Development/Plasticity/Repair

Induction of Neuregulin Signaling in Mouse Schwann Cells

In Vivo Mimics Responses to Denervation

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Neuregulins play crucial roles in early development of Schwann cells (SCs), but their roles in the activities of SCs during denervation and reinnervation of muscle are less clear. In the present study, the Tet-On system has been used in transgenic mice to enable inducible expression of a mutant, constitutively active neuregulin receptor (ErbB2) in SCs. This induction simulates neuregulin signaling to these cells. Reporter transgenes were used to show a tightly regulated, SC-selective expression in muscle. Induction leads to a number of changes in SCs at neuromuscular junctions that mimic the response to muscle denervation/reinnervation. These include process extension, soma migration, and proliferation. SCs also come to express nestin, a protein characteristic of their reaction to muscle denervation. This activation of SCs results in the sprouting of nerve terminals, and these sprouts follow the extensions of the SCs. However, these sprouts and their associated SCs disappear after the removal of the inducer. Last, induction of the active receptor is sufficient to rescue SCs in neonatal muscle from denervation-induced apoptosis. These findings show that the responses of SCs in muscle to denervation can be explained by induction of an autocrine/paracrine neuregulin signaling cascade suggested by previous molecular studies.

Key words: neuromuscular junction; terminal Schwann cell; S100B; GGF; ErbB receptor; Neu; Tet-On; rtTA; nestin; cell migration; sprouting; cell proliferation

Introduction

Schwann cells (SCs) play pivotal roles in the response to muscle denervation. They are activated by nerve injury, creating an environment supporting axon regeneration and muscle reinnervation. At normal neuromuscular junctions (NMJs), terminal SCs (tSCs) cap the synaptic contacts. Myelinating SCs wrap the motor axon entering each such contact. After muscle denervation, SCs grow long processes that serve as substrates for nerve regeneration, both within the endoneurial tubes of the intramuscular nerves as well as between synaptic contacts in the muscle (Son and Thompson, 1995a,b; Love and Thompson, 1998; Kang et al., 2003; Koirala et al., 2003). Our understanding of the molecular and cellular signals that regulate these responses of SCs is incomplete.

One strategy for increasing understanding is to manipulate the expression of genes known to be involved in SC function. Neuregulin-1 (Nrg-1) is a family of alternatively spliced factors that are secreted or membrane-bound and signal through the ErbB family of receptor tyrosine kinases. Peripheral neurons are known to synthesize Nrg-1 (Buonanno and Fischbach, 2001). SCs possess ErbB receptors. Neuregulin activity is responsible for the survival, proliferation, migration, and differentiation of SCs and their precursors (Marchionni et al., 1993; Dong et al., 1995; Meyer and Birchmeier, 1995; Mahanthappa et al., 1996; Zanazzi et al., 2001; Jessen and Mirsky, 2005; Lyons et al., 2005). The expression of a cell surface Nrg-1 isoform on axons has been shown recently to determine how SCs wrap axons and whether they produce myelin (Garratt et al., 2000; Michailov et al., 2004; Taveggia et al., 2005). Denervation of muscle in neonatal rats results in rapid, apoptotic death of tSCs, apparently attributable to the interruption of the supply of Nrg-1 to SCs. Exogenous application of a soluble Nrg-1 isoform, glial growth factor II (GGFII), rescues these cells (Trachtenberg and Thompson, 1996). Application of GGF also results in SC proliferation, migration, and process growth (Trachtenberg and Thompson, 1997). These responses, together with nerve sprouting, are also seen during GGF application to normally innervated neonatal rat muscle (Trachtenberg and Thompson, 1997). Neones that receive GGF also suffer a severe disruption of NMJs and muscle denervation. However, because ErbB receptors are also present on axons (Pearson and Carroll, 2004) and muscle fibers (Moscoso et al., 1995; Zhu et al., 1995; Trinidad et al., 2000), all or some of these responses may not be attributable to GGF acting...
directly on SCs. To clarify which responses are attributable to Nrg-1 action on SCs and to further investigate the responses of SCs in vivo to Nrg-1 signaling, we expressed a transgene for a mutant, constitutively active erbB2 receptor (caerbB2) in SCs. To achieve temporal control of expression, we used the Tet-On system developed by Kistner et al. (1996).

Results presented here show that expression of active erbB2 can be induced in SCs in living mice via administration of the antibiotic doxycycline. After such induction, SCs extend processes and migrate away from NMJs. In many cases, axon sprouts follow these SC processes. In addition, SCs proliferate. During removal of the doxycycline, SCs not associated with synapses disappear and axon sprouts retract. Induction can also rescue SCs from denervation-induced apoptosis. Thus, most of the phenotypes obtained from GGF application to neonatal muscle can be mimicked by activation of the signaling receptor selectively in SCs. These provide direct evidence that activation of SCs promotes the growth of associated axons. Together, our results suggest that the generation of the second responder line that expressed a nuclear-localized erbB2 receptor selectively in SCs. These provide direct evidence that activation of SCs promotes the growth of associated axons.

Materials and Methods

Animal care and surgical procedures. Mice were deeply anesthetized for survival surgeries by intraperitoneal injections of ketamine–xylazine as described previously (Zuo et al., 2004) or by intraperitoneal injections of Nembutal (200 mg/kg) for terminal experiments. For muscle denervation, the sciatic nerve was exposed in the thigh and a 1 mm piece of the nerve resected; the wound was closed with 6-0 silk suture. Surgeries were conducted in accordance with National Institutes of Health guidelines.

Construction of transgenes and generation of mice. A 9.4 kb portion of the upstream regulatory sequence for human S100B was inserted upstream of the coding sequence for the reverse tetracycline transactivator (rTATA) and human growth hormone polyadenylation sequences (Zuo et al., 2004). The coding sequence for the rTATA was optimized for codon usage in the mouse (Valencik and McDonald, 2001) and kindly provided by Dr. John McDonald (University of Utah, Salt Lake City, UT). The 10.9 kb transgene was isolated from a vector backbone, gel purified, and used to prepare transgenic mice (Zuo et al., 2004). Founders and their transgenic offspring were identified by PCR for a 295 bp portion of the transgene using the following primers: forward, 5'-CACACAGTTCGCCTCTTTGA-3’; reverse, 3'-ATCAAATCCAGGCCAAGAG-5’. Each of the five founders identified were then backcrossed to C57BL/6J mice.

To determine which of the founder mice were capable of doxycycline-regulated expression of target transgenes in Schwann cells, each was crossed to a “transponder” mouse, a line designated G9 (Krestel et al., 2001), kindly provided by Dr. Heinz Krestel (University of Zurich, Zurich, Switzerland). In these mice, the TetO operator sequence is linked bicistronically through the minimal cytomegalovirus promoter to each the coding sequence for β-galactosidase (β-gal) and the coding sequence for a humanized version of the S65T mutant of green fluorescent protein (GFP). We found histochemical or immunological staining for β-gal gave us an excellent ability to detect doxycycline-regulated expression (see below); GFP expression, in contrast, was much weaker and difficult to detect. On the basis of β-gal expression, we identified one of the S100-rTATA lines that gave the highest expression in Schwann cells in muscles; one of the other lines that gave weak β-gal expression and the three remaining lines that gave no detectable expression were discarded. We confirmed the doxycycline-induced expression in this line using a second responder line that expressed a nuclear-localized β-gal (Furth et al., 1994). In both cases, mice carrying either of the transponder transgenes were identified by PCR using primers for LacZ as described previously (Furth et al., 1994).

TetO-constitutively active erbB2 mice (TetO-NeuNT) mice have been described previously (Moody et al., 2002). In addition to coding for a constitutively active erbB2 receptor, the transgene has an internal ribosomal entry site (IRES) and the coding sequences for a luciferase reporter. Mice bearing this transgene were identified by PCR for a 295 bp product in the luciferase portion of the transgene using the following primers: forward, 5’-CCTCTTGGCCAAAGACTC-3’, reverse, 3’-CACACAGTTCGCCTCTTTGA-5’.

Doxycycline administration. For induction of adult animals, 600 mg of doxycycline HCl (Dox) (Hovione, Taipa, Macau) and 5 g of sucrose were first dissolved in 60 ml of water and then mixed with 100 g of pulverized mouse breeder chow. Mice were fed ad libitum, and Dox food was made fresh two to three times per week. No attempt was made to precisely monitor the amount of food consumed; variability in consumption may account for some of the variability in levels of induction seen, although variability was noted even in animals that were injected with Dox. Adult S100–rTATA/TetO–NeuNT mice (~1 month old) were fed Dox food for 3, 5, 7, or 10 d. For studies in neonatal mice, pups were injected intraperitoneally once daily for 3 consecutive days beginning at postnatal day 5 (P5) with 20 µl of 10 mg/ml Dox in 0.9% sterile saline.

Bromodeoxyuridine administration. Bromodeoxyuridine (BrDU) (203806; Calbiochem, La Jolla, CA) dissolved in 0.9% NaCl containing 0.007N NaOH was administered via three intraperitoneal injections given at intervals of 4 h at a dose of 1 mg/10 gm body weight. For adult animals, BrDU was administered for 3 d via the drinking water (1.5 mg/ml). BrDU was detected by immunostaining as described previously (Love and Thompson, 1999).

Histochemical detection of β-galactosidase at NMJs. Animals were perfused through the heart with PBS, pH 7.4. The sternomastoid and soleus muscles were dissected, fixed in 4% phosphate-buffered formaldehyde, pH 7.4, for 15 min, and rinsed in three changes, 10 min each, of PBS. The muscles were then incubated at 37°C in PBS solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (BP1615-100; FisherBiotech, Fair Lawn, NJ), 4 mM Fe(CN)6, 4 mM K3Fe(CN)6, 2 mM MgCl2. Twelve hours later, muscles were washed in three changes of PBS of 10 min each. ACh receptors (AChRs) were labeled with tetramethylrhodamine isothiocyanate (TRITC)–α-bungarotoxin as described below. A sheet of the upper two to three fiber layers was dissected from each of the muscles and mounted as whole mounts in aqueous fluorescence mounting medium.

Fluorescent labeling of muscle whole mounts. Muscles were processed as reported previously (Trachtenberg and Thompson, 1997). Briefly, Schwann cells were labeled with rabbit anti-cow S100 (Z0311) at 1:400; DakoCytomation, Carpinteria, CA) and an anti-rabbit secondary antibody conjugated to TRITC (55671, at 1:400; Cappel). ACh receptors (AChRs) were labeled with tetramethylrhodamine isothiocyanate (TRITC)–α-bungarotoxin as described below. A sheet of the upper two to three fiber layers was dissected from each of the muscles and mounted as whole mounts in aqueous fluorescence mounting medium.

Filter sets used were standard HQ sets (catalog #41001 for FITC and GFP, #41002 for TRITC, and #41003 for Cy5) for spectral overlap with GFP. Bleed-through between TRITC and Cy5, between FITC and TRITC, and between DAPI and FITC was not a problem at the

camera exposures used in the study. Minimal bleed-through was observed between CFP and GFP, but, as shown in the figures, the structures labeled were different and, in any case, the phenomena involving SC proliferation, migration, and process growth could be verified in single transgenic S100−GFP animals. Figures were constructed using Adobe Photoshop (Adobe Systems, San Jose, CA) with adjustments made only in image brightness and contrast.

Luciferase assays. Luciferase activity was analyzed using the luciferase assay system following the instructions of the manufacturer (E4030; Promega, Madison, WI). Snap-frozen tissues were dounce homogenized in 500 or 1000 μl 1× reporter lysis buffer. Tissue lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and supernatants were pipetted into new tubes. Twenty microliters of supernatant were mixed with luciferase assay substrate, and luciferase activity was read in a Lumitrex luminometer. Luciferase activity levels were normalized to total protein as determined using the Bradford method per the instructions of the manufacturer (Bio-Rad, Hercules, CA). For analysis of muscle endplate zones, a region comprising ~60% of the central belly of the sternomastoid muscle was dissected.

S100−enhanced GFP and Thy1−enhanced GFP transgensics. To facilitate fluorescence imaging of terminal Schwann cells and motor nerve terminals, the S100−rtTA/TetO−NeuNT mice were mated with mice double transgenic for S100−enhanced GFP (EGFP) and Thy1.1−CFP (Zuo et al., 2004). These last two transgenes encode GFP in Schwann cells and CFP in motor axons. In this way, we were able to identify Schwann cells and motor axons in the muscles under fluorescence in addition to AChR at endplates via application of rhodamine−α-bungarotoxin (see above).

**Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.** Schwann cell apoptosis was detected by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) using the ApopTag Red In Situ Apoptosis Detection kit (S7165; Chemicon, Temecula, CA) per the instructions of the manufacturer. For quantitative assessment of SC apoptosis, TUNEL-positive (TUNEL+) nuclei were counted only if they were present in cells identified as SCs by GFP expression from the S100−EGFP transgene.

**Physiology.** Soleus muscles were dissected and pinned to a Sylgard-coated dish and superfused with oxygenated Ringer’s solution containing 500 or 1000 μl 1× reporter lysis buffer. Tissue lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and supernatants were pipetted into new tubes. Twenty microliters of supernatant were mixed with luciferase assay substrate, and luciferase activity was read in a Lumitrex luminometer. Luciferase activity levels were normalized to total protein as determined using the Bradford method per the instructions of the manufacturer (Bio-Rad, Hercules, CA). For analysis of muscle endplate zones, a region comprising ~60% of the central belly of the sternomastoid muscle was dissected.

**Expression of the reporter can be induced within days.** To determine how rapidly induction occurs in the tSCs at the NMJ, young adult S100−rtTA/TetO−LacZ mice were fed Dox-containing food for 3, 5, or 7 d. When the sternomastoid and soleus muscles were examined histochemically, we found that the fraction of endplates with X-gal-labeled tSCs increased the longer the induction. After 3 d of induction, 60−70% of endplates had X-gal-labeled tSCs, whereas at 5 and 7 d, this fraction rose to 80−90% of endplates (Table 1). Longer periods of induction (10 or 21 d) did not result in any additional increase in the fraction of endplates with labeled tSCs. When labeled tSCs were present at an endplate, there appeared to be a tendency for all SCs at these endplates to be labeled. For example, after 3 or 7 d of induction, the number of labeled tSC cell bodies at individual endplates in the sternomastoid muscle (3.1 ± 0.2 or 3.4 ± 0.1, respectively) approached the number of tSCs identified by transgenic expression of GFP in uninduced muscles of the same age that lacked the Tet-On transgenes (3.7 ± 0.1). An additional measure of induction was the intensity of label seen, particularly in the processes of the tSCs covering the nerve terminals. With 3 d Dox induction, label was most prominent within the cell bodies, and the processes covering the nerve terminal were weakly labeled (an example of this type of labeling can be seen after longer induction in Fig. 1B, white arrowheads). In most cases, 5 and 7 d of Dox

**Results**

**Regulatory elements of the S100B gene confer doxycycline-inducible regulation of a LacZ reporter transgene in Schwann cells.** Previous work has shown that the Tet-On gene expression system can be used to conditionally regulate transgene expression in a variety of tissues (Mansuy et al., 1998; Xie et al., 1999; Chang et al., 2000; Diamond et al., 2000; Tichelaar et al., 2000; Liu et al., 2001; Moody et al., 2002; Ludwig et al., 2004; Suarez et al., 2004; Ponomareva et al., 2006). In these cases, a cell-specific promoter was used to drive expression of a transgene that codes for the transcription factor rtTA. The rtTA protein promotes the expression of a second transgene whose regulatory elements contain sequences that bind this protein when a tetracycline antibiotic is administered to the mice. In an attempt to regulate expression of transgenes in SCs in an inducible manner, we prepared a construct, designated S100−rtTA, in which regulatory elements of the human S100B gene were coupled to sequences coding for rtTA. We used this construct to prepare transgenic mice.

To evaluate the inducibility and specificity of the regulation conferred by the S100−rtTA transgene, we mated progeny of each of five S100−rtTA founders with a line of reporter mice transgenic for a LacZ reporter gene linked to tetracycline regulatory elements (Krestel et al., 2001). Double transgenic offspring (S100−rtTA/TetO−LacZ) were induced with the tetracycline Dox administered in the food of their dams and their own food from birth to P30. Mice derived from one of the transgenic founders displayed robust histochemical labeling for the β-galactosidase reporter within a zone in the center of muscle in the expected position of the endplates. Labeling was also present in the intra-muscular nerve (Fig. 1A). Double transgenic mice that were not induced showed no detectable histochemical labeling (Fig. 1C). To determine whether the label was present in SCs, we immunostained muscles using a polyclonal antibody to the S100B protein to identify SCs and a monoclonal antibody to β-galactosidase. SCs along motor axons and tSCs at motor nerve endings were positive for both markers (Fig. 1D, E). Labeling of other cell types was not present. These experiments show that this S100−rtTA mouse line can be used to regulate the expression of a second transgene containing tetracycline-dependent regulatory sequences in a Dox-dependent manner. In muscle, this induced expression is highly selective for SCs.
treatment produced such intense labeling of tSC processes that only the fluorescence at the edge of the receptor plaque could be visualized after application of rhodamine–bungarotoxin (an example of this type of labeling after longer induction can be seen in the uppermost junction in Fig. 1B). tSCs along preterminal motor axons were also labeled after Dox induction, and, as in the case of tSCs, label was initially confined to the cell body but, with longer induction, was present along entire internodes (Fig. 1B, arrow). Overall, these data suggest that induction results in expression in most tSCs within 3–5 d. Some tSCs, however, appear to be refractory to induction; such refractoriness may be a result of cell-to-cell variability in the activity of S100-promoter-driven transgenes, as has been reported previously (Zuo et al., 2004).

A mutant, constitutively active ErbB2 receptor can be induced selectively and reversibly in Schwann cells

To produce mice in which we could test the consequences of activating neuregulin signaling in SCs, we crossed our S100–rtTA line with another mouse line having a tetracycline-regulated transgene that encodes a mutant, constitutively active form of the ErbB2 receptor (designated caErbB2 or NeuNT) (Moody et al., 2002).

To indirectly assess the induction of mutant ErbB2 receptors, we assayed the expression of the reporter enzyme luciferase encoded from an IRES in the ErbB2 transgene (Moody et al., 2002). We harvested tissues from young adult mice after 5 d of Dox administration and assayed these tissues for luciferase activity (Fig. 1F). Tissues from un-induced animals of the same genotype were also examined, and the background of the assay itself was determined by assaying the same tissues taken from animals lacking any luciferase gene. The background luciferase activity was not significantly different from that found in double transgenic animals that were given no Dox. This suggests that, in the absence of induction, expression of the luciferase transgene (and by inference, the mutant ErbB2 receptor) is not detectable. Conversely, 5 d of Dox induction gave a ~20-fold increase above background in luciferase activity in endplate regions dissected from sternomastoid muscles (Fig. 1F). This likely underestimates the level of induction, because the SCs constitute only a small fraction of the total protein isolated for such assays. To assay a more pure population of Schwann cells, luciferase activity was measured in homogenates of peripheral nerves. Here, on average, a >600-fold increase in baseline enzyme activity was detected (Fig. 1F). A much smaller induction (less than twofold) was detected in the spinal cord, brain (excluding the cerebellum), salivary gland, skin, small intestine, and liver (data in legend to Fig. 1F). Trans-
gene expression was induced at low levels in cerebellum and thymus (legend to Fig. 1F), sites of expression of S100 transgenes (Zuo et al., 2004). The low levels of induction in other S100-expressing tissues and the selectivity of transgene expression in SCs in muscle suggest that the results we report below are likely the consequence of SC transduction of the mutant ErbB2 receptor that occurs within 3–5 d of administration of the inducer and that this induction is reversible. Although Ponomareva et al. (2006) have used this same transgenic system to show that the induction of the luciferase reporter occurs at the same time as the levels of mutant ErbB2 and phospho-ErbB2 increase, there is no reason to believe the two will be linearly correlated, because one of the proteins is translated from an IRES and both proteins are likely to be degraded at different rates.

**Induction of active ErbB2 in SCs alters the relationship between SCs and nerve terminals at NMJs**

Observation of the SCs in the transgenic animals also shows the inducible control of transgene expression and nerve growth. To facilitate examination of neuromuscular synapses, we interbred mice to introduce two additional transgenes into the S100–rtTA/TetO–NeuNT mice: an S100–GFP transgene that makes SCs fluoresce green and a Thy1–CFP transgene (Feng et al., 2000) that makes the axons fluoresce blue. The postsynaptic AChRs were also visualized by the application of rhodamine–bungarotoxin to the fixed muscles (Fig. 2).

Transgenic mice that did not receive Dox had NMJs that resembled in all respects (position of the somata of SCs, coverage of the underlying nerve terminal by SC processes, low frequency of short SC processes extending from junctions, low frequency of nerve sprouts extending from junctions, and apposition of nerve terminals to AChR) the NMJs in muscles that lacked the S100–rtTA transgene (Fig. 2A–C; Table 2). These observations show that the un-induced animals have normal neuromuscular synapses and suggest, especially in light of the results below, that leakage expression of the mutant ErbB2 in the double transgenic animals is minimal.

In contrast, major alterations in the morphology of neuromuscular synapses were seen in transgenic mice after induction with Dox. In adult mice induced for 3 or 5 d, 27.1 and 62.7% of the junctions, respectively, had SCs that were no longer restricted to covering the nerve terminals but had extended lamellar-like processes into the spaces between the AChR-containing synaptic gutters (Fig. 2D,G,J; Table 2). In control animals, only 7.5 ± 2.5% of junctions were found to have terminal SCs that had any of these features. At many junctions after induction, SC processes continued to spread until the entire junctional area was covered by a “mat” of SC processes (Fig. 2D). These data suggest that terminal SCs begin to alter their relationships to nerve terminals within 3 d of Dox-induced expression of active ErbB2.

**Induction of active ErbB2 induces growth of terminal Schwann cell processes outside the synaptic area and axonal sprouting**

After induction, SCs extended processes from the junction into perijunctional areas (Fig. 2G,J; arrows) in addition to those extended into the spaces between gutters. After 3 or 5 d of induction, 34 and 83%, respectively, of junctions in the sternomastoid had SC processes that left the junction. This fraction was higher than that seen in control animals (17%), and, in addition, the processes were longer and more profuse from individual junctions (Table 2). Moreover, many SC processes were associated with sprouts growing from nerve terminals (Fig. 2H,K; arrows); indeed, these sprouts were always found in association with SC processes. After 3 or 5 d of induction, 16 and 30% of the junctions with SC processes, respectively, had terminal sprouts. These fractions were higher than those seen in control animals (11%), and, moreover, the sprouts were longer (Table 2). Similar observations were made in the soleus muscle (Table 2). After induction

**Figure 2.** Induction of active ErbB2 in Schwann cells results in their extension of processes, their migration from the NMJ, and nerve sprouting. NMJs in sternomastoid muscles of adult mice transgenic for inducible expression of mutant ErbB2 (S100–rtTA/TetO–NeuNT) visualized by transgenic expression of GFP in SCs (A, D, G, J), transgenic expression of CFP in their motor axons and nerve terminals (B, E, H, K), and by labeling AChR with rhodamine–bungarotoxin (BTx) (C, F, I, L). A–C, Junction from an un-induced animal showing typical arrangement of SCs, nerve terminals, and AChR. D–L, Junctions from animals induced with Dox for 5 d. D, Extension of lamellar-like processes (arrow) into the spaces between the AChR-containing synaptic gutters. G, Extension of SC processes from the junction (arrow); arrowhead marks the soma of an SC that has migrated from the junction. Arrow in H marks a sprout that has extended from the terminal. J, K, Terminal (arrows in J, K) and preterminal sprouts (arrowhead in K). Scale bar (in L): G–I, 25 µm; A–F, J–L, 50 µm.
suggest that, in contrast to GGF administration to muscles, evoked twitch or tetanic frequencies (data not shown), even when muscle contractile response to stimulation of the muscle nerve at either position of nerve terminals to AChR (Fig. 2).

For 10 d, many of the sprouts and their associated SC processes were so long that they commonly moved outside the plane of focus and were difficult to follow (Fig. 3). Most of these SC processes and the terminal sprouts grew parallel to the axis of the muscle fibers toward the myotendinous junctions. Despite this growth from the junction, no new accumulations of AChRs were found along these terminal sprouts, suggesting that no new synaptic sites were formed. Although rare, “preterminal” axonal sprouts were also found that arose at or near the last heminode of the myelinated preterminal axon. These grew along the outside of SCs along the motor axon (Fig. 2, arrowhead).

Like the responses reported above for induction in adult muscles, induction in neonatal animals produced SC growth and nerve sprouting. However, the extent of sprouting of nerve terminals was less. For example, induction for 14 d from birth resulted in only 6.7 ± 1.8% of the junctions having sprouts in the sternomastoid muscle (n = 4 animals, 1098 junctions examined), much less than the 30% of junctions reported above for 5 d of induction in the adult. Induction in the neonate also appeared to prolong the retention of polyneuronal innervation as judged by the number of preterminal axons entering synaptic sites (data not shown).

Despite the sprouting observed in these adult and neonatal muscles, we observed no obvious change in the bungarotoxin-labeled AChR within the synaptic sites (Fig. 2F, I, L). No areas within the sites had an obviously diminished intensity of AChR labeling as was reported previously after administration of GGF to neonatal rat muscles (Trachtenberg and Thompson, 1997). Similarly, there were no obvious changes in the fidelity of apposition of nerve terminals to AChR (Fig. 2E, F, K). Muscles dissected from animals after induction showed no deficits in their contractile response to stimulation of the muscle nerve at either twitch or tetanic frequencies (data not shown), even when muscles showed considerable nerve sprouting. These observations suggest that, in contrast to GGF administration to muscles, evoking neuregulin signaling selectively in SCs causes SC growth and nerve terminal sprouting but no obvious change in the morphology or physiology of the synaptic contact itself. That the muscle fibers remain effectively innervated offers an explanation for why the sprouts fail to form new synapses (Jansen et al., 1973). However, our analyses do not exclude the possibility of subtle changes in the original synaptic contacts.

**Induction of active ErbB2 produces migration and proliferation of terminal Schwann cells**

After Dox induction in young adult mice, SCs migrated into the extrajunctional space directly adjacent to the endplate (Fig. 2G, arrowhead). Such cells also extended processes that grew along muscle fibers (Fig. 2G, arrow) and also contacted the endplate from which they appeared to have migrated. By 5 d of Dox induction, >20% of endplates in the sternomastoid and soleus muscles...
given once daily intraperitoneal injections of 200 SCs. This was supported by BrdU labeling (Fig. 4). Neonates the signaling from the mutant ErbB2 had caused proliferation of many cases, such cells covered the entire endplate zone (Fig. 3). In neonatal or adult muscles. In adults, BrdU incorporation was not quantified. In both neonates and considerably after induction in the adult (Fig. 4). The number of labeled cells increased much lower than in the neonate, as reported previously (Love et al., 1998). The number of labeled cells increased as a consequence of the action of neuregulin on SCs.

**Figure 4.** Induction of active ErbB2 results in SC mitosis. A, B, Two views of an NMJ in a postnatal day 7 soleus muscle after 3 d of Dox induction; BrdU was given by intraperitoneal injections during the 3 d of induction. Arrow in B indicates one BrdU-labeled SC. C, D, Two views of an NMJ in an adult sternomastoid muscle after 10 d of Dox induction; BrdU was given in the drinking water during the last 3 d of induction. A polyclonal antibody to S100 was used to identify SCs (A, C), and monoclonal antibody to BrdU was used to identify nuclei that had incorporated BrdU (B, D). In both the neonatal and the adult muscle, the induction of mutant ErbB2 resulted in BrdU-labeled SC nuclei. BrdU-labeled cells are indicated by arrows in C and D. Scale bar, 30 μm.

had terminal SCs whose cell bodies had migrated a short distance away from the synapse (Table 2).

Large numbers of GFP + cells were observed in the extrajunctional regions of Dox-induced neonatal or adult muscles. In many cases, such cells covered the entire endplate zone (Fig. 3A), suggesting that, in addition to causing migration from endplates, the signaling from the mutant ErbB2 had caused proliferation of SCs. This was supported by BrdU labeling (Fig. 4). Neonates given once daily intraperitoneal injections of 200 μg of Dox for 3 consecutive days had an increase in the total number of junctions containing BrdU + tSCs compared with un-induced animals (52.2 ± 5.2 vs 27.2 ± 3.1%; p < 0.05) (Fig. 4A, B). In adults, the level of BrdU labeling in SCs in the un-induced muscles was much lower than in the neonate, as reported previously (Love and Thompson, 1998). The number of labeled cells increased considerably after induction in the adult (Fig. 4C,D), but the extent of the labeling was not quantified. In both neonates and adults, BrdU + tSCs were also encountered within the intramuscular nerves of induced animals (data not shown). We conclude that the proliferation seen in rat tSCs after application of exogenous GGFII is a consequence of the action of neuregulin on SCs.

**Induction of active ErbB2 in SCs produces expression of a cytoskeletal marker for the reactive state found in SCs after denervation**

Denervation of NMJ results in changes in gene expression in tSCs (cf. Astrow et al., 1994; Georgiou et al., 1994) in addition to process extension. One easily detected change in “reactive” tSCs is the upregulation of the cytoskeletal protein nestin (Kang et al., 2001). Because SCs expressing the mutant ErbB2 exhibit many behaviors typical of these reactive SCs, we asked whether the SCs in the induced animals also changed their expression of nestin. As shown in Figure 5, both the cell bodies (excluding the nuclei) and the processes of tSCs at the junction become immunopositive for nestin after induction of active ErbB2. These changes occur despite the fact that these tSCs remain in contact with motor nerve terminals and nerve sprouts. Thus, induction of ErbB2 signaling in SCs causes upregulation of a prominent marker that is typical of SCs that have lost contact with axons.

**Doxycycline withdrawal reverses ErbB2-induced changes in SCs and sprouting and leads to withdrawal of axons**

To evaluate whether SCs, SC processes, and axon sprouts that form during induction require the continued expression of the active ErbB2, we induced with Dox for several days and then withdrew Dox and examined the muscles 10–30 d later. The muscles were dramatically changed from their status at the end of the induction period. First, the mat of SCs that were not associated with nerve sprouts disappeared (Fig. 3D,E), although those associated with sprouts remained. After longer periods of withdrawal, even these SCs and their associated sprouts began to disappear (Fig. 3G,H).

During Dox withdrawal, many of the nerve sprouts that were still present terminated in swollen bulbs, a morphology suggestive of the retraction bulbs seen during the loss of axonal inputs to NMJs during postnatal synapse elimination (cf. Bishop et al., 2004). Moreover, these sprouts commonly had a beaded appearance along their length. Both of these features were substantially different from sprouts seen during the outgrowth phase of induction when the tips were commonly tapered (Fig. 6A). Moreover, in at least 12 cases in which a remnant nerve sprout was presumed to be in the process of retraction, small pieces of CFP-labeled axonal material were present in the area of SCs beyond the end of the swollen bulb (Fig. 6C). Such cases suggest the nerve sprout is consumed by the SCs as has been reported during the formation of “axosomes” during withdrawal of axonal inputs from synaptic sites during neonatal synapse elimination (Bishop et al., 2004). Together, these results suggest that active ErbB2 causes SCs to assume a state in which they promote nerve growth; subsequent removal of the active ErbB2 reverses this situation so that the SCs no longer promote growth and appear to promote axon withdrawal. Because SC processes commonly remain along the pathway of the withdrawing axons, it appears that SC withdrawal is not required for this axon withdrawal. These results also suggest that SCs formed during the period of induction are not maintained in the absence of active ErbB2.

**Denervation of neonatal mouse sciatic nerve induces Schwann cell apoptosis**

In neonatal rat, tSCs undergo programmed cell death (apoptosis) after denervation of muscle (Trachtenberg and Thompson, 1997). To determine whether neonatal tSCs in the mouse also die after denervation, we examined soleus muscles for morphological evidence of SC apoptosis after sciatic nerve transection at P5. After 24 h, approximately two-thirds of the endplates were devoid of all SCs (Table 3); after 48 h, 94% of endplates were devoid of these cells. SCs missing from these endplates appear to have undergone apoptosis, because cells with blebbing membranes, masses of condensed chromatin (DAPI label; data not shown), and prominent vacuoles were found adjacent to bungarotoxin-labeled synaptic sites. To further substantiate that the cells were disappearing as a consequence of apoptosis, TUNEL (Gavrieli et al., 1992) was performed (Fig. 7). Approximately 75% (Table 3) of the cells that appeared to be dying on the basis of the above morphological criteria were also TUNEL +. Surprisingly, nearly every apoptotic SC was located adjacent to and not above an endplate (Fig. 7C,E, arrow), suggesting that these cells had moved...
from the endplate before their death. In addition to those located some distance from the endplates, some tSCs were found one to two cell body lengths away from denervated synaptic sites (Table 3). Those that were not apoptotic were bipolar in shape and extended highly branched processes that contacted endplates and extended toward the muscle ends (data not shown). By 48 h, most of these nearby cells had also disappeared. Although we observed some SC death along intramuscular nerves and terminal branches from these nerves, they remained populated with SCs 48 h after denervation (data not shown). Evidence of SC death was rare in the endplate zone 48 h after denervation, suggesting that most of the cells destined to die had done so by this time. These observations show that, as in the neonatal rat, neonatal mouse terminal Schwann cells undergo denervation-induced apoptosis. Moreover, unlike their adult counterparts that remain located above the endplate for several days after denervation (our unpublished observations), the neonatal tSCs rapidly abandon the synaptic site.

### Conditional expression of active ErbB2 rescues neonatal Schwann cells from denervation-induced apoptosis

Because denervation-induced apoptosis in the neonatal rat can be prevented by exogenous application of GGFII (Trachtenberg and Thompson, 1996), we asked whether inducing neuregulin signaling selectively in SCs would similarly effect a rescue. Active ErbB2 was induced 48 h before denervation on postnatal day 5. The fate of SCs was then monitored morphologically and by TUNEL. The induction of the mutant ErbB2 appeared to rescue many tSCs from apoptosis because the number of S100+ cells in the endplate region that were TUNEL+ was much reduced 24 h after denervation (Fig. 7, compare C, D; Table 3). However, this rescue did not result in the tSCs remaining directly above the endplates; an equivalent number of endplates devoid of tSCs was found in both the induced and un-induced animals (Table 3). Evidently, tSCs in the neonate move from the endplates after denervation. Most S100+ cells and the TUNEL+ and S100+ cells are found not above the endplates but adjacent to them in the un-induced animals (Table 3). What induction appears to have done is to reduce the number of these migrating tSCs that then undergo apoptosis.

### Discussion

Experiments involving cell culture and mouse knock-outs have shown that neuregulins promote the survival, differentiation, migration, and proliferation of Schwann cells. Here we have taken a gain-of-function approach to examine some of these roles in vivo, concentrating on the SCs at the NMJ. One motivation for the present experiments was the observation of dramatic changes in NMJs seen after exogenous application of the neuregulin isoform GGFII to muscle in neonatal rats. This application induced SC proliferation, SC migration and SC process growth, nerve sprouting, withdrawal of nerve terminals from synapses, the dispersion of AChRs and synaptic failure, and rescued SCs from denervation-induced apoptosis (Trachtenberg and Thompson, 1997). However, all cellular components of the synapse, muscle fibers, motor neurons, and SCs, are reported to have receptors for neuregulins. It is therefore not clear which cellular target explains the consequences of neuregulin application. Indeed, recent experiments by Ponomareva et al. (2006) suggest that critical aspects of the disruption of NMJs in these experiments are explained by the action of neuregulin directly on muscle fibers. To

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**Figure 5.** Induction of active ErbB2 results in upregulation of nestin expression in tSCs. Active ErbB2 was induced in SCs in an adult mouse by 7 d of Dox administration. **A,** SCs visualized by the transgenic expression of GFP. **B,** Nestin visualized by application of a polyclonal antibody and a rhodamine secondary. **C,** AChRs visualized by the transgenic expression of CFP. **D,** AChRs visualized by application of Alexa 647–bungarotoxin. The nestin label is present in tSCs present at the junction and in processes extended by them (arrow in **A, B**); one of these processes is clearly associated with a nerve sprout (asterisks in **C**). Nestin label is also present in an extrajunctional SC (**B,** arrowhead). Scale bar, 30 μm.

**Figure 6.** Growth of nerve sprouts along SCs during induction of ErbB2 signaling differs from retraction of these sprouts after withdrawal of the inducer. Nerve sprouts visualized by transgenic expression of CFP (**A, C**) and SCs visualized by transgenic expression of GFP (**B, D**) in animals in which mutant ErbB2 had been induced for 10 d (**A, B**) or for 7 d and the Dox withdrawn over the subsequent 11 d (**C, D**). Sprouts advance without obvious filopodia (arrowhead in **A**) but withdraw by leaving pieces of CFP label located behind and disconnected from the retracting axon (arrowheads in **C**). These pieces lie along the SCs. Retreating axons often end with bulbous enlargements. Scale bar, 50 μm.
of these cells that have apparently left the endplate are dying in the induced animals. SCs are found immediately adjacent to the endplates and at greater distances from them. Many fewer

Table 3. Induction of active ErbB2 rescues many SCs at neonatal NMJs from denervation-induced apoptosis

<table>
<thead>
<tr>
<th>SCs</th>
<th>Endplates</th>
<th>Animals</th>
<th>Endplates devoid of SCs (%)</th>
<th>Endplates with an immediately adjacent SC (%)</th>
<th>Apoptotic SCs per endplate (n)</th>
<th>TUNEL+ SCs per endplate (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denervated, not induced</td>
<td>1134</td>
<td>10</td>
<td>70.8 ± 6.1</td>
<td>13.7 ± 1.2</td>
<td>0.46 ± 0.03</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>Denervated, induced</td>
<td>599</td>
<td>4</td>
<td>66.3 ± 6.8</td>
<td>15.5 ± 3.8</td>
<td>0.16 ± 0.02*</td>
<td>0.14 ± 0.02*</td>
</tr>
</tbody>
</table>

Soleus muscles were denervated by nerve resection on postnatal day 5 and examined at day 6. For the animals that were induced with Dox, induction began on day 3. SCs were identified by immunolabeling for S100. *p < 0.05, significant difference from animals that were not induced (Student’s t test). Endplates in both sets of muscles have lost SCs to the same extent. SCs are found immediately adjacent to the endplates and at greater distances from them. Many fewer of these cells that have apparently left the endplate are dying in the induced animals.

test which effects might be attributable to neuveglin action directly on SCs, we have taken the approach of inducibly activating neuveglin signaling in these cells using transgenes in mice. One transgene, coding for the transcription factor rtTA, was constructed with the S100B promoter so that it is expressed in SCs. A second transgene, coding for a neuveglin receptor mutated to be constitutively active (caErbB2), was placed under the control of promoter elements that bind rtTA in the presence of the inducer, the antibiotic Dox. We show that Dox-induced expression of caErbB2 produces TSC migration and proliferation, the extension of SC processes, and nerve terminal sprouting. These experiments show that all of these responses observed in response to neuveglin application can be explained by SCs. Furthermore, these experiments provide direct evidence that manipulating neuveglin signaling in SCs in vivo is sufficient to induce the growth of axons.

Neuregulins and reactive glia

After nerve injury, glial cells enter a so-called reactive state in which they express genes believed to play roles in promoting axonal regeneration, at least in the case of SCs in the PNS. Analysis of reactive SCs has shown that they upregulate expression of neuveglin (Raabe et al., 1996; Carroll et al., 1997; Rosenbaum et al., 1997) and that receptors for neuveglin in SCs become activated (Raabe et al., 1996; Carroll et al., 1997; Rosenbaum et al., 1997). Therefore, a stimulus for the reactive state could arise from autocrine/paracrine neuveglin signaling in SCs. This paracrine/autocrine signaling could supplant the neuveglin signal normally provided by the nerve either quantitatively or qualitatively (e.g., the isoform of neuveglin or its presentation as a cell-attached or soluble factor).

Our experiments suggest that SCs become reactive after activation of neuveglin signaling, although they remain in contact with undamaged axons. The cells extend processes and come to express a marker (nestin) for reactive glia. These processes, like those of SCs after denervation, support the growth of nerve sprouts. Indeed, we did not encounter examples of nerve sprouts that were not associated with these processes.

However, the reactive state of SCs expressing caErbB2 is not identical to that after denervation. Although process extension of SCs activated by expression of mutant ErbB2 appears as robust as that observed after denervation/partial denervation of muscles, the number of these processes occupied by sprouts appears less. Many junctions appear refractory to axonal sprouting, even 10 d after Dox induction, when the LacZ reporter shows that transgenes have been activated in most SCs. This suggests that additional stimuli arising from nerve degeneration and muscle denervation are present that either boost the reactive state of the glia or promote nerve growth. The existence of additional signals would not be surprising; given all of the possible trophic and cell surface interactions
between axons and SCs, it seems unlikely that denervation and the disruption of all of these interactions would yield the same phenotype as activation of a single signaling pathway, the one for neuregulin.

Migration
The motile responses of SCs during development are mediated in part by ErbB receptor activation (Mahanthappa et al., 1996; Lyons et al., 2005). Once established at junctions, the cell bodies of tSCs appear to be generally stable in their position above synapses. Our data suggest that, in addition to migrating along axonal sprouts or escaped fibers, caErbB2 expression can cause the cell bodies of SCs to migrate along muscle fibers, a substrate they would normally not follow.

Do neonatal and adult SCs respond similarly to caErbB2?
Our observations suggest that nearly all of the cellular responses seen in SCs after mutant ErbB2 expression are shared between neonatal and adult animals with one exception: we observed fewer nerve terminal sprouts in neonatal muscles despite extensive SC process growth. An explanation for this difference is that neonatal SCs express lower levels of the mutant receptor or lower levels of factors that induce nerve sprouting. However, several studies show that SCs harvested from neonatal rats can induce neurite outgrowth (Brockes et al., 1979; Bixby et al., 1988; Seeleheimer and Schachner, 1988; Peng et al., 2003; Ullian et al., 2004; Bampton et al., 2005). Another possibility is that neonatal motor axons are more refractory to growth signals, a possibility consistent with their enlarged terminal arbors in muscle and the pruning of these arbors that is occurring during postnatal synapse elimination.

Rescue of neonatal SCs from denervation-induced apoptosis
The precursor cells that give rise to SCs require ErbB receptor activation for survival (Meyer and Birchmeier, 1995; Erickson et al., 1997; Meyer et al., 1997; Riethmacher et al., 1997; Britsch et al., 1998; Morris et al., 1999; Woldeyessus et al., 1999; Garratt et al., 2000; Wolpowitz et al., 2000). That neuregulins continue to play important roles in cells after their commitment to the SC lineage was suggested by the observation of SC apoptosis in neonatal (but not adult) animals after nerve section and degeneration of axons (Grinspan et al., 1996; Trachtenberg and Thompson, 1996). SC apoptosis was particularly pronounced at rat NMJs in tSCs (Trachtenberg and Thompson, 1996). Evidence that nerve-supplied neuregulin is critical for the survival of these cells came from the rescue of these cells from denervation-induced apoptosis by application of GGFII. However, the applied neuregulin could have acted indirectly through a variety of other tissues that possess neuregulin receptors, e.g., muscle fibers. To determine whether neuregulin signaling in SCs could rescue these cells from apoptosis in vivo, we induced the expression of caErbB2 selectively in SCs after denervation. We found that tSCs as well as SCs within the nerves in neonatal mice underwent apoptosis after nerve injury, just as in the rat. Expression of active ErbB2 can rescue these cells from apoptosis after nerve injury. This suggests that a supply of this factor from axons is normally sufficient to prevent the execution of an apoptotic program. What is somewhat puzzling is that the cells are incapable of rescuing themselves via upregulation of neuregulin expression and autocrine signaling. This suggests that either the autocrine/paracrine pathway is insufficient to rescue neonatal SCs or that this signaling is activated too slowly to prevent the cells from committing to apoptosis.

SC proliferation and regulation of SC number
After denervation in adult animals, two waves of proliferation are found in SCs in distal nerve segments. The first wave occurs at the time of denervation and is believed to be stimulated by the degeneration process itself; this appears not to affect tSCs because they do not proliferate on denervation. The second wave occurs on contact of the SCs by regenerating axons. This latter wave of mitosis is believed to be mediated by nerve-derived neuregulin (Morrissey et al., 1995). Our experiments show that activation of neuregulin signaling can induce SC mitosis in nerves and at junctions.

An important feature of nerve development is the numerical matching of SCs with axons. During the early development of nerves, SCs proliferate so as to ensure that an adequate supply of these cells is available to ensheath axons and to cover nerve terminals. Other investigators have suggested that the initial proliferation is followed by a period of SC death such that SCs that do not come to be associated with axons are removed (Grinspan et al., 1996). A similar numerical matching must occur during nerve regeneration in the adult. Our experiments show that the extra SCs that appear in adult muscles after induction of active ErbB2 that lack association with nerve sprouts quickly disappear after removal of the inducer. Those newly generated SCs that are associated with nerve sprouts appear to survive for a longer time. The SCs generated during the induction as well as those generated during reinnervation in vivo arise from the division of adult SCs that are independent of neuregulin. We hypothesize that the proliferation of these cells restores a period of dependence on neuregulin signaling for survival. This would explain why adult SCs that are initially independent of nerve-derived signals become dependent on them once again and how a numerical matching between axons and their SCs could be reestablished.

References


