination efficiency, Southern blot analysis of Schwann cells isolated from the sciatic nerve of P5 P0 CRE/TβRII $^{f/+}$ and P0 CRE/TβRII $^{f/f}$ mice was performed (Fig. 1*B*). Densitometric analysis of the Southern blot indicated that the recombination efficiency, normalized for cell purity (~90% by S100 staining) (data not shown), was >92% in both heterozygous and homozygous floxed mice, a value that should guarantee that the effects of the lack of TGFβ signaling are not masked by the presence of unrecombined cells.

To confirm this result, we also used an indirect approach. TGFβ binding to the type II receptor results in type I receptor recruitment and phosphorylation, which in turn phosphorylates R-Smads (Smad1, 2, 3, 5, and 8) (for review, see Shi and Massague, 2003; Izzo and Attisano, 2004). Phosphorylated R-Smads then form a heterodimeric complex with the Co-Smad, Smad4. The activated complex is translocated to the nucleus, in which, in collaboration with cofactors, it regulates gene transcription. If the TGFβ type II receptor was lost, TGFβ should not be able to drive Smad into the nucleus. We therefore cultured P5 Schwann cells from TβRII $^{f/f}$ (from here on called wild type), P0 CRE/TβRII $^{f/+}$, and P0 CRE/TβRII $^{f/f}$ mice (the latter from here on often called mutant). After 24 h, the cells were stimulated with 10 ng/ml TGFβ-1 for 30 min, fixed, and immunolabeled with antibodies against Smad2 and Smad4 (Fig. 1*C*). In the cells isolated from P0 CRE/TβRII $^{f/f}$ mice, there was essentially no nuclear localization when compared with wild-type and P0 CRE/TβRII $^{f/+}$ cells, indicating that at P5 the recombination is almost complete.

Mutant mice myelinate correctly

Previous studies indicated that, in DRG neuron/Schwann cell cocultures *in vitro*, TGFβ-1 inhibits Schwann cell proliferation, suppresses P_0 and galactocerebroside induction, and blocks myelination (Einheber et al., 1995; Guenard et al., 1995a,b). Therefore, we asked whether TGFβs were negative regulators of myelination *in vivo*. To answer this, we tested whether myelination occurred prematurely or more extensively in mice without TGFβ receptor using EM. Figure 2 shows that at P1, Schwann cells had started segregating the large-caliber axons into a 1:1 relationship

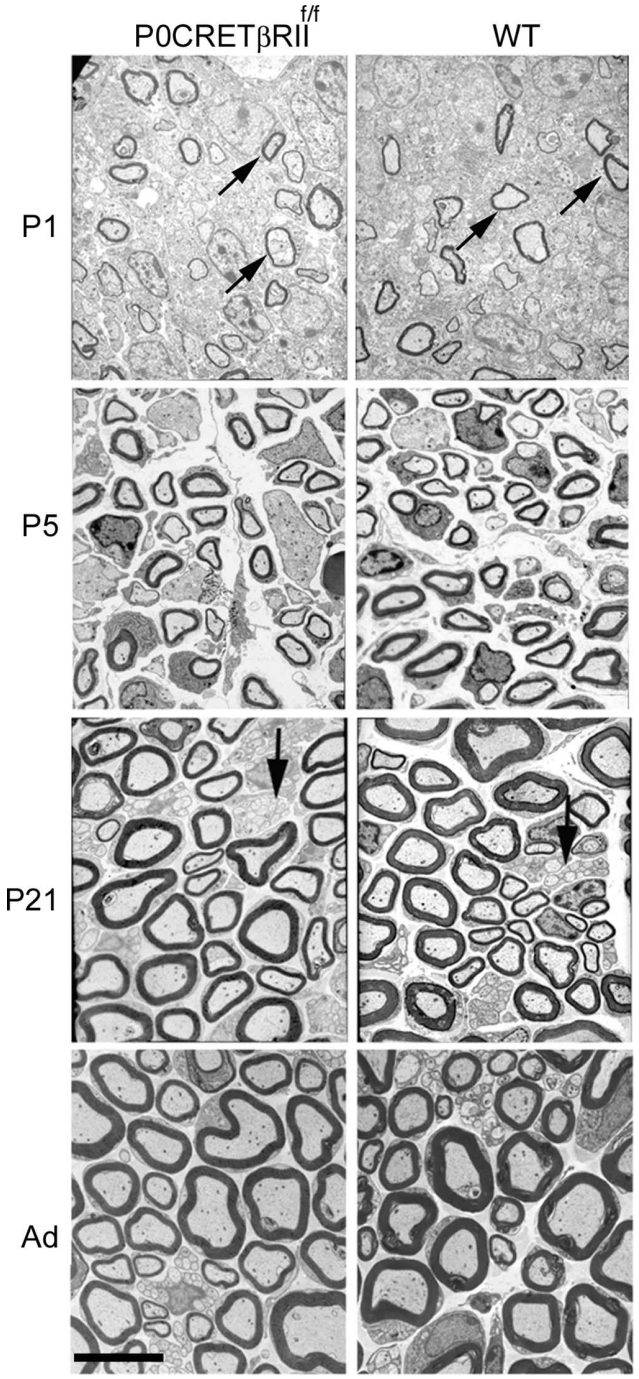

Figure 2. P0 CRE/TβRII $^{f/f}$ mice myelinate normally. Transverse sections of sciatic nerves from P1, P5, P21, and adult (Ad; 4–6 months) wild-type (WT) and mutant mice were analyzed by EM. At every age, the mutant nerve appears indistinguishable from the wild type. Similarly to the wild type, in the mutant nerve at P1, Schwann cells are starting to correctly segregate the large-caliber axons in a 1:1 relationship, and a thin myelin sheath is already visible around some of them (arrows). At P5, in both wild-type and mutant nerves, large-caliber axons have reached the 1:1 relationship with Schwann cells, and most of them are surrounded by a thickening myelin sheath. At P21, myelination is almost complete. Bundles of nonmyelinated axons are segregated from each other and surrounded by nonmyelinating Schwann cells, forming Remak fibers (arrows). Similarly, the adult nerve does not show any difference from the wild type. Scale bar, 10 μm.

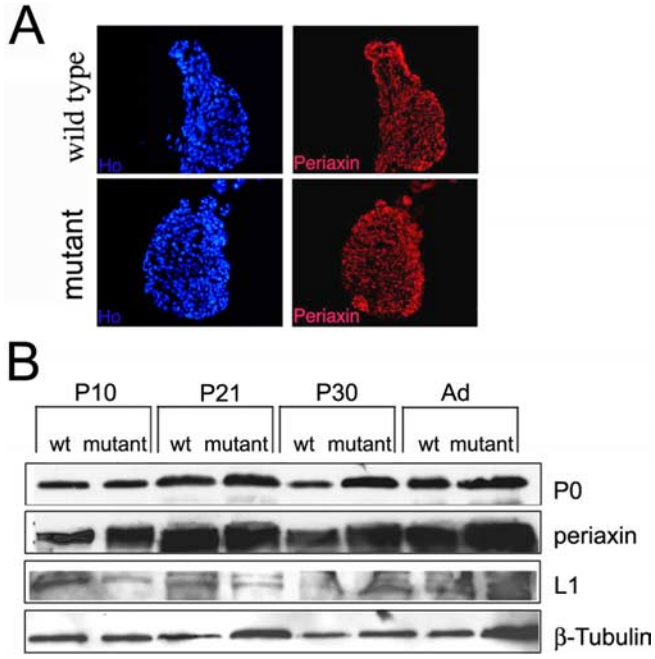

Figure 3. Expression of Schwann cell-specific proteins is normal in the P0 CRE/TβRII $^{f/f}$ mouse. ***A***, Transverse sections of newborn mutant and control wild-type sciatic nerves were immunolabeled with antibodies against the myelinating Schwann cell-specific protein periaxin (red). Hoechst (Ho) staining is shown in blue. No differences were observed between mutant and wild type in periaxin, indicating that precocious myelination in the mutant is unlikely. ***B***, Western blot of protein extracts from mutant and wild-type (wt) mice at P10, P21, P30, and adult. No striking differences (as measured by densitometry, normalized with β-tubulin) are detectable in the expression of the myelin proteins P_0 and periaxin and of the nonmyelinating Schwann cell-specific protein L1.

and that in many cases myelination had started, with one or more loops of myelin already formed. No significant differences between the wild-type and the mutant mice were detectable. By P5, essentially all large-caliber axons were segregated, and myelination was proceeding correctly. At P21, in both wild-type and mutant nerves, myelination was nearly completed, and smaller caliber axons were segregated into bundles surrounded by a single nonmyelinating Schwann cell. In addition, the myelin sheath appeared morphologically normal and stable in the adult (up to 1 year old), with no signs of demyelination or onion bulbs. Moreover, measurement of the g-ratio (the ratio between the axon diameter and the diameter of the axon plus myelin) showed that it was not significantly different between wild-type and mutant mice (data not shown).

Myelin-related protein expression is normal in the mutant mice

As mentioned previously, TGFβ-1 suppresses the expression of myelin-related proteins such as P_0 and galactocerebroside *in vitro* (Morgan et al., 1994; Einheber et al., 1995). Lack of TGFβ signaling could therefore be expected to result in precocious or increased expression of myelin-related proteins, although the EM analysis showing apparently normal myelin (above) renders this less likely. To test this, we immunolabeled newborn sciatic nerves from wild-type and mutant mice with an antibody against the myelinating Schwann cell proteins periaxin and P_0. No obvious differences were detectable between the wild-type and P0 CRE/TβRII $^{f/f}$ mice (Fig. 3*A* and data not shown). Similarly, no differences were seen when we immunolabeled newborn sciatic nerves with an antibody against p75NTR and L1 (data not shown). Moreover, Western blot experiments performed on sciatic nerve extracts during the active phases of myelination (P10, P21, and P30) and in the adult showed that wild-type and mutant mice

expressed similar amounts of the myelin-specific proteins P_0 and periaxin and of the nonmyelinating Schwann cell-specific protein L1 (Fig. 3*B*).

Together, these structural and biochemical data show that TGFβ signaling through type II TGFβ receptors does not have a major role in the regulation of myelination in developing nerves, although they do not exclude the existence of subtle myelin abnormalities.

In the absence of TGFβ receptors, Schwann cell death is suppressed in developing and injured nerves

Previous experiments demonstrated that TGFβ-1 can induce apoptosis in freshly isolated Schwann cells from newborn rat nerves and that injection of TGFβ-1 into injured neonatal nerves increases Schwann cell death (Skoff et al., 1998; Parkinson et al., 2001). These observations raise the possibility that TGFβ functions as a death signal during Schwann cell development. To test this hypothesis, we immunolabeled longitudinal sections of sciatic nerves from E18 wild-type and mutant mice with the TUNEL technique, to detect nuclear fragmentation. These experiments revealed that the percentage of TUNEL-positive nuclei was considerably lower in mutant nerves than in wild-type ones, decreasing from 0.41 to 0.16% (Fig. 4*A*) ($*p < 0.005$). Similarly, Schwann cell apoptosis in P2 nerves was significantly reduced, from 0.34% in the wild-type controls to only 0.09% in mutant mice (Fig. 4*A*) ($**p < 0.001$).

In normal neonatal nerves, Schwann cell survival is likely to depend on the combined action of axonally derived neuregulin-1 and autocrine signals (Meier et al., 1999). If the neonatal nerve is transected, the survival of Schwann cells is sustained only by autocrine signals, and, although most of the cells survive, death by apoptosis is greatly increased (Grinspan et al., 1996). Moreover, if TGFβ is injected in the distal stump of a wild-type transected nerve, the number of TUNEL-positive nuclei is increased further, whereas death is suppressed by injection of blocking TGFβ antibody (Parkinson et al., 2001). This indicates that TGFβ also contributes to Schwann cell death in injured nerves.

To test this conclusively, we performed TUNEL staining on nerve sections from wild-type and P0 CRE/TβRII$^{f/f}$ mice 24 h after transection of P1 nerves. This showed that in the wild-type mice, cell death increased 13.85-fold, from 0.34 to 4.71%, in agreement with previous results (Grinspan et al., 1996; Syroid et al., 1996, 2000; Parkinson et al., 2001). Similarly, in the mutant nerves, Schwann cell death increased 13.22-fold, from 0.09 to 1.19%, which means that injury-induced Schwann cell death remained fourfold lower than in the wild-type mice ($**p < 0.001$) (Fig. 4*B*,*C*).

These experiments, in conjunction with previous data, show that TGFβ acts as a death signal both in normal Schwann cell development and during the wave of Schwann cell death that follows injury in newborn nerves.

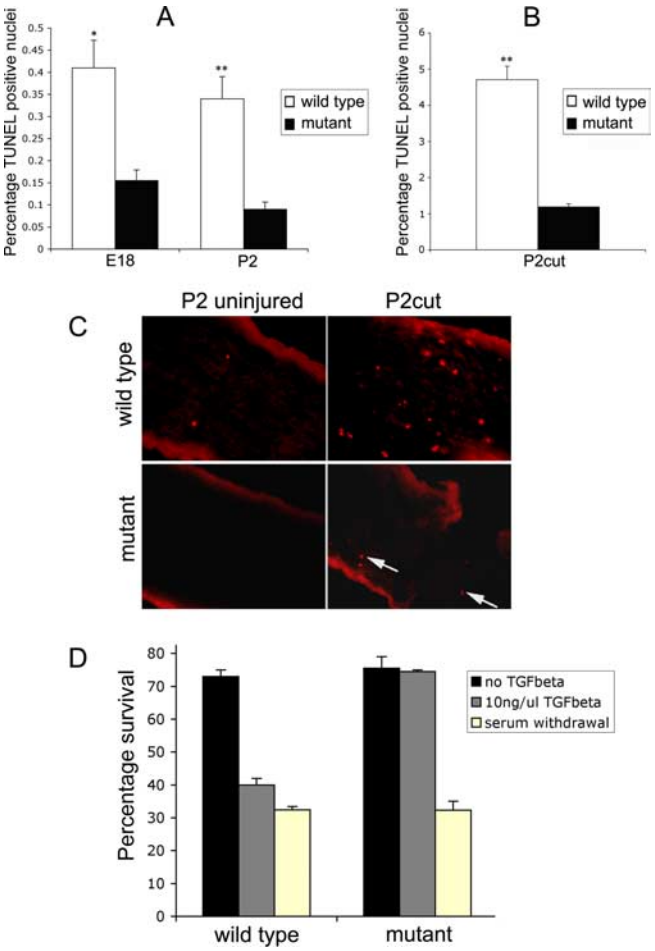

Figure 4. Schwann cell death is reduced during development and after injury in neonatal nerves of P0 CRE/TβRII$^{f/f}$ mice. *In vitro*, Schwann cells from mutant mice are not sensitive to TGFβ killing, although they die normally after serum withdrawal. ***A***, Percentages (mean ± SEM; $n = 10$) of TUNEL-positive nuclei in transverse sections of E18 and P2 sciatic nerves from wild-type and mutant mice. At both time points, the rate of death is significantly reduced in the mutant compared with the wild type (see Results). ***B***, ***C***, Sciatic nerves from P1 wild-type and mutant mice ($n = 7$) were transected, and after 24 h, TUNEL was performed on longitudinal sections. In the distal stump of the transected nerve, Schwann cell death increased significantly in both the wild type and the mutant (arrows in ***C***). However, in the mutant, it remained 3.95-fold ($**p < 0.001$) lower than in the wild type (see Results). ***D***, Death of mutant cells *in vitro*. When exposed to TGFβ, only 40 ± 2% of the wild-type cells survived after 24 h, whereas 73 ± 2% of the mutant cells were still alive ($n = 3$; $*p < 0.005$). This is identical to survival of the untreated cells, showing that the mutant cells are completely protected from TGFβ-induced death. In contrast, when wild-type or mutant cells were subjected to 10% serum withdrawal, death rates were similar, suggesting that the reduced cell death in the mutant was a specific response to TGFβ signaling ($n = 3$). P2cut, P2 nerve transfected at P1.

In vitro, mutant Schwann cells are resistant to TGFβ killing, although they die normally when deprived of serum

To test the hypothesis that the reduction in cell death in perinatal nerves of P0 CRE/TβRII$^{f/f}$ mice is caused by the loss of sensitivity of individual Schwann cells to TGFβ killing, we performed an *in vitro* survival assay in the presence of TGFβ. Freshly isolated Schwann cells from the sciatic nerves of newborn mutant and wild-type mice were plated at a density of 3000 cells per coverslip on a laminin substrate. Cells were allowed to attach to the substrate for ~3 h and were then exposed to 10 ng/ml TGFβ-1 for 1 d. At the end of the experiment, the cultures were fixed and stained with Hoechst dye to visualize cell nuclei. The number of living cells is expressed as survival percentage, that is, the number of living cells present at the end of the experiment as a percentage of the number of cells present (i.e., that had attached) on sister coverslips after 3 h (Parkinson et al., 2001). We observed that TGFβ killed more than half of the wild-type cells during the 24 h period, as expected from previous work. In contrast, TGFβ-induced death was completely blocked in cells from P0 CRE/TβRII$^{f/f}$ mice (Fig. 4*D*). A different result was seen when wild-type and mutant cells were subjected to 10% serum withdrawal, a treatment known to induce Schwann cell apoptosis (Syroid et al., 1996; Parkinson et al.,

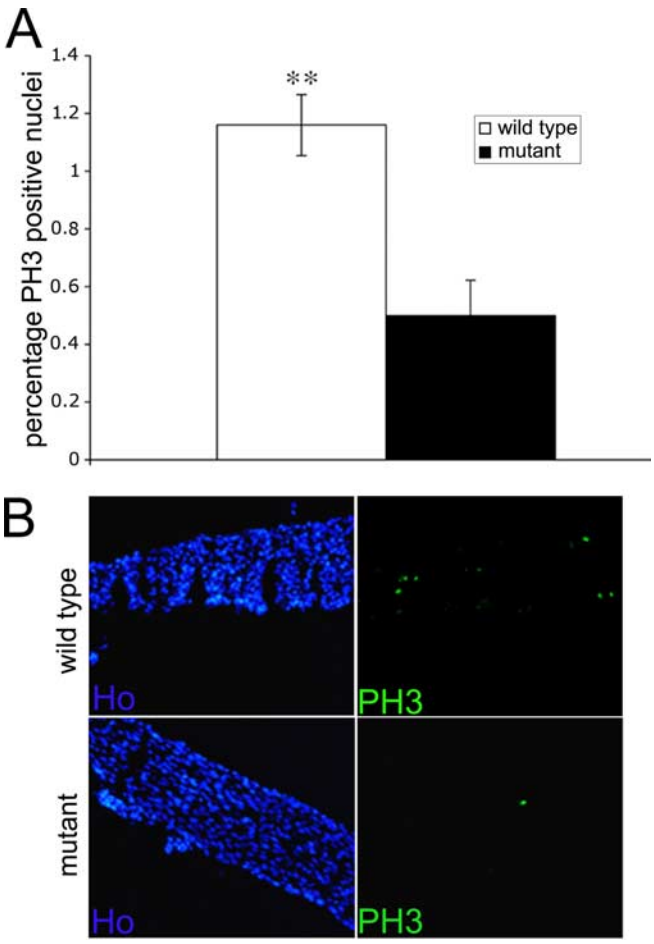

Figure 5. Schwann cell proliferation is reduced during development in P0 CRE/TβRII$^{f/f}$ mice. The final number of cells is similar in mutant and wild-type nerves. Longitudinal sections from E18 sciatic nerves were immunolabeled with an antibody to PH3, which specifically marks the cells that are dividing. ***A***, In the mutant nerve, the number of dividing cells (mean ± SEM; $n = 10$) is reduced from 1.15 ± 0.1 to 0.45 ± 0.12% ($n = 8$; $^{**}p < 0.001$). ***B***, Comparison between a wild-type and a mutant nerve: whereas in the wild type, several dividing nuclei are identified, in the mutant nerve, the number of PH3-positive nuclei is significantly reduced. Ho, Hoechst dye.

2001). In this case, similar death rates were seen in both types of cell (Fig. 4*D*).

These experiments indicate that the reduced death of mutant cells *in vitro* and *in vivo* was seen specifically in response to TGFβ and that general cell death mechanisms were unaffected by the loss of type II receptors.

TGFβ drives Schwann cell proliferation in developing nerves

In purified Schwann cell cultures, TGFβ, in the presence of serum or cAMP-elevating agents or both, promotes Schwann cell DNA synthesis and proliferation (Eccleston et al., 1989; Ridley et al., 1989). We therefore asked whether TGFβ controlled Schwann cell proliferation *in vivo*. This was tested by labeling longitudinal sections of E18 sciatic nerves from wild-type and mutant mice with an antibody against PH3, which specifically labels those cells that are actively dividing. Figure 5 shows that in the mutant nerves, the percentage of PH3-positive nuclei is reduced 2.3-fold when compared with the wild-type controls ($^{**}p < 0.001$).

These results indicate that *in vivo* TGFβ is not only a killing signal but paradoxically also a mitogen. If this is true, the final number of cells in wild-type and mutant nerves might not be very different, because these two processes would tend to cancel each other out. To test this, we compared the number of cells present in P15 nerves by labeling a series of transverse sections from wild-type and mutant mice with Hoechst dye and used ImageJ to measure the intensity of staining, which is directly proportional to the number of nuclei present. In the wild-type nerves, we found that total fluorescence per micrometer was very similar from section to section and animal to animal, as expected if this method is a faithful indicator of the number of nuclei (i.e., cells) present (data not shown). This experiment showed that P15 nerves of wild-type and mutant mice contained comparable numbers of cells (data not shown).

TGFβ receptor type II recombination has already occurred at E18

For the observed reductions in Schwann cell death and proliferation in mutant mice to be attributable to the loss of type II receptor and therefore of TGFβ signaling, we needed to verify that by E18 the recombination of the floxed segment had already occurred. To do so we used the indirect approach already used at P5 and immunolabeled cultured Schwann cells from E18 wild-type and mutant mice with Smad2 and Smad4 antibodies after treatment with TGFβ. Schwann cells from mutant and wild-type E18 embryos were plated at 3000 cells per coverslip in DM plus 0.5% FCS, treated for 30 min with 10 ng/ml of TGFβ1, fixed, and immunolabeled with antibodies to Smad2 or Smad4. These experiments showed that in the mutant mice there was essentially no nuclear localization of Smad proteins (Fig. 6), indicating that the recombination had already occurred at least as early as E18, as expected from other studies with the P0 CRE mice used here (Feltri et al., 2002; Saito et al., 2003). This is consistent with the view that the phenotypes described above are attributable to the absence of type II TGFβ receptors.

TGFβ cooperates with neuregulin-1 in promoting Schwann cell proliferation *in vitro*

The experiments above show that there is a significant reduction in Schwann cell proliferation in mice lacking TGFβ signaling to Schwann cells. The simplest interpretation is that TGFβ is a mitogen in developing nerves. Although the evidence at present derives only from cell cultures, neuregulin-1 is often considered the major axonally derived Schwann cell mitogen (for review, see Jessen and Mirsky, 2004, 2005). This raised the possibility that neuregulin-1 and TGFβ cooperate in driving Schwann cell proliferation. To test this, we used cultures of Schwann cells from neonatal nerves to compare the proliferative response of wild-type and mutant cells to neuregulin-1. This revealed that the mutant cells synthesized less DNA than the wild-type ones (Fig. 7*A*). Schwann cells express and secrete TGFβ *in vitro* (Stewart et al., 1995), and it seemed possible that the reduced DNA synthesis was caused by the absence of cooperative TGFβ input in the mutant cells. To test this, wild-type cells were exposed to neuregulin-1 in the presence of TGFβ receptor blocker. This reduced DNA synthesis to levels comparable with those seen in mutant cells (Fig. 7*A*). This indicated the presence of TGFβ in the culture medium and implied that TGFβ and neuregulin-1 cooperate in driving Schwann cell DNA synthesis. To show this directly, cultured Schwann cells from wild-type P3 nerves were exposed to increasing concentrations of neuregulin-1, first in the presence and then in the absence of TGFβ. This showed that TGFβ strongly promoted DNA synthesis induced by low concentrations of neuregulin-1 (Fig. 7*B*).

These results, together with the *in vivo* observations (above),

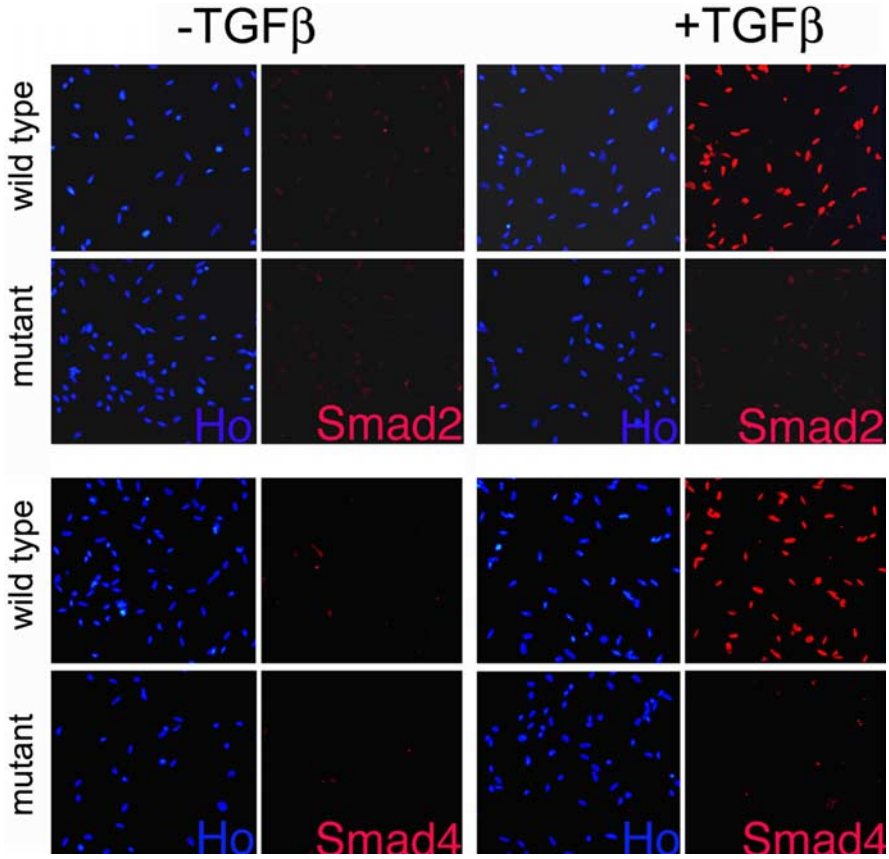

Figure 6. TGFβRII recombination has already taken place by E18. Schwann cells from mutant and wild-type mice were labeled with antibodies to Smad2 or Smad4 (red). Essentially no nuclear localization of Smad2 or Smad4 is detectable in the mutant cells from E18 animals, suggesting that recombination has already taken place at this time. Nuclei were visualized with Hoechst dye (Ho; blue).

raise the possibility that in perinatal nerves, neuregulin-1 from axons and TGFβ from the Schwann cells themselves and/or axons act together to stimulate Schwann cell division.

Schwann cell death and proliferation are not affected in adult nerves from P0 CRE/TβRII$^{f/f}$ mice in normal circumstances or after axotomy

In the normal adult nerve, Schwann cells are present in a quiescent state. After nerve injury, they undergo a wave of proliferation that is part of the process of Wallerian degeneration. In contrast to what happens in the perinatal nerve (above), however, Schwann cells in the adult nerve do not undergo increased apoptosis after axotomy (Scherer et al., 1993; Grinspan et al., 1996; Scherer and Salzer, 2001). Nevertheless, TGFβ-1 mRNA levels are upregulated in the distal stump after axotomy (Scherer et al., 1993), suggesting that TGFβ could have a role in the early events after nerve injury. Therefore, we tested cell death and proliferation in normal and axotomized nerves from P0 CRE/TβRII$^{f/f}$ and wild-type mice. No apoptosis or proliferation was detectable in the uninjured adult nerves of either wild-type or mutant mice (data not shown). Moreover, 7 d after nerve cut, there was essentially no detectable apoptosis in the wild-type or the mutant nerves (data not shown). In contrast, nerve transection clearly triggered proliferation, as indicated by PH3 staining (Fig. 8*A*). However, no significant difference in the proliferation rate was detectable between the wild-type and mutant nerves.

These experiments show that TGFβ is unlikely to have a significant role in the control of cell death and division in injured adult nerves.

Myelin-related proteins are correctly downregulated in mutant nerves after nerve injury

Previous experiments indicated that TGFβ, at least in DRG neuron/Schwann cell cocultures, can inhibit myelin protein expression (Einheber et al., 1995; Guenard et al., 1995a). Moreover, after nerve injury while TGFβ-1 mRNA is upregulated, myelin-related gene mRNAs and proteins are strongly downregulated in the distal stump. We therefore wanted to test whether TGFβ has a role in controlling the downregulation of myelin genes in injured adult nerves. First, we immunolabeled transverse sections of normal and transected (2 and 7 d after axotomy) adult nerves with antibodies against P_0 and periaxin. We could not detect any clear difference in the levels of expression of the two proteins between mutant and wild-type nerves in any of the conditions analyzed (data not shown). We then used Western blotting to examine the levels of the major Schwann cell myelin protein P_0 in the distal stump of crushed adult sciatic nerves at 7 d after injury, again finding no clear differences between wild-type and mutant nerves (Fig. 8*B*) (data not shown). Last, we used semiquantitative RT-PCR to compare the levels of mRNA for P_0 and the myelin-related protein periaxin in the distal stump 7 d after nerve crush. No differences were detected between wild-type and mutant nerves (Fig. 8).

Discussion

The issue of how cell numbers are controlled is a major question in developmental biology. In the PNS, the number of Schwann cells must be adjusted precisely to enable these cells to reach a 1:1 relationship with axons before myelination, and nonmyelinating Schwann cells also attain a fixed range of numerical ratios with unmyelinated axons. Which growth factors or growth factor receptors carry out this important control of cell numbers during normal development *in vivo* is essentially unknown. The present work indicates that type II TGFβ receptors and, by implication, signaling by TGFβ, are involved in regulating Schwann cell numbers in developing nerves by controlling both proliferation and apoptosis. In contrast, lack of TGFβ did not affect other processes in which TGFβ had been implicated in previous *in vitro* and *in vivo* studies, such as myelination, myelin maintenance, or the response of adult nerves to injury.

The action of TGFβ *in vitro* and the generation of transgenic mice to define the role of TGFβ *in vivo*

In the PNS, TGFβ is expressed in some DRG neurons, and Schwann cells express TGFβ-1, -2, and -3 (Unsicker et al., 1991; Scherer et al., 1993; Stewart et al., 1995).

Here we have examined the function of TGFβ in these cells by generating a conditional knock-out mouse in which the func-

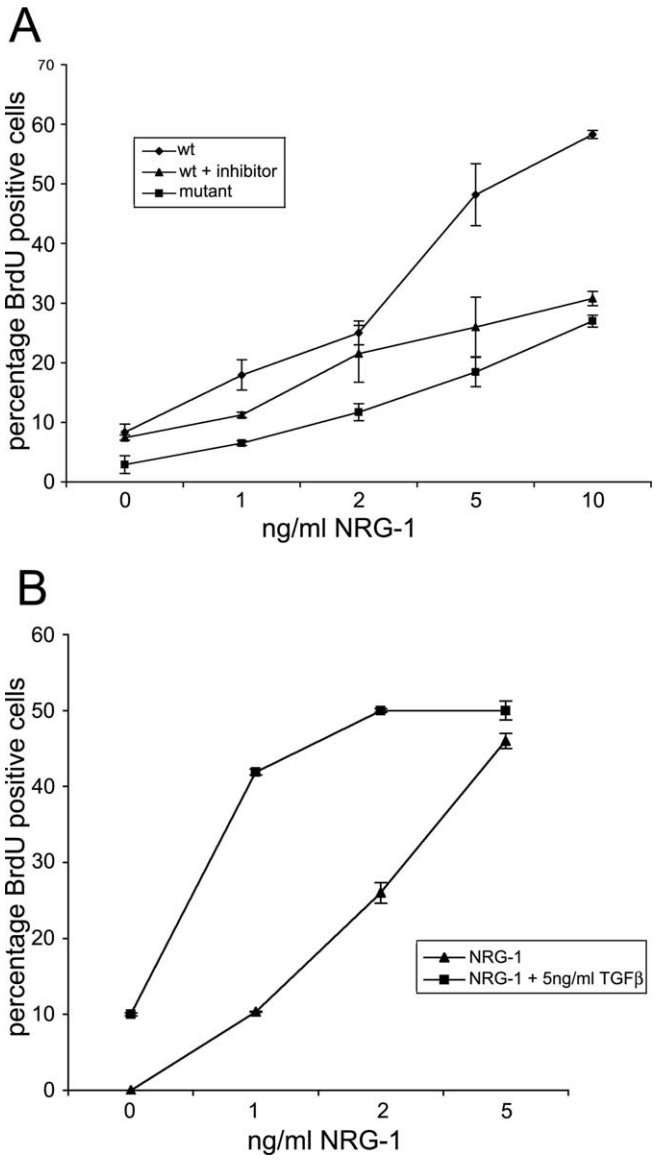

Figure 7. TGFβ cooperates with neuregulin-1 in promoting Schwann cell proliferation *in vitro*. ***A***, Schwann cells from wild-type (wt) and mutant mice were isolated, exposed to increasing concentrations of neuregulin-1 (NRG-1) for 10 h, and tested for BrdU incorporation. This showed that mutant cells proliferate at a reduced rate, which is similar to that seen when wild-type cells were exposed to neuregulin-1 in the presence of SB431542 (10 μM), a TGFβ receptor blocker [averages of three separate experiments, each using triplicate coverslips; wild type and P0 CRE/TβRII$^{f/f}$ represent littermates from three separate litters]. ***B***, BrdU incorporation in wild-type cells exposed to increasing concentrations of neuregulin-1 in the presence or absence of 5 ng/ml TGFβ. Note that TGFβ strongly promotes the proliferation induced by low concentrations of neuregulin-1 (averages of three separate experiments, each using triplicate coverslips).

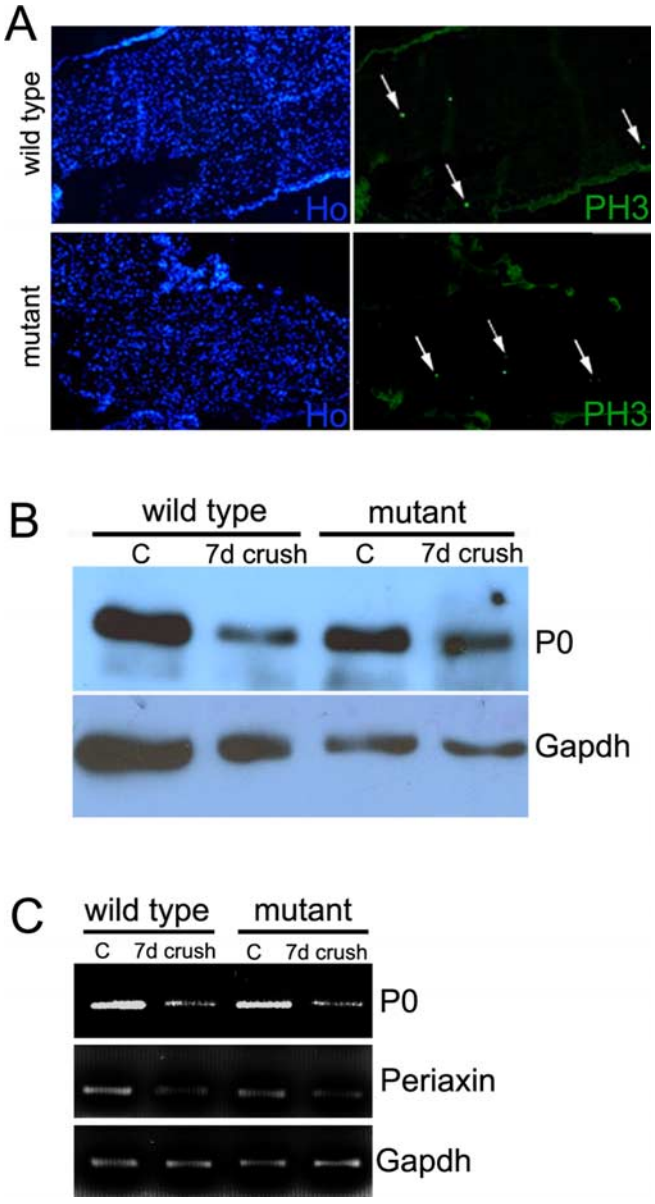

Figure 8. No differences in cell proliferation or in regulation of myelin genes are detectable between mutant and wild-type mice after adult sciatic nerve injury. ***A***, Adult sciatic nerves from wild-type and mutant mice were cut and, 7 d after transection, the distal stumps of the cut nerves were labeled with PH3 to visualize cell proliferation. In both wild-type and mutant nerves, there was active proliferation, but there was no significant difference in the number of PH3-positive nuclei (arrows). Nuclei were visualized with Hoechst dye (Ho). ***B***, Western blot on extracts from wild-type and mutant sciatic nerves. Adult sciatic nerves were crushed and tested for P_0 and periaxin (data not shown) protein expression. Protein levels decreased in a similar manner, namely by 4.1-fold in the wild type and 3.4-fold in the mutant (normalized to GAPDH and determined by densitometric analysis). ***C***, Similarly, the mRNA levels for P_0 and periaxin were equal 7 d after nerve crush, indicating that TGFβ is not essential for these events. C, Contralateral nerve; 7d crush, 7 d after nerve crush.

tional TGFβ receptor type II is specifically deleted in Schwann cells. Southern blot analysis from DNA obtained from P5 sciatic nerves indicates that the recombination efficiency was probably >90%, which should guarantee that the effects caused by lack of TGFβ signaling are not masked by the presence of unrecombined cells. This was confirmed indirectly by experiments in which we tested for Smad nuclear localization. We demonstrated that in P0 CRE/TβRII$^{f/f}$ mice at both E18 and P5, there is essentially no nuclear localization of the Smad proteins Smad2 and Smad4 in response to TGFβ. This strongly suggests that the recombination of exon 3 of the type II receptor has already occurred by E18. Moreover, *in vitro* experiments on Schwann cells dissociated from P1 mutant and wild-type mice showed that, at this developmental time point, the cells from P0 CRE/TβRII$^{f/f}$ mice are not sensitive to TGFβ-mediated apoptosis, another indirect indication that the recombination has occurred successfully.

Lack of TGFβ signaling does not impair myelination and myelin protein expression *in vivo*

One of the most intriguing effects observed *in vitro* in DRG-neuron/Schwann cell cocultures treated with TGFβ is the block of myelination (Einheber et al., 1995; Guenard et al., 1995a), indicating that TGFβ is potentially a negative regulator of myelination. Our morphological analysis *in vivo* showed, however, that myelination does not start prematurely in mice lacking TGFβ signaling. Moreover, myelination proceeds correctly, and myelin maintenance, as assessed by EM, is not abnormal, even in 1-year-old mice.

This contrasts with findings in adult (6- to 13-week-old) TGFβ$^{-/-}$ nude mice, which show abnormalities in myelin compaction, with tomacula-like and "honeycomb" structures, particularly frequent at the node of Ranvier region (Day et al., 2003). However, in this study, only 11 TGFβ$^{-/-}$ nude pups were found out of nearly 1000 pups genotyped, indicating that most of the double-transgenic mice die during embryonic development and suggesting that any phenotype observed may not be completely Schwann cell autonomous.

In addition to blocking myelination in DRG/Schwann cell cocultures, treatment with TGFβ suppresses the cAMP-induced expression of myelin genes (Mews and Meyer, 1993; Morgan et al., 1994; Einheber et al., 1995; Guenard et al., 1995b; Stewart et al., 1995). If we assume that TGFβ signaling is ongoing in developing nerves, this leads to the expectation that myelin proteins might be overexpressed in our mutant mice. This was not borne out, however, because periaxin and P_0 expression, tested by immunohistochemistry and Western blotting, was comparable in wild-type and mutant mice.

Together, these results argue against an important function for TGFβ signaling via the type II receptor during myelination of developing nerves *in vivo*.

In the absence of TGFβ type II receptors, Schwann cell death and proliferation are reduced during development and after injury

We have shown that TGFβ has the potential to actively kill Schwann cells isolated from the nerves of newborn animals and tested immediately after plating *in vitro* (Parkinson et al., 2001). This effect can be blocked *in vitro* through enforced expression of the transcription factor Krox-20, which inactivates the JNK–c-Jun pathway (Parkinson et al., 2001, 2004), an observation that could explain why early myelinating cells are resistant to death *in vivo* (Grinspan et al. 1996). These findings led to the suggestion that TGFβ was a negative regulator of Schwann cell survival in developing nerves (Parkinson et al., 2001). We confirm this notion, finding that in P0 CRE/TβRII$^{f/f}$ mice, Schwann cell death is significantly decreased at E18 and perinatal stages during normal development. Previously we also found that the Schwann cell death response seen in transected newborn nerves is increased by injection of TGFβ, whereas injection of blocking TGFβ antibodies reduces it (Parkinson et al., 2001). In agreement with this, we find that injury-induced death in neonatal nerves of mutant mice is also reduced. Interestingly, although Schwann cell death after injury remained nearly fourfold lower in the mutant than in the wild type, it increased substantially from baseline. It is likely that this injury-induced Schwann cell death, even in the absence of TGFβ signaling, is caused by the action of the p75NTR pathway, which also mediates Schwann cell apoptosis after neonatal nerve injury (Syroid et al., 2000).

In agreement with previous studies that showed that TGFβ has mitogenic effects on Schwann cells, the observed reduction in Schwann cell death was accompanied by a reduction in Schwann cell proliferation. Moreover, quantitative assessment of nuclear fluorescence confirmed that there were no marked differences in the overall number of Schwann cells between wild-type and mutant mice. This indicates that in terms of total numbers, the killing and mitogenic effects tend to balance out.

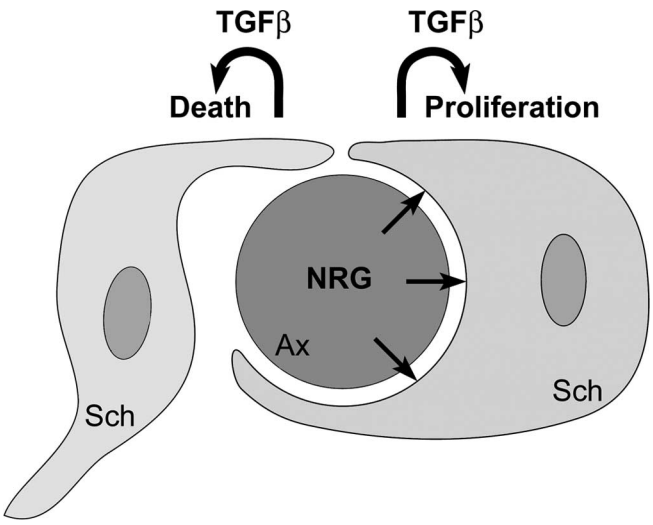

Figure 9. A model of TGFβ function in developing nerves. TGFβ kills those cells that lose out in competition for axonal neuregulin-1 (NRG) but is a mitogen for cells with adequate axonal association. TGFβ is therefore a bifunctional amplifier that strengthens both the positive and negative consequences for Schwann cell (Sch) numbers that result from a competition for a limited amount of axonal survival support. Ax, Axon.

We also examined the proliferative effects of TGFβ on cultured Schwann cells and related this to the effects of neuregulin-1, a Schwann cell mitogen that is often thought to represent the axonal mitogen that drives Schwann cell division in developing nerves. We exploited the fact that TGFβ rapidly loses the ability to kill Schwann cells during the first few days after birth (Parkinson et al., 2001). Thus, in the present experiments using cells obtained from 3- to 4-d-old mice cultured for 2–4 d, we found that adding TGFβ alone did not induce death but stimulated some DNA synthesis. Furthermore, in cultures that already contained neuregulin-1 and were therefore proliferating, adding TGFβ stimulated DNA synthesis still further. This mitogenic effect of TGFβ was particularly strong at low neuregulin-1 concentrations. Unexpectedly, Schwann cells lacking the TGFβ receptor showed reduced mitogenic response to neuregulin-1 alone, and this effect was mimicked by a TGFβ receptor blocker. This indicates that in mouse Schwann cell cultures, the medium contains significant levels of active TGFβ, presumably secreted by the Schwann cells themselves (Guenard et al., 1995b; Stewart et al., 1995).

Conclusions

Together, the *in vitro* and *in vivo* data on TGFβ indicate that it acts as a killer or a mitogen, depending on context. We showed previously that when cells are tested straight after plating from newborn nerves, TGFβ killing is blocked by addition of neuregulin-1, provided the cells are plated at sufficient density also to receive support from autocrine signals, a situation likely to prevail in developing nerves (Parkinson et al., 2001). Here we found that under these conditions and in the presence of

neuregulin-1, TGFβ promoted Schwann cell DNA synthesis. Together, these *in vitro* experiments suggest a model in which the function of TGFβ is controlled by neuregulin-1: in the absence of neuregulin-1, TGFβ induces apoptosis, whereas in the presence of neuregulin-1, TGFβ stimulates proliferation (Fig. 9).

In developing nerves, it is now well established that axon-bound neuregulin-1 type III is necessary for Schwann cell precursor survival (Wolpowitz et al., 2000; Leimeroth et al., 2002; Jessen and Mirsky, 2005), and this signal is likely also to take part in promoting the survival and proliferation of early immature Schwann cells (Grinspan et al., 1996; Parkinson et al., 2001). We therefore speculate that *in vivo* TGFβ acts as a mitogen for those Schwann cells that receive sufficient neuregulin-1 from axons that they associate with, whereas TGFβ also ensures that supernumerary cells with less effective axonal association are killed (Fig. 9). In this model, therefore, TGFβ is a bifunctional amplifier that boosts either proliferation or death depending on the degree of axonal contact.

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