Despite advances in microsurgical techniques, patient outcome after severe peripheral nerve injury is still often poor. Functional recovery depends on many factors, including the lesion site and the interval of time between the injury and the surgical repair.

Concerning the lesion site, the more proximal the injury is, the more time is required for nerve regeneration and target reinnervation, because axons have to regenerate over longer distances. The rate of axonal regrowth is estimated to be approximately 1 mm per day. As a conse-
quency, when the nerve lesion is more distal (e.g., median or ulnar nerve injuries close to the wrist), the functional recovery of the hand muscles is expected to be reached within a few months. On the contrary, when the nerve lesion is more proximal (e.g., brachial plexus injuries close to the spinal cord), axons require 2–3 years to regrow before reaching target muscles.25,29,53

The timing of surgical intervention depends on the nerve injury type. In cases of sharp nerve transection, when the wound is clean and there are no other major injuries, primary nerve repair is usually the best choice (within 72 hours to 7 days).8,48 When the nerve injury is more severe (e.g., injury caused by a gunshot) and an immediate repair is not possible due to other concomitant and complicating injuries, the nerve reconstruction is delayed until all other tissues are accurately restored.48

In both cases (proximal injury or delayed nerve repair), a full functional recovery rarely occurs. This poor outcome can be attributed to many factors, which include the following: 1) the inability of denervated muscle to accept reinnervation and to recover from muscle atrophy; 2) the reduced ability of injured axons to regenerate after a long-term axotomy; and 3) the loss of the Schwann cell capability to support regeneration.25

This issue has been widely studied over the last several years.14,24,32,44,45,51,53 It has been shown that a decrease in the number of regenerating motoneurons and myelinated axons is accompanied by a decline in Schwann cell marker expression and an increase of fibrosis in the sciatic distal nerve stump following delayed nerve grafting.25 Also, previous studies, in which after a delay the tibial nerve was cross-sutured to the common peroneal nerve with a denervated nerve autograft, showed a decrease in the number of regenerated motoneurons, demonstrating that chronically denervated Schwann cells have an inhibitory effect on nerve regeneration.24

Moreover, recent studies that have focused on the expression of different molecules in the chronically axotomized distal nerve stump as well as in the delayed repaired nerves, have provided a neurobiological explanation for the poor functional outcome. A progressive downregulation of some regeneration-associated genes—namely α1-tubulin, actin, and GAP-43—has been recently shown in chronically axotomized motoneurons,23 even after the transient increase observed after a refreshment axotomy. Brain-derived neurotrophic factor and glial cell line–derived neurotrophic factor mRNA are significantly upregulated in transected distal tibial nerve following denervation (up to 6 months), whereas neurotrophin-3 and nerve growth factor mRNA levels are comparable to control levels.38 After delayed repair, Schwann cells in the distal stump significantly overexpress cleaved caspase 3 and downregulate ATF3.44,45

Previous data from our laboratory and others demonstrated that the neuregulin 1 (NRG1)/ErbB system is strongly and selectively regulated after acute peripheral nerve injury and during the early phases of regeneration,42,50 but little is known about expression changes of this system during chronic denervation and delayed repair. NRG1 is one of the most important factors regulating the activity of Schwann cells, both during their develop-

ment (Schwann cell survival, proliferation, differentiation, and migration) and during nerve regeneration after injury in adulthood (Schwann cell survival, axon guidance, and remyelination).12,16,20,26,41,42,46,54 The different NRG1 isoforms, generated through the use of different promoters and alternative exon splicing,1 can be soluble or transmembrane. Type I/II isoforms are produced either as soluble peptides ready to signal in a paracrine or autocrine manner or as transmembrane precursors that need proteolytic cleavage to release a soluble fragment into the extracellular environment. Type III isoforms are produced as transmembrane proteins that are ready to signal in a juxtacrine manner or that need proteolytic cleavage to be active.9

The aims of the current study were therefore as follows: 1) to study the expression of the NRG1/ErbB system during long-term degeneration (up to 9 months); and 2) to investigate—at functional, morphological, and biochemical levels—whether chronically denervated distal nerve stump can still sustain nerve regeneration of freshly axotomized axons.

Methods

Surgical Procedure

A total of 36 adult female Wistar rats, weighing approximately 200 g each, were used in this study. All animals were housed in a room with controlled temperature and humidity, with light/dark cycles of 12:12 hours, and had access to food and water ad libitum. All procedures were approved by the Bioethics Committee of the University of Turin, the Institutional Animal Care and Use Committee of the University of Turin, and the Italian Ministry of Health, in accordance with the European Communities Council (Directive 2010/63/EU).

Animals were operated under general anesthesia induced by intramuscular injection of 3 mg/kg tiletamine plus zolazepam (Zoletil). Surgical procedures were performed with rats placed supine with the legs wide apart.

Using a linear incision from the nipple to the elbow crease, the left median nerve was exposed, isolated, and transected approximately in the middle of the exposed part; the proximal nerve stump was buried in the pectoral muscle to prevent regeneration; the distal stump was sutured to adjacent innervated muscles and allowed to degenerate for 3 or 6 months.

Three or 6 months after median nerve axotomy, cross suture of the proximal stump of freshly axotomized ulnar nerve and the distal stump of the degenerated median nerve was performed with 2 ETHILON 9/0 stitches (3-month delayed repair group, n = 7, and 6-month delayed repair group, n = 7). Before the suture between the ulnar and median nerves, the first 5 mm of the distal degenerated median nerve stump was harvested in each rat. In another 7 animals, immediate cross suture between the median and ulnar nerves was performed (immediate repair group) (Fig. 1).

To prevent interference during the grasping test, the right median nerve was transected at the middle one-third of the brachium, and its proximal stump was sutured to the pectoralis major muscle to avoid spontaneous reinnervation.

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All animals were euthanized 6 months after the surgery by an anesthetic overdose, and the regenerated nerves were harvested. Healthy median nerve segments (n = 5), 9-month degenerated nerves (n = 5), and 3-month regenerated end-to-end–repaired median nerves (n = 5) from other animals were also harvested.

Grasping Test

The strengths of the flexor digitorum sublimis muscle and the flexor digitorum profundus muscle, which are both innervated by the median nerve in rats, were assessed by the grasping test. This test was done every 3 weeks, from 3 weeks postsurgery to 24 weeks (6 months). The grasping test was performed according to the same procedure previously described, using the BS-GRIP Grip Meter device (2Biological Instruments). Each animal was tested 3 times, and the average value was recorded.

Isolation of RNA, Preparation of cDNA, and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using TRIzol Reagent (Invitrogen), according to the manufacturer’s instructions. Reverse transcription (RT) of 0.4 μg total RNA was performed in a 25-μl reaction volume containing 1 × RT-Buffer, 0.1 μg/μl bovine serum albumin, 0.05% Triton, 1 mM deoxynucleoside triphosphate; 7.5 μM Random Hexamer Primers; 40 U RiboLock, and 200 U RevertAid Reverse Transcriptase (all RT ingredients were provided by Thermo Scientific). The reaction was performed at 25°C for 10 minutes, 42°C for 90 minutes, and 70°C for 10 minutes. Quantitative real-time polymerase chain reaction (PCR) was performed using an ABI Prism 7300 (Applied Biosystems, Life Technologies Europe BV) detection system. The cDNA was diluted 10-fold in nuclease-free water, and 5 μl (corresponding to 15 ng starting RNA) were analyzed in a 20-μl reaction volume containing 1 × iQaq universal SYBR Green supermix (Bio-Rad) and 300 nM forward and reverse primers. Dissociation curves were routinely performed to verify the presence of a single peak corresponding to the required amplicon. Analyses were performed in technical and biological triplicate. Degenerating nerve data from the real-time PCR experiments were analyzed using the −ΔΔCt method for the relative quantification to appreciate both upregulation (ranging from 0 to +∞) and downregulation (ranging from 0 to −∞). Meanwhile, relative quantitative data of regenerating nerve were analyzed as 2^−ΔΔCt (ranging from 0 to +1).

The threshold cycle number (Ct) values of calibrator and samples of interest were normalized to the geometrical average of 2 endogenous housekeeping genes: ANKR2D7 (ankyrin repeat domain 27) and RICTOR (RPTOR independent companion of MTOR complex 2). The average of uninjured nerves was used as a calibrator. Primers were designed using AnnHyb software (http://www.bioinformatics.org/annhyb/) and synthesized by BMR Genomics. Primer sequences for ErbB2, ErbB3, NRG1 Type II/III, and MBP were previously published. Primer sequences for S100, p75, and glial fibrillary acidic protein (GFAP) are as follows. S100: forward 5′-GGG TGACAAGCACAAGCTGAGAA-3′, reverse 5′-TTTG TCC ACCACTTTCTGCTTATG-3′; p75: forward 5′-AGC AGACCCATACGCAAGCT-3′, reverse 5′-TCTTAC TCTTCCACTGCTTGG-3′; and GFAP: forward 5′-GAG GCATGGCCACCAGTAAACAG-3′, reverse 5′-GGA AGCAACGTCTGAGGCTCG-3′.

Total Protein Extraction and Western Blot Analysis

Total proteins were extracted using the TRizol Reagent (Invitrogen) after RNA extraction, according to the manufacturer’s instructions. In the final passage, the protein pellet was resuspended in Laemmli buffer (2.5% sodium dodecyl sulfate, 0.125 M Tris-HCl, pH 6.8) at 100°C. Protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Sigma-Aldrich) on 1:4 proportion of proteins.
diluted proteins to avoid detergent interference. Proteins (50 μg/sample) were resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis; for the analysis of NRG1 isoforms, 4%–15% precast gels were used (Bio-Rad). Western blot analysis was performed as previously described.17 Primary antibodies used included antitotal HER2/ErbB2 (#sc-284) and antitotal HER3/ErbB3 (#sc-285) (diluted 1:1000, both purchased from Santa Cruz); anti-NRG1 Type III N terminal (#AB5551) from Chemicon International, diluted 1:1000; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#AM4300) diluted 1:20,000 from Ambion. Secondary antibodies used were horseradish peroxidase–linked antirabbit (#NA934) and antimouse (#NA931) diluted 1:40,000 (GE Health).

Statistical Methods
Statistical analysis was performed using SPSS software. All data (stereological analysis and gene expression analysis) were statistically analyzed using the t-test or 1-way ANOVA, and post hoc analysis was done with the Bonferroni test. Regression lines were analyzed by the Student t-test using the Prism Software Package (GraphPad).

Results

Degenerating Nerve

We first focused on studying the degenerating median nerve from a morphological and biomolecular point of view, to understand its characteristics before repairing the chronically degenerated nerve with a freshly axotomized nerve.

The chronically degenerated median nerves (3, 6, and 9 months after axotomy) were analyzed by transmission electron microscopy. Three months after the axotomy, neither myelinated nor unmyelinated fibers were detected, and axonal and myelin debris had been cleared. De-differentiated Schwann cells, as well as fibroblasts, colonized the distal part of the degenerating nerve (Fig. 2A). Some inflammatory cells (mast cells) were also still detected (Fig. 2A'). After 6 months, atrophic Schwann cells were observed (Fig. 2B), as well as endoneurial tubes (the basal laminae of Schwann cells) with some debris inside (Fig. 2B'). Similar morphological features were also detected after 9 months of nerve degeneration (Fig. 2C and C').

Quantitative real-time PCR analysis was performed to evaluate mRNA expression during long-term median nerve degeneration of the following Schwann cell–specific markers: myelin basic protein (MBP), S100, low-affinity nerve growth factor receptor (p75), and GFAP.

As expected, the MBP mRNA level (Fig. 2D) strongly decreased 3 months after injury, and its level remained strongly downregulated 6 and 9 months later, reflecting the chronic degeneration phase detected at the morphological level (Fig. 2A–C, A'–C'). The expression pattern of S100 is similar to that of MBP; it strongly decreased and remained downregulated for all of the analyzed time points (Fig. 2E). On the contrary, p75 was intensely upregulated during long-term degeneration (Fig. 2F). Finally, GFAP was downregulated 3 and 9 months after the injury (Fig. 2G).

Next, we focused on the NRG1/ErbB system, which is known to have an important role during nerve degeneration/regeneration. In particular, mRNA and protein expression levels were examined by quantitative real-time PCR and Western blot analysis in long-term degenerating median nerves (Fig. 3).

Although ErbB2 and ErbB3 mRNA expression did not change after axotomy (Fig. 3A and B), ErbB2 and ErbB3 proteins were still present at 3 and 6 months. Three months after axotomy, mRNA expression of soluble NRG1 (Type I/II) was downregulated, and this downregulation was even more accentuated after 6 and 9 months (Fig. 3C). After 9 months, ErbB2 and ErbB3 proteins were no longer detectable (Fig. 3D).

For NRG1 protein expression, an antibody directed to...
the cytoplasmic N-terminus common to all transmembrane Type III isoforms was used. As previously described, this antibody recognizes different bands; among these, a band can be detected at approximately 75 kDa, which can be the product of NRG1 Type III cleavage by the α-secretase ADAM17/TACE (the α-N-terminal fragment [NTF], with a lower molecular weight) or the product after cleavage by the β-secretase BACE1 (β-NTF, with a higher molecular weight). In the control nerve, α-NTF is expressed. During chronic degeneration, no bands recognized by this antibody (α-NTF or β-NTF) were detected.

Regenerating Nerve

Immediately after median nerve axotomy (immediate repair group), 3 months after the axotomy (3-month delayed repair group), or 6 months after the axotomy (6-month delayed repair group), the distal median nerve stump was sutured to the freshly axotomized ulnar nerve, and axons were allowed to regenerate into the median nerve distal stump for 6 months (as displayed in Fig. 1). During the postoperative period, animals were tested every 3 weeks for flexor digitorum muscle function by means of the grasping test. One rat in the immediate repair group had already started to recover 6 weeks after nerve repair. Starting from Week 12, 3 animals began to recover, and by the end of the postoperative period (24 weeks), 4 of 7 animals showed a functional recovery (Fig. 4A). However, the grasping force, expressed in grams (Fig. 2).
4B), was lower compared with healthy control animals, where it was approximately 450 g (data not shown). No animals in the 2 delayed repair groups (3- and 6-month delays) showed functional recovery over time.

The structure of the nerve was evaluated, and the total number of myelinated fibers was estimated. All samples were quantitatively analyzed, as well as nerves withdrawn from animals that did not show functional recovery. Morphological evaluation on toluidine blue–stained semi-thin sections revealed that 6 months after nerve repair (compared with uninjured healthy control nerves [Fig. 5A]), regenerated nerves from the 3 experimental groups (immediate repair [Fig. 5B], 3-month delayed repair [Fig. 5C], and 6-month delayed repair [Fig. 5D]) exhibited regrowth of many myelinated fibers organized in microfascicles with a well-defined axoplasm and well-organized myelin sheaths. As expected, from a qualitative point of view, they were smaller compared with uninjured healthy control nerves. Moreover, the amount of connective tissue is greater in the experimental groups compared with the healthy control nerves.

Quantitative analysis of the semi-thin sections showed that after immediate repair, the cross-sectional area (mm²) of the regenerated nerve is larger than in the healthy control group (Fig. 5E). On the contrary, no difference was observed in the cross-sectional area for the 2 delayed repair groups compared with both the healthy control group and the immediate repair group. The total number of myelinated fibers, obtained by stereological methods, was higher in the immediate repair group compared with the healthy control group. The 2 delayed repair groups showed fewer fibers than the immediate repair group, but there were no discernible differences in the total number of myelinated fibers between these 2 groups and the healthy control group (Fig. 5F).

With respect to axon and fiber size, a difference was detectable between the healthy control group and the 3 experimental groups. Moreover, the 2 delayed repair groups showed smaller axon and fiber diameters when compared with the immediate repair group (Fig. 6A). As expected, the mean myelin thickness was decreased in the 3 experimental groups compared with the healthy control group (Fig. 6B). No differences were seen among the 3 experimental groups. Finally, the g-ratio (axon diameter/fiber diameter) did not differ among the 4 groups.

In Fig. 6C–F, the frequency distributions of fiber diameters are shown. Healthy control nerve fiber diameters showed a bimodal distribution with peaks at 5–6 μm and 9–11 μm, whereas all 3 experimental groups showed a unimodal distribution of the fiber diameters. In particular, all of these groups showed a peak at 2–3 μm, but the 2 delayed repair groups showed a higher frequency of distribution of very small fibers (1–2 μm) compared with the immediate repair group.
Finally, we analyzed the g-ratio/axon diameter correlation of individual fibers by means of scatterplots, evaluating the differences in linear regression (Fig. 6G–J). As shown in the figure, the linear regression line for the healthy control nerve displays a flatter slope compared with the regenerated nerves.

Quantitative assessment of Schwann cell nuclei was performed by electron microscopy analysis on the distal regenerated nerve stumps. In particular, we quantified only myelinating Schwann cells (that is, Schwann cells for which the nucleus was associated with an axon). Figure 7 shows the results of the quantification, with representative electron micrographs. Data are expressed as the ratio between the total number of myelinated fibers and the total number of myelinating Schwann cell nuclei. In the healthy control group, this ratio was 13.65 ± 1.26, meaning that for each counted Schwann cell nucleus, there were 13.65 myelinated fibers. In the immediate repair group, the ratio was 11.61 ± 0.62, whereas in the 3- and 6-month delayed repair groups, the ratios were 7.00 ± 0.28 and 5.53 ± 0.91, respectively. These results demonstrate that, in proportion to the total number of regenerated myelinated fibers, there are more Schwann cells in the 2 delayed repair groups compared with both the healthy control and immediate repair groups.

To understand whether the expression levels of Schwann cell–specific markers (MBP, S100, p75, and GFAP) changed after delayed nerve regeneration, we performed quantitative real-time PCR analysis on the regenerated distal nerve stumps. We focused on the worst case, which was the 6-month delayed repair group. We compared these real-time PCR results with the results obtained after 6 months of degeneration (as a negative control) and with healthy control nerves (as a positive control). These last 2 samples are the same as those shown in Fig. 2. Moreover, as an additional positive control for regeneration, we used a 3-month regenerated distal segment of an end-to-end–repaired median nerve. As expected, the MBP mRNA level (Fig. 8A) increased after delayed nerve regeneration, we performed quantitative real-time PCR analysis on the regenerated distal nerve stumps. We focused on the worst case, which was the 6-month delayed repair group. We compared these real-time PCR results with the results obtained after 6 months of degeneration (as a negative control) and with healthy control nerves (as a positive control). These last 2 samples are the same as those shown in Fig. 2. Moreover, as an additional positive control for regeneration, we used a 3-month regenerated distal segment of an end-to-end–repaired median nerve. As expected, the MBP mRNA level (Fig. 8A) increased after delayed nerve regeneration compared with the 6-month degenerating nerve; however, its expression was still different compared with both the healthy control values and the end-to-end–repaired median nerve.

Intriguingly, S100 (Fig. 8B) mRNA expression in the 6-month delayed repair group remained downregulated.
compared with both the healthy control nerve and the end-to-end-repaired median nerve; however, its expression was higher than the 6-month degenerating nerve. Expression of \( p75 \) (Fig. 8C) strongly increased after nerve degeneration, and it was still upregulated after delayed nerve regeneration when compared with the healthy control group. Expression of \( GFAP \) (Fig. 8D) was lower than in the 2 positive control groups.

Finally, we evaluated the regulation of the NRG1/ErbB system after delayed nerve repair. We observed that \( ErbB2 \) and \( ErbB3 \) mRNA expression was slightly upregulated after delayed regeneration compared with the 6-month degeneration and the end-to-end–repaired median nerve (for \( ErbB2 \); Fig. 9A) or the healthy control group (for \( ErbB3 \); Fig. 9B). Intriguingly, mRNA expression of soluble \( NRG1 \) (Type I/II), for which expression strongly decreased after nerve degeneration, was still strongly downregulated after delayed regeneration. Indeed, its level was comparable to the 6-month degeneration expression level and differed from the levels in the healthy control and the end-to-end–repaired median nerve (Fig. 9C).

For the protein analysis (Fig. 9D), we observed a very low \( ErbB2 \) expression in all experimental groups. The \( ErbB3 \) protein seemed to be slightly upregulated during

FIG. 6. Histograms show the results of histomorphometric evaluation (performed on Reg samples, Fig. 1). Axon diameter and fiber diameter (A) and myelin thickness and g-ratio (axon diameter/fiber diameter) (B) are represented. Values in the graphs are expressed as the mean + SEM \( (p \leq 0.001 \) vs all experimental groups; \( \star p \leq 0.05 \)). The 2 delayed repair groups showed smaller axon and fiber diameters when compared with both the immediate repair group and the healthy control nerve. Histograms show the percentage of the nerve fiber diameter distribution of control nerves \( (n = 432) \) (C) and of regenerated nerves after immediate repair \( (n = 1117) \) (D), 3-month delayed repair \( (n = 1004) \) (E), and 6-month delayed repair \( (n = 735) \) (F). The 2 delayed repair groups have a higher percentage of very small fibers \( (1–2 \mu m) \) compared with the immediate repair group. Scatter plots display the g-ratio of individual myelinated axons as a function of the respective axon diameter in the control nerve (G) and in the regenerated nerves after immediate repair (H), 3-month delayed repair (I), and 6-month delayed repair (J). Linear regression lines and the relative equation are also represented, showing that the healthy control nerve displays a significantly flatter slope compared with the regenerated nerves.

FIG. 7. Representative transmission electron micrographs of ultrathin cross sections of a control nerve (A) and regenerated nerves in the immediate repair (B), 3-month delayed repair (C), and 6-month delayed repair (D) groups (corresponding to Reg samples, Fig. 1). Bar = 10 \( \mu m \). White asterisks indicate myelinating Schwann cell nuclei. Histograms show the ratio between myelinated fibers and myelinating Schwann cell nuclei (E). Values in the graphs are expressed as the mean + SEM \( (\bigstar p < 0.01 \) vs control group; \( \$ p < 0.01 \) vs immediate repair group). The 2 delayed repair groups show a fiber/Schwann cell ratio lower than both healthy control and immediate repair groups, corresponding to a higher density of Schwann cells.
delayed regeneration compared with the degenerating nerve, but it was still lower than the 2 positive controls. With respect to NRG1 protein expression, we did not detect any bands after delayed regeneration, whereas in the end-to-end–repaired median nerve, a barely detectable band was present.

Discussion

In this study, we focused on 1) the molecular changes in the chronically degenerated nerve, with specific attention paid to the NRG1/ErbB gliotrophic system; and 2) the ability of the chronically degenerated nerve to sustain regeneration of freshly axotomized axons. This study demonstrated that chronic degeneration of the distal nerve stump compromises nerve regeneration in terms of functional recovery, as well as the number and size of regener-ated myelinated fibers. The inability of the distal nerve stump to sustain regeneration could be due to impaired Schwann cells that are not able to completely recover from atrophy. Indeed, we hypothesized that the downregulation of NRG1 still observed after delayed regeneration negatively affects Schwann cell activities and, consequently, nerve regeneration.

Surgical Paradigm Choice

We used the surgical paradigm of the cross suture between the chronically denervated median nerve distal stump and the freshly axotomized ulnar nerve proximal stump. A similar paradigm was used in studies by Sulaiman and Gordon and by Sulaiman et al., where the denervated common peroneal nerve was cross sutured with the freshly axotomized tibial nerve.51,52 We used a similar surgical procedure (but shifted from the hindlimb to the forelimb) for 3 main reasons. First, we wanted to mimic the clinical surgical procedure, in which the proximal nerve stump is usually refreshed before repairing a nerve injury23 (in our experiments, the ulnar nerve axotomy mimics this event). Second, before the cross suture, we withdrew degenerating median nerve segments from the same animals, to reduce the number of animals used in the study. Indeed, “Reduction” (with “Replacement” and “Refinement”) is 1 of the 3 R principles. “Reduction” means to minimize the number of animals used in the study by obtaining more information from the same animal. Indeed, we were able to study the long-term degenerating process and the regeneration after delayed repair in the same animals. Third, this technique needs a single suture, whereas the grafting technique requires 2 sutures to repair the nerve. In this way, it is possible to limit the variability due to the surgery (indeed, a double suture means more variability in terms of regeneration, because regrowing axons must cross 2 consecutive sutures).

Nevertheless, it is important to remember that the translation from animal models to humans is sometimes unreliable for nerve regeneration, due to the differences between rats and humans. These differences are reflected in a dif-

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**FIG. 8.** Quantitative real-time PCR results of MBP (A), S100 (B), p75 (C), and GFAP (D) mRNA expression in the 6-month delayed repair group, after 6 months of regeneration (Reg, Fig. 1). This result was compared with the expression level obtained in the 6-month degenerating nerve (DegM, Fig. 1) and in the control nerves (already shown in Fig. 2, but now expressed as 2−ΔΔCt instead of −ΔΔCt). An additional positive control for regeneration (end-to-end–repaired median nerve, 3 months after regeneration) is shown. Data were normalized to the geometrical mean of 2 endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphs are expressed as the mean ± SEM (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). After 6 months of delayed regeneration, Schwann cell markers were regulated differently compared with the healthy control nerve and the end-to-end–repaired median nerve.
ferent outcome after nerve repair. Depending on the surgical paradigm, peripheral nerves regenerate faster and better in rats than in humans, and rats may completely recover, unlike humans. However, this is a general issue that is not unique to nerve regeneration studies, because most in vivo research is performed in animal models.33

Long-Term Degenerating Nerve Strongly Downregulates Soluble NRG1

In the first part of our study, we analyzed the chronically denervated median nerve distal stumps. In particular, we evaluated the expression of Schwann cell markers and the NRG1/ErbB gliotrophic system, to understand which molecular changes occur after long-term degeneration, before repairing the median nerve with a freshly axotomized ulnar nerve proximal stump.

The analysis of MBP expression was used as an internal control for degeneration, as well as the ultrastructural analysis performed by electron microscopy. As expected, the MBP mRNA level strongly decreased during degeneration, reflecting the absence of myelinated fibers, which were also no longer detected at the morphological level.

Because the differentiation status of Schwann cells has been demonstrated to change after injury (that is, when the Schwann cell–axonal contact is lost), we analyzed the expression of the most common Schwann cell markers (S100, p75, and GFAP). The marker S100 is known to be expressed by both immature and mature myelinating and nonmyelinating Schwann cells.6 Immunofluorescence analysis has shown a progressive decrease of S100-positive cells in long-term denervated (4 or 6 months) distal stumps.36,59 Our results, obtained by quantitative real-time PCR mRNA analysis, are consistent with these data, and we hypothesize that S100 downregulation is due to Schwann cell atrophy.

The p75 receptor is a member of the tumor necrosis factor receptor family and binds neurotrophins with low affinity. Neurotrophins and their receptors are known to be involved in peripheral nerve regeneration. In particular, p75 is expressed in axotomized motoneurons after injury and is upregulated in Schwann cells after nerve injury; this upregulation has been associated with Schwann cell migration and apoptosis.10,59 The subsequent downregulation is generally associated with target reinnervation.59

FIG. 9. Quantitative real-time PCR results of ErbB2 (A) and ErbB3 (B) receptors and soluble Type I/II NRG1 isoform (C) expression in the 6-month delayed repair group, after 6 months of regeneration (Reg, Fig. 1). This result was compared with the expression obtained in the 6-month degenerating nerve (DegM, Fig. 1) and in the control nerves (already shown in Fig. 3, but now expressed as 2^−ΔΔCt instead of −ΔΔCt). An additional positive control for regeneration (end-to-end–repaired median nerve, 3 months after regeneration) is shown. Data were normalized to the geometrical mean of 2 endogenous housekeeping genes (ANKRD27 and RICTOR). Values in the graphs are expressed as the mean ± SEM (p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). Soluble NRG1 (Type I/II) was still strongly downregulated after 6 months of delayed nerve regeneration. D: Western blot analysis of proteins extracted from the 6-month delayed repair group and probed with antibodies for ErbB2, ErbB3, and NRG1 (AB5551 antibody, which can recognize 2 bands of approximately 75 kDa [γ-NTF and β-NTF]); GAPDH was used as a loading control. The positions of the detected proteins are indicated on the left; size markers are indicated on the right. The NRG1 was not detectable after delayed regeneration.
Herein, we observed a strong increase of p75 mRNA during long-term degeneration. Because no axons are present in the chronically degenerated distal nerve stump (axons are degenerated within 3 months and are not more visible by electron microscopy) and no regeneration occurs, p75 is more likely upregulated by Schwann cells that progressively undergo atrophy.

Finally, it has been demonstrated that GFAP, a Schwann cell–specific cytoskeleton constituent expressed by non-myelinating Schwann cells, is upregulated after damage (3–5 days after injury). After long-term denervation (up to 9 months), we observed a downregulation of GFAP, which follows an overexpression pattern similar to S100 and MBP. In this case also, the downregulation was probably due to Schwann cells undergoing atrophy after chronic denervation.

Previous studies demonstrated that soluble NRG1 (Type I/II), expressed by Schwann cells, is strongly upregulated in the rat median nerve in response to acute injury. However, this upregulation is transient, and soluble NRG1 mRNA expression returns to control values within 28 days. In this study, we demonstrated that soluble NRG1 mRNA expression strongly decreases during chronic degeneration, starting 3 months after nerve axotomy. We also analyzed axonal transmembrane NRG1 at the protein level, using an antibody directed to the cytoplasmic N-terminus common to all transmembrane Type III NRG1 isoforms. This antibody recognizes different bands, which can be the product of NRG1 Type III cleavage by the α-secretase ADAM17/TACE or the β-secretase BACE1.

In previous work, we hypothesized that there is a switch from ADAM17/TACE to BACE1 proteolytic cleavage of Type III NRG1 under regenerative conditions. From a functional point of view, this change means a switch from inhibition of the myelination (mediated by ADAM17/TACE cleavage of Type III NRG1) to promotion of the myelination (mediated by BACE1 cleavage of Type III NRG1). Here we observed that during chronic degeneration (3, 6, and 9 months), transmembrane NRG1 is totally missing, whereas in the control healthy nerves, only the NRG1 fragment produced by the ADAM17/TACE cleavage is detected. These results suggest that both transmembrane Type III NRG1 isoforms (mainly expressed by axons) and soluble Type I/II NRG1 isoforms (mainly expressed by Schwann cells) are no longer expressed during long-term nerve degeneration, probably because axons are no longer present in the chronically denervated distal nerve stump and Schwann cells are atrophic.

Next, we focused on the heterodimer ErbB2-ErbB3, which is the NRG1 receptor expressed by Schwann cells. The ErbB2 and ErbB3 mRNA did not change in the chronically denervated nerves (they remained similar to healthy control nerve values). However, ErbB2 and ErbB3 proteins followed a different expression pattern compared with mRNA because a decrease was detected after 9 months of degeneration. A discrepancy between mRNA and protein expression has already been described and might be explained by microRNA-mediated post-transcriptional regulation. Previous studies reported that ErbB2 mRNA was still expressed in Schwann cells that had been denervated for 1 month, whereas it was no longer expressed after long-term denervation, in accordance with our data. We suggest that this downregulation at the protein level could be due to Schwann cell atrophy.

Nerve Regeneration Is Impaired After Delayed Repair

As already demonstrated, healthy Schwann cells are indispensable for nerve regeneration. In the last several years, different studies tried to understand whether, and how, chronic denervation of Schwann cells affects axonal regeneration. It has been demonstrated that after delayed repair, Schwann cells in the distal stump of a long-term degenerated nerve lose their ability to support nerve regeneration, suggesting that they need to be reinnervated in a timely manner. Indeed, these cells progressively undergo atrophy, downregulate the expression of factors that sustain nerve regeneration, and upregulate the expression of molecules that inhibit axon regeneration.

In our study, we also demonstrated that long-term chronic denervation dramatically reduces nerve regeneration from different points of view. First, we did not observe any functional recovery in the 2 experimental groups in which nerve repair was delayed (both 3- and 6-month delayed repair groups). Nevertheless, we observed only a partial functional recovery of the immediate repair group; at the end of the observation period (24 weeks), only 4 of 7 animals showed a functional recovery (but none of the animals reached healthy values of approximately 450 g).

Moreover, this recovery was also lower compared with the end-to-end–repair median nerve, where animals attained values of approximately 350 g. This incomplete recovery can be explained by the complexity of the surgical model (cross suture between ulnar and median nerves), which requires a reorganization of projections from the CNS. Indeed, it has already been widely demonstrated that following a peripheral injury, there is a reorganization of cortical representation, as well as plastic changes in subcortical structures such as the thalamus, brainstem, or spinal cord.

Moreover, in our surgical paradigm, axons belonging to the ulnar nerve, which in the rat innervate the intrinsic muscles of the paw, after nerve repair regenerate into the median nerve distal stump, which in the rat innervates extrinsic flexor muscles of the forelimb digits. This means that regenerated axons (from the ulnar nerve to the median nerve) have to innervate different muscles, and the time required to learn this new skill is longer compared with a direct suture.

Despite this negative functional recovery, we observed many myelinated fibers in all experimental groups (not only in the immediate repair group where there was a partial functional recovery, but also in the 2 delayed repair groups). However, by performing stereological and morphometric analysis, we observed a significantly lower number of myelinated fibers in the delayed repair groups compared with the immediate repair group. On the other hand, only the latter showed a significantly higher fiber number compared with healthy control nerves. However, this is a typical condition of peripheral nerve regeneration, where axons sprout from a single regenerating fiber. Indeed, also in the end-to-end–repair median nerve, the number of myelinated fibers increased compared with the healthy control nerve.

Size parameters revealed that myelinated fibers (as well
as axons) of the delayed repair groups were smaller compared with the immediate repair group. These data were even clearer when we observed the fiber diameter distribution. Despite the fact that the distribution pattern was similar among the 3 experimental groups (they showed a unimodal distribution, whereas in the healthy control nerve a typical bimodal distribution could be observed), in the 2 delayed repair groups, the percentage of very small fibers (1–2 μm) was higher compared with the immediate repair group. Finally, we calculated the g-ratio (inner axonal diameter divided by the outer axonal diameter), a common tool to evaluate myelinlation (that is, a lower g-ratio indicates thicker myelin and vice versa). The mean g-ratio did not change among groups.

However, when we plotted the g-ratio of individual fibers in relation to the respective axon diameter, we observed that the slope of the linear regression was higher in the 2 delayed repair groups compared with the immediate repair and healthy control groups. The slope of the linear regression is an indirect parameter of nerve fiber maturation—the higher the slope, the higher the ratio between the g-ratio and the axon diameter and the lower the fiber maturation.58

Taken together, these results confirm that when the repair was delayed (by 3 and 6 months), myelinated fibers that regenerated within the degenerated distal stump were less numerous, smaller, and less mature compared with fibers that were allowed to regenerate immediately after axotomy, demonstrating that a poor regeneration occurred.

**Poor Outcome After Delayed Repair Is Due to Impaired Schwann Cells**

To explain the reduced regeneration, we focused on Schwann cells. The ratio between myelinated fibers and Schwann cells showed that, in proportion to the total number of regenerating fibers, Schwann cells were more numerous in the 2 delayed repair groups compared with the healthy control nerve and immediate repair groups. We hypothesized that a greater number of Schwann cells might reflect a shorter internodal length. Indeed, it has been demonstrated that internodes are shorter after regeneration.58

In demyelinating pathologies, the repair of demyelinating regions is accompanied by proliferation of supernumerary Schwann cells and formation of new shorter internodes.37 Also during nerve regeneration, a higher number of Schwann cells has been described; there is an overproduction of Schwann cells, which envelop the regrowing axons, followed by a decrease 10 months after the nerve repair.28,47

It is well known that internodal distance affects nerve impulse conduction velocity;58 shorter internodes might reflect slower conduction velocity, which might explain the negative functional recovery in the 2 delayed repair groups.

Next, we analyzed Schwann cells from a biomolecular point of view. To identify the molecular changes between degeneration and regeneration conditions, we focused on the worst case (6-month delay), and we compared expression profiles with its negative control (6 months of degeneration). Moreover, the end-to-end–repaired median nerve was used as a positive control for regeneration. We observed an expected increase of MBP mRNA expression after delayed nerve regeneration (of course, its expression was still different compared with the healthy control and the end-to-end–repaired median nerve, due to the presence of smaller fibers with thinner myelin). Expression of p75 was still upregulated, whereas SI100 mRNA expression was still strongly downregulated compared with healthy control nerves.

These data confirm previous work in which a decrease in SI100 level was shown in the distal stump of 3- and 6-month delayed repaired nerves.32 It is interesting to note that downregulation of SI100 does not reflect a decrease in the relative number of Schwann cells. Therefore, we hypothesized that after chronic degeneration, Schwann cells would be impaired in their activities and characteristics, even after 6 months of regeneration. This hypothesis was also supported by the analysis of the NRG1/ErbB system. The mRNA expression of the soluble NRG1 isoform (Type I/II) was still strongly downregulated after nerve regeneration, even though its receptors were slightly upregulated (probably to compensate for the lack of ligand).

To the contrary, in the end-to-end–repaired median nerve, the soluble NRG1 expression level was similar to that in the healthy control nerve. At the protein level, the axonal transmembrane NRG1 Type III was still missing after delayed regeneration, even though axons partially regenerated, as shown by morphological and stereological analysis. Indeed, it has been demonstrated that axonal transmembrane NRG1 Type III is a rate-limiting factor for nerve remyelination in the early phases after injury, whereas at later stages other signaling pathways seem to compensate. This suggests that axonal NRG1 is not necessary for long-term nerve remyelination.11

**Conclusions**

This study demonstrates that the chronic degeneration of the distal nerve stump compromises nerve regeneration. Motor functional recovery, number, and size of regenerated myelinated fibers are reduced. This poor outcome might be explained by impaired Schwann cells (NRG1 and other Schwann cell markers are deregulated after delayed regeneration) that are not able to properly support nerve regeneration.

Our results support the view that soluble NRG1 could be a good candidate for improving peripheral nerve regeneration. Manipulating its expression (by overexpressing NRG1 in denervated Schwann cells) might lead to better results. Future studies are necessary to elucidate whether this strategy could be promising for improving the outcome after delayed nerve repair.

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**References**

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47. Schröder JM: [Supernumerary Schwann cells during remyelination of regenerated and segmentally demyelinated axons in peripheral nerves.] Verh Dtsch Ges Pathol 52:222–228, 1968 (Ger)

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