ARTICLE

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Lentiviral-Mediated Transfer of CNTF to Schwann Cells within Reconstructed Peripheral Nerve Grafts Enhances Adult Retinal Ganglion Cell Survival and Axonal Regeneration

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We recently described a method for reconstituting peripheral nerve (PN) sheaths using adult Schwann cells (SCs). Reconstructed PN tissue grafted onto the cut optic nerve supports the regeneration of injured adult rat retinal ganglion cell (RGC) axons. To determine whether genetic manipulation of such grafts can further enhance regeneration, adult SCs were transduced with lentiviral vectors encoding either ciliary neurotrophic factor (LV-CNTF) or green fluorescent protein (LV-GFP). SCs expressed transgenes for at least 4 weeks after transplantation. There were high levels of CNTF mRNA and CNTF protein in PN grafts containing LV-CNTF-transduced SCs. Mean RGC survival was significantly increased with these grafts (11,863/retina) compared with LV-GFP controls (7064/retina). LV-CNTF-transduced SCs enhanced axonal regeneration to an even greater extent (3097 vs 393 RGCs/retina in LV-GFP controls). Many regenerated axons were myelinated. The use of genetically modified, reconstituted PN grafts to bridge tissue defects may provide new therapeutic strategies for the treatment of both CNS and PNS injuries.

Key Words: axonal regeneration, retinal ganglion cells, lentiviral vectors, ciliary neurotrophic factor, Schwann cells, tissue engineering, gene therapy

INTRODUCTION

Neurons of the adult mammalian central nervous system (CNS) exhibit poor spontaneous regenerative growth after injury unless provided with an appropriate microenvironment. Such an environment can be created by addition of exogenous trophic factors [1,2] and/or by implantation of cellular substrates or tissue bridges such as peripheral nerve (PN) grafts [3]. In the visual system and spinal cord of adult rodents, damaged axons have been shown to regrow through autologous PN grafts or into various types of Schwann cell (SC) implant [1,4–10].

The clinical use of multiple PN autografts after spinal cord injury has been reported [11]; however, this therapeutic approach may not always be practicable due to difficulties in obtaining sufficient host material and, perhaps most importantly, the additional functional deficits that ensue from harvesting autologous PN tissue. To develop an alternative to autologous PN grafts, we reconstituted allogeneic PN sheaths with autologous or congeneic SCs and transplanted these chimeric PNs onto transected optic nerve (ON) [12]. The reconstituted grafts supported the regrowth of adult retinal ganglion cell (RGC) axons; however, compared to PN autografts the number of RGCs regenerating axons into chimeric PNs was relatively low. Further improvements are thus required to achieve a greater and more consistent level of regrowth, optimizing the potential for functional recovery.

RGCs express receptors to ciliary neurotrophic factor (CNTF) [13,14] and become increasingly responsive to this cytokine as they mature [15,16]. We have shown that intravitreal injections of CNTF, but not neurotrophins

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(brain-derived neurotrophic factor (BDNF), neurotrophin-4/5, neurotrophin-3 (NT-3)), significantly increase long-distance axonal regeneration of adult RGCs into PN grafts [12,17]. Previous studies have successfully used viral vectors to transfer BDNF, NT-3, or glial cell line-derived neurotrophic factor genes directly to injury sites or into fibroblasts or PN segments/glial cells that were later applied to injury sites [18–22]. In the present study, lentiviral vectors encoding CNTF (LV-CNTF) or green fluorescent protein (LV-GFP) were used to transduce purified adult SCs *ex vivo* prior to PN reconstruction. We examined whether long-term, stable production of an enhanced level of CNTF around axonal growth cones within the reconstituted PN grafts [12] would increase long-distance regeneration of adult RGC axons. We found that viability and axonal regeneration of injured adult RGCs were significantly enhanced 4 weeks after LV-CNTF intervention, associated with increased levels of CNTF in the chimeric PN grafts. These reconstituted grafts were even more effective than autografts in promoting RGC axonal regeneration. This approach thus provides a new and potentially important therapeutic alternative for CNS or peripheral nervous system (PNS) repair that increases axonal regeneration and at the same time reduces the need for obtaining autologous PN tissue from injured patients.

RESULTS

Successful and Rapid Transduction of Schwann Cells *in Vitro*

The LV vector used in this work was highly efficient in transducing SCs. After LV-GFP transfection, the reporter GFP gene was expressed rapidly by transduced SCs *in vitro*; the proportion of GFP-expressing SCs increased from less than 20% at 24 h to over 90% at 48 h posttransduction (Figs. 1A and 1B). In contrast, when immunostained with an anti-CNTF antibody, about 30% of SCs were CNTF-positive 48 h after LV-CNTF transduction (Figs. 1C and 1D). While the difference in transgene expression may reflect different levels of transduction efficiency, it is possible that the observed difference was due to lack of sensitivity of the CNTF immunofluorescence procedure. Furthermore, most of the CNTF is released from trans-

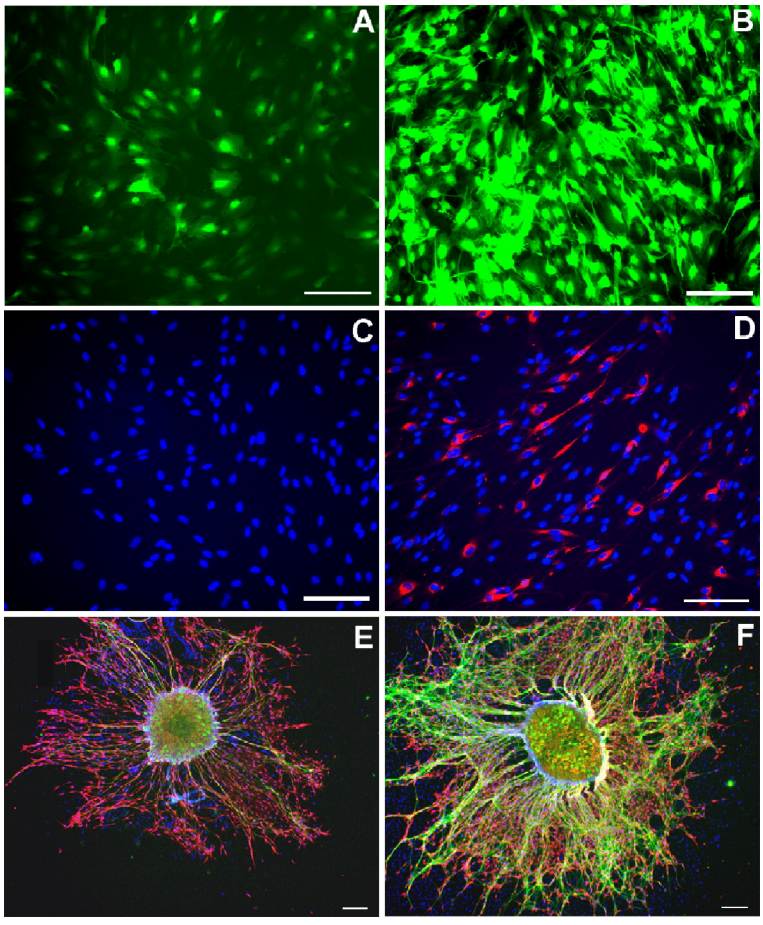

FIG. 1. Direct fluorescence images of reporter gene GFP expression in adult SCs (A) 24 and (B) 48 h after LV-GFP transduction: over 90% of SCs were expressing GFP 48 h after transduction. Immunostaining with anti-CNTF antibody of (C) normal adult SCs and (D) adult SCs 48 h after LV-CNTF transduction: no detectable CNTF-positive SCs were seen in normal SCs but about 30% of CNTF-positive SCs (red in D) were seen 48 h after LV-CNTF transduction. Bioactivity assay of supernatants from (E) LV-GFP- and (F) LV-CNTF-transduced SCs on E15 DRG: significantly more neurite outgrowth and cellular migration were seen after LV-CNTF supernatant treatment. Green, panneurofilament-positive neurites; red, S-100-positive SCs; blue, Hoechst 33342-labeled nuclei. Scale bars, 100 μm in A–D and 200 μm in E and F.

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duced cells; thus immunostaining will stain only the fraction that remains in the cytoplasm.

CNTF Released from LV-CNTF-Transduced Schwann Cells Is Biologically Active

We compared neurite outgrowth from fetal dorsal root ganglion (DRG) explants in conditioned medium from LV-GFP- and LV-CNTF-transduced SC cultures. Three days after conditioned medium treatment, compared with explants in LV-GFP supernatant, immunostaining with panneurofilament antibody revealed a clear increase in the number of neurites expressed by all three embryonic DRG explants treated with supernatant from LV-CNTF-transduced SCs (Figs. 1E and 1F). Due to the large number and density of neurites after LV-CNTF supernatant treatment, we were unable to count the number of neurites and thus quantitatively compare neurite expression between the two groups. These data confirmed that the CNTF produced and released by LV-CNTF-transduced SCs was biologically active. Interestingly, as revealed by immunoreaction with S-100 antibody and Hoechst 33342 staining, there was increased SC migration out of DRGs treated with LV-CNTF supernatant. The cellular migration distances of Hoechst 33342-labeled cells were significantly different ($P < 0.01$, two-tailed Student t test) between these two treatment groups (average area of migration and standard deviation 5.66 ± 1.14 mm^2 in LV-GFP group vs 10.88 ± 1.017 mm^2 in LV-CNTF group).

Enhanced CNTF mRNA Expression after LV-CNTF Transduction *in Vitro* and *in Vivo*

We carried out reverse transcription PCR analysis of CNTF mRNA expression levels in LV-GFP- and LV-CNTF-transduced SCs 2 days after transduction *in vitro* and in engineered PN grafts 4 weeks after transplantation *in vivo*. We detected low levels of CNTF mRNA expression in LV-GFP-transduced SCs 2 days after transduction (Fig. 2A) and in LV-GFP SCs in reconstituted PNs 4 weeks after transplantation (Fig. 2B). There was, however, a significant increase in the level of CNTF mRNA expression in LV-CNTF-transduced SCs (Fig. 2A) and in PN grafts containing LV-CNTF SCs (Fig. 2B), indicating successful incorporation of the CNTF gene into adult SCs and prolonged expression of the transgenic mRNA after *in vivo* transplantation. We also saw CNTF mRNA expression in normal cellular PNs (data not shown). Our data suggest that the level of endogenous CNTF is low in cultured adult SCs, a result consistent with some previous reports [23,24].

Increased CNTF Production as Revealed by ELISA

ELISA of CNTF protein in transduced SCs *in vitro* and engineered PN grafts 4 weeks after *in vivo* transplantation revealed a consistent increase in CNTF production after LV-CNTF treatment. We found significant increases in CNTF protein levels in both supernatant and cultured SCs 48 h after LV-CNTF transduction. It is known that SCs

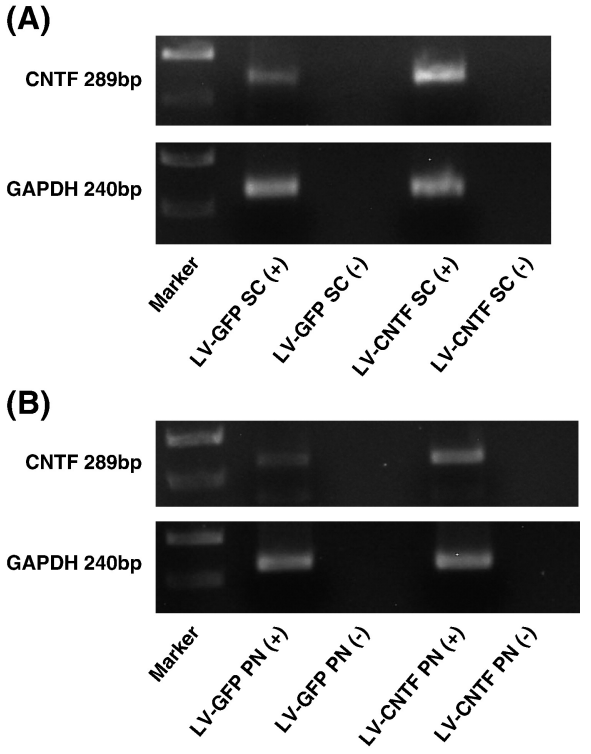

FIG. 2. RT-PCR of CNTF mRNA (A) from transduced SCs 2 days after *in vitro* transduction and (B) from engineered PN grafts 4 weeks after *in vivo* transplantation. Low levels of CNTF mRNA expression were detected in control SCs 2 days after transduction *in vitro* or in PNs 4 weeks after *in vivo* transplantation. Significantly higher levels of CNTF mRNA were seen in SCs *in vitro* or PNs *in vivo* after LV-CNTF treatment at corresponding times. +, with reverse transcription polymerase; −, without reverse transcription polymerase.

produce various neurotrophic factors, including CNTF, but the natural form of CNTF is usually cytosolic and apparently not readily released from cells. Thus, as expected, the level of CNTF in the supernatant of LV-GFP-transduced SCs was undetectable. The level of endogenous CNTF in these SCs was generally very low. Of the six ELISA tests on LV-GFP-transduced SCs, no detectable CNTF was seen in five, but in one other run there was an unexpectedly high level of CNTF at 10 ng per 10^6 LV-GFP-transduced SCs, thus resulting in an average level of cytosolic CNTF of 1.7 ± 1.6 (SEM) ng per 10^6 LV-GFP-transduced SCs.

CNTF produced after LV-CNTF transduction contained the human growth hormone release sequence and could thus be released from the transduced SCs. Consistent with this, 1×10^6 SCs transduced with LV-CNTF secreted an average of 94 ± 14 ng of CNTF/48 h into the culture medium. LV-CNTF-transduced SCs also contained, on average, 22 ± 5.6 ng of cytosolic CNTF per 1×10^6 cells; thus while the level of CNTF secretion was very high from these transduced cells, there were still relatively large amounts of CNTF in the cytoplasm compared to the LV-GFP-transduced cell population.

In vivo, 4 weeks after transplantation, we detected a significant difference in CNTF content between the two types of transduced PN grafts ($P < 0.05$). This CNTF was presumably mostly cytosolic in origin. The average CNTF content per milligram of total protein was 5.7 ± 1.2 ng in LV-CNTF-engineered PN grafts, while the content was 1.8 ± 0.6 ng/mg in LV-GFP-engineered PN grafts. Measurement of total CNTF content in each LV-CNTF-transduced PN graft revealed an average level of 1.785 ng CNTF per graft. Based on our *in vitro* data, the levels of secreted CNTF were likely to be at least four or five times this amount.

Immunostaining of Reconstituted PNs Reveals Long-Term Schwann Cell Viability

One week after injection of SCs into PN sheaths *in vitro* (Fig. 3A), or 4 weeks after PN transplantation *in vivo* (Fig. 3B), we saw GFP-expressing and CNTF-immunopositive SCs dispersed along the entire length of the PN grafts, indicating the presence of many viable SCs. The majority of these cells congregated toward the middle of the grafts, forming a longitudinal core of cells along the axis of the PN implants. This is consistent with our previous findings [12]. Laminin immunostaining did not reveal any obvious differences between LV-GFP- and LF-CNTF-reconstituted grafts. Importantly, 4 weeks after PN transplantation, large numbers of transduced S-100-positive SCs expressed high levels of GFP (Figs. 3C, 3D, and 3E) or CNTF (Fig. 3F). In addition, in the PN graft from the long-term survival rat, expression of reporter gene GFP was still visible after 12 weeks (Fig. 3G), indicating the potential for prolonged transgene expression in the grafts.

Increased RGC Survival after LV-CNTF Treatment

Examples of viable, TUJ1-immunopositive RGCs after LV-GFP or LV-CNTF treatment are shown in Figs. 3I and 3K. The average numbers and SEMs of TUJ1-positive surviving RGCs were 7064 ± 1057/retina in LV-GFP and

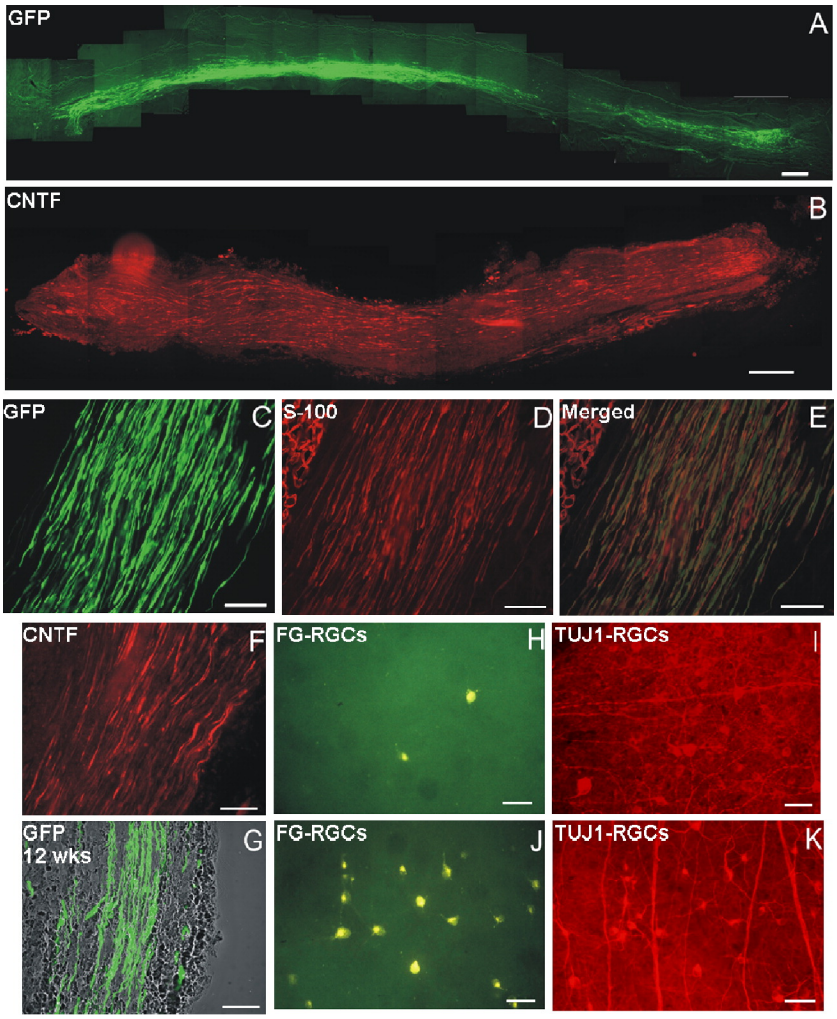

FIG. 3. Photomontages of PN grafts reconstituted with (A) LV-GFP 7 days *in vitro* and (B) LV-CNTF-transduced SCs 4 weeks after *in vivo* transplantation. PN sections from LV-CNTF-engineered animals were immunoreacted with anti-CNTF antibodies to show CNTF-positive SCs (red), while GFP-expressing SCs were visible directly under the fluorescence microscope. Transduced SCs were seen to be viable and disperse along the entire length of the PN segments. Higher power images of a PN graft reconstituted with (C) LV-GFP-transduced SCs showing GFP and (D) S-100-positive adult SCs 4 weeks after transplantation. (E) Merged image of C and D. (F) Higher power image of a PN graft reconstituted with LV-CNTF-transduced SCs showing CNTF-positive adult SCs 4 weeks after transplantation. (G) Long-term (12 weeks) transgene (GFP) expression. (H) FG-labeled regenerating and (I) TUJ1-positive viable RGCs in an animal that received an LV-GFP-manipulated PN graft (H and I, same field). (J and K, same field) FG-labeled regenerating (J) and TUJ1-positive viable (K) RGCs in an animal that received an LV-CNTF-manipulated PN graft. Scale bars, 400 μm in A and B and 100 μm in C–J.

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11,863 ± 1263/retina in LV-CNTF PN-grafted animals (Fig. 4), an increase of 68% in the latter group. This difference was statistically significant ($P < 0.02$, two-tailed Student t test).

Increased Number of RGCs Regenerating an Axon after LV-CNTF Treatment

Examples of fluorogold (FG) retrogradely labeled axon-regenerating RGCs after LV-GFP or LV-CNTF treatment are shown in Figs. 3H and 3J. There was about an eightfold increase in the mean number of RGCs regenerating an axon into the graft after LV-CNTF intervention (3097 ± 1409/retina) compared with LV-GFP intervention (393 ± 227/retina) (Fig. 4). The difference between these two groups was significant ($P < 0.01$, two-tailed Student t test). In an earlier study we reported an average of 299 RGCs/retina regenerating axons into PN sheaths repopulated with congeneic, non-genetically modified SCs [12]. Thus the expression of GFP in reconstituted PN grafts did not have any obvious detrimental effects on the SCs nor on their capacity to support RGC axonal regrowth. In one of the nine LV-CNTF-treated animals there were very high numbers of both surviving (18,753) and regenerating (13,748) RGCs; in this animal, over 70% of viable RGCs regenerated an axon into this PN graft. Examination of this PN graft also revealed a large number of panneurofilament-positive regrowing axons in the graft (see below).

In summary, both RGC survival and RGC axonal regeneration were increased after transplantation of PN grafts containing LV-CNTF-engineered SCs. Compared to LV-GFP-grafted PNs, the relative influence of LV-CNTF on RGC survival (68% increase) was less than the effect on axonal regeneration (788% increase). Only 5.6% of viable adult RGCs were FG labeled in the LV-GFP group compared with 26.1% in the LV-CNTF group, suggesting that the major therapeutic effect of sustained supply of CNTF in the PN grafts was in the promotion of long-distance axonal regeneration.

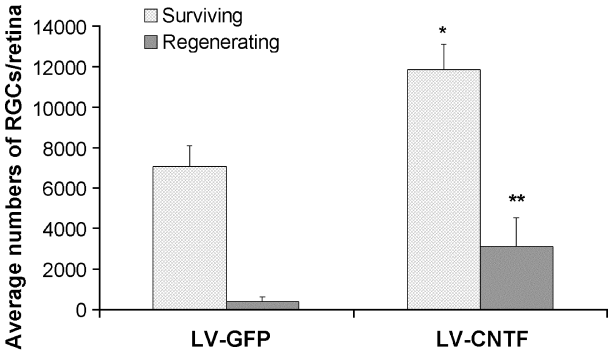

FIG. 4. The average numbers and the standard errors of the mean of TUJ1-positive surviving and FG-labeled regenerating RGCs after genetic manipulation of the reconstituted PN grafts using LV-GFP or LV-CNTF. Significantly higher numbers of both surviving and regenerating RGCs were seen in LV-CNTF-manipulated animals (*$P < 0.02$; **$P < 0.01$; two-tailed Student t test).

Immunohistochemical Analysis Confirmed Axonal Regrowth in Reconstructed PN Grafts

Consistent with the FG-labeled RGC counts obtained from retinal whole mounts, immunohistochemical staining of longitudinal PN sections with panneurofilament antibodies revealed numerous regenerating axons in all LV-CNTF-treated PN grafts. In the animal with an especially high number of FG-labeled regenerating RGCs after LV-CNTF treatment, a large number of regrowing axons were present along the whole length of the PN graft, although we saw substantially more axons in proximal parts of the graft close to the ON–PN interface (Figs. 5A and 5B). There were consistently higher average numbers of regenerating axons in LV-CNTF-treated PN grafts compared to LV-GFP-treated PN grafts along the length of the reconstructed PN grafts (Fig. 5C). There was thus a clear beneficial effect of enhanced CNTF expression on sustained axonal regrowth in PN tissue. Interestingly, in both groups there were similar numbers of regenerating axons at the most proximal end (axon entry site) of the PN grafts, but in grafts containing LV-GFP-transduced SCs there was a greater decrease in axonal numbers with increasing distance along the grafts (Fig. 5C). A comparable fall-off in axonal number at the distal end of PN grafts has also been described in blind-ended PN autografts [25,26] and in immunosuppressed allografts [27]. It remains to be seen whether this fall-off in axonal numbers after ON–PN transplantation is seen in grafts that are inserted into retinorecipient target sites in the brain [3,7,10].

Electron Microscopy of Adult SC-Repopulated PN Grafts

Examination of semithin and ultrathin transverse sections of adult SC-repopulated PN 4 weeks after PN–ON transplantation revealed that, in accordance with immunohistochemical observations, regenerating axons were usually located toward the center of the grafts and often formed close-packed axon fascicles (Figs. 6A and 6B). We made no attempt to quantify the number of regenerating axons in this material. Consistent with our previous study [12], we found SCs in large numbers and they had a normal ultrastructural appearance (Fig. 6C), indicative of their continued viability 4 weeks after injection into PN sheaths and transplantation to host ON. SCs myelinated regenerating RGC axons (Fig. 6C). The myelin was densely packed, typical of normal PN myelination. Some of the axons were small and unmyelinated, often grouped together in fascicles, and sometimes clearly associated with a single SC (Fig. 6C). As described previously [12], a consistent and intriguing difference between normal and reconstructed PN was the presence of substantially larger amounts of collagen in the extracellular space of the reconstituted nerves (Fig. 6C).

Recombinant CNTF enhances myelin formation by oligodendrocytes in culture [28], and in the injured PNS the cytokine accelerates SC myelination of regenerating axons [29,30]. It was therefore surprising that at 4

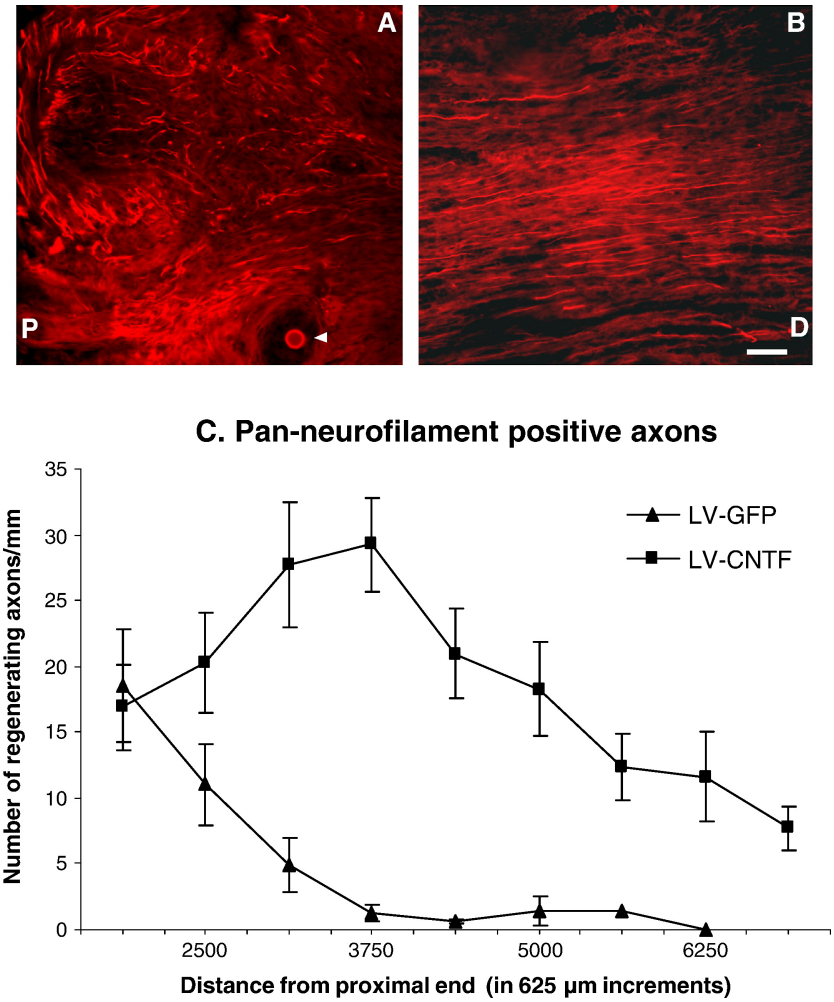

FIG. 5. Panneurofilament staining showing regenerating RGC axons in the (A) proximal and (B) distal parts of a PN graft repopulated with LV-CNTF-transduced SCs. Numerous axons were seen throughout the whole length of the PN graft. Arrowhead points to a suture that was used to connect the PN graft with the ON stump. P, proximal to the ON stump; D, distal to ON stump. Scale bar, 50 μm. (C) Immunohistochemical data from LV-GFP- and LV-CNTF-engineered PN grafts 4 weeks after transplantation. The average number of panneurofilament-positive axons across the PN is plotted against incremental distance from the PN–ON interface. While the numbers of regenerating axons were similar at the entry zone between the two groups, they decreased gradually toward the distal part of the PN grafts under both conditions.

weeks after transplantation, we saw no qualitative differences in axon myelination between the LV-CNTF- and the LV-GFP-treated PN grafts. Perhaps the myelination of adult RGC axons by SCs in the reconstructed PN grafts is slow and is not complete at this time, and longer survival times might reveal a difference between the two groups.

Discussion

It is well established that viable SCs are essential for the regrowth of axons in PN autografts [3,31]. SCs promote axonal regeneration in both the CNS and the PNS due to their capacity to provide growing axons with a variety of adhesion molecules and neurotrophic factors [32,33]. Using *in vitro* expanded populations of SCs we recently developed a way of reconstructing PN segments, which, after transplantation, was shown to support long-distance regeneration of adult RGC axons [12]. In the present study we used a gene therapy approach to modify the SCs in the chimeric PN constructs and examined whether this new type of engineered bridge could further promote survival and axonal regeneration of injured RGCs.

We show here that LV is efficient in *ex vivo* transduction of adult SCs and that the CNTF produced and secreted by these transduced SCs is biologically active. A sustained supply of CNTF by genetically modified SC in reconstituted PN segments can be achieved over an extended period after transplantation to the cut ON. Importantly, these reconstituted PN segments containing LV-CNTF-transduced SCs promoted both the survival and especially the long-distance axonal regeneration of injured adult RGCs. Transduced SCs expressed the transgene for at least 4 weeks, and enhanced RGC survival and axonal regeneration were correlated with increased CNTF production in PN grafts.

When injected intraocularly as a recombinant protein, CNTF has previously been shown to be effective in augmenting the survival and regrowth of adult RGC axons into PN grafts [17,34,35]. We now demonstrate, for the first time, that supply of CNTF in the PN bridges themselves can also exert beneficial biological actions on

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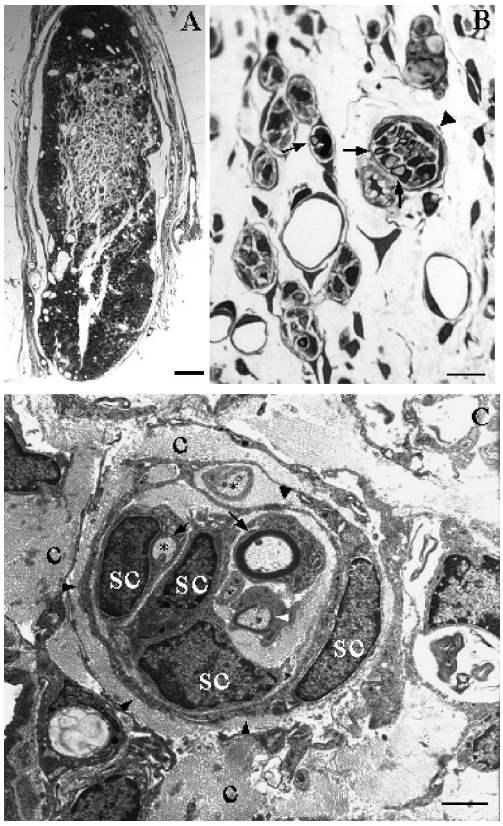

FIG. 6. Representative (A) semithin, (B) ultrathin, and (C) electronic microscopic images of LV-CNTF-transduced SC-reconstituted PNs 4 weeks after transplantation. Repopulating SCs (sc in C) survived and were of healthy appearance. Numerous myelinated (arrows in B and C) and unmyelinated (asterisks in C) regenerating RGC axons, which were often in clusters (arrowhead in B), were observed in the reconstituted PN grafts. Dense myelin sheaths wrapping regenerating axons were seen throughout the transverse sections (arrows in B and C). Sometimes axon myelination in progress was evident, as the axons were only partially wrapped by a myelin sheath (white arrowhead in C). Basal lamina formation (black arrowheads in C) and a substantial amount of collagen deposition were also visible (c in C). Scale bars, 100 μm in A, 10 μm in B, and 2 μm in C.

adult CNS neurons and with similar or even greater efficiency. Thus, after multiple intraocular CNTF injections in rats with PN autografts, we previously showed that there were, on average, 15,574 surviving RGCs of which 23.6% (3678) were retrogradely labeled with FG and had regenerated an axon to the distal end of the graft [35]. This compares with the present data using LV-CNTF-transduced SCs: a mean of 11,863 surviving RGCs of which 26.1% (3097) had regenerated an axon. The outcomes using this new gene therapy approach are especially impressive compared with our previous chimeric PN study [12]. Using unmodified adult SCs in reconstituted PNs, even after intraocular CNTF injections, we found on average only 999 regenerating RGCs per grafted animal.

Recently CNTF has been shown to act via retrograde signal transduction to achieve its biological function in facial motoneurons [36], and in peripheral nerves at least, the retrograde transport of CNTF has been reported to be increased by nerve injury [37]. RGCs express CNTFRα [13,14,38] and expression of this receptor in the retina is transiently increased after ON injury [14]. It is thus possible that the observed enhancement of RGC survival and increase in axonal regeneration were achieved by CNTF retrograde signaling from the engineered SCs in the reconstituted PN grafts to RGC soma in the retinas. On the other hand, local environmental modulation of growth cone pathfinding and elongation by sustained supply of CNTF within the PN grafts may also have contributed to the observed axonal regeneration. Importantly, we observed that similar numbers of axons were growing into the proximal ends of LV-GFP- and LV-CNTF-engineered PN grafts, indicating that the potential for injured axons to enter the PN grafts is similar under both conditions. This, together with the vast difference in the number of long-distance regrowing axons in the two types of PNs (Fig. 6C), suggests that CNTF exerts its primary effect on axon elongation rather than on local sprouting at the proximal PN–ON interface. In future studies, we plan to test the effect of combining this new PN approach with genetic transfer of growth-promoting genes directly into the injured adult RGCs themselves using viral vectors [39–44].

Previous regeneration studies have successfully used viral vectors to transfer growth factor genes directly into fresh PN segments [21,22]. The present approach allows the specific *ex vivo* transduction of a purified population of adult SCs and their subsequent placement within cell-free PN sheaths. Potentially, SCs engineered with different genes can be mixed together to optimize axonal regeneration even further in such reconstituted grafts. The use of these chimeric, genetically modified PN grafts has several important advantages over PN autografts: (1) this approach results in prolonged but potentially modifiable transgene expression after *in vivo* transplantation and (2) from a therapeutic point of view, it eliminates the need to harvest the patients' own PNs as bridging materials, a procedure that would likely result in additional functional deficits to the individual. Remarkably, the extent of RGC axonal regeneration in the LV-CNTF-modified PN grafts (mean of 3097 FG-labeled RGCs) is almost threefold higher than the figure we previously reported for PN autografts in the absence of any other interventions (mean of 1116 RGCs) [35]. The present study thus provides a basis for the development of new therapeutic alternatives for the treatment of traumatic CNS injuries, alternatives that may also be of benefit in the fields of plastic surgery and peripheral nerve repair.

MATERIALS AND METHODS

Adult (8- to 10-week-old) Fischer 344 (F344) and Sprague–Dawley (SD) rats were used in this study (source: Animal Resource Center, WA, Australia). SD

rats were used to provide the PN sheaths. We and others have previously shown that allogeneic, cell-free PN sheaths do not elicit an immunogenic reaction in the host, and when repopulated with congeneic or autologous SCs these chimeric grafts support axonal regeneration [12,45,46]. The allogeneic PN sheath approach was used again in this study to provide proof of principle that donor PNs can be used in the clinical context. All surgical procedures were performed under halothane anesthesia (induction 5%, maintenance 2% in 1/3 O_2/N_2O mixture) and animals received a subcutaneous injection of buprenorphine (0.02 mg/kg, Temgesic; Reckitt & Colman, Hull, UK). Experiments conformed to Australian NHMRC guidelines and were approved by the UWA Animal Ethics Committee.

Production of acellular PN sheaths. Acellular PN conduits were prepared from the peroneal nerve of euthanized (ip, Nembutal, sodium pentobarbitone; Rhone Merieux, Pinkenba, Australia) SD rats [12]. Segments of peroneal nerve (1.5 cm in length) were dissected out and immediately freeze–thawed five times to kill endogenous cells [45–47]. This procedure does not significantly disrupt the basal lamina scaffold within the nerves [12,13].

Adult Schwann cell cultures. The preparation of adult SCs to a high purity has been described previously [12,48]. For detailed information see the supplementary information. In brief, adult SCs were obtained from sciatic nerve explants that had been depleted of fibroblasts. After dissociation and when they had reached confluence, SCs were passaged into new dishes prior to LV transfection.

Production of lentiviral vectors. See supplementary information. Briefly, PCR was used to amplify the rat CNTF fragment, which contains the signal sequence required for the release of human growth hormone, out of plasmid HRC-5AS. The CNTF fragment was cloned into pRRLsin-PPThCMV-MCS-wpre and LV-CNTF stocks were produced by cotransfection of the vector, packaging, and envelope plasmids into 293T cells. After 2 days, medium with viral particles was harvested and, if needed, was concentrated by ultracentrifugation. For LV-GFP stocks, the number of transducing particles was defined by infecting 293T cells and counting the number of GFP-expressing cells after 48 h. Titers were expressed as transducing units (TU) per milliliter and concentrated stocks ranged on the order of 10^8 to 10^9 TU/ml.

Transduction of Schwann cells with lentiviral vectors. When SCs reached 70–80% confluence, replication-deficient LV-GFP [22] or LV-CNTF vectors were added to the dish at a multiplicity of infection of 1:50 for 24 h. Transduced SCs were used after 3 days *in vitro* when high levels of transgene expression were achieved. Expression of the reporter gene GFP was visible directly under a fluorescence microscope. Immunoreaction with anti-CNTF antibody (R&D Systems, Minneapolis, MN, USA) was carried out to reveal CNTF-positive SCs 48 h after transduction. Goat IgG control immunoglobulin or omission of primary antibody was used as negative control (see supplementary information for details).

Bioactivity assay. To determine whether CNTF produced by the LV-CNTF is biologically active, conditioned medium from LV-GFP- and LV-CNTF-transduced SCs was collected daily and stored immediately at −80°C. The detailed bioactivity assay procedure is provided in the supplementary information. In brief, conditioned media were tested on embryonic dorsal root ganglion explants and the media were replenished every day. Neurite outgrowth was assessed using immunohistochemistry and a monoclonal panneurofilament antibody (Zymed, San Francisco, CA, USA).

Cellular reconstitution of freeze–thawed nerves. Prior to injecting LV-transduced adult SCs into freeze–thawed PN sheaths, we put the acellular PN segments into a dish containing a confluent culture of appropriate cells (i.e., adult SCs transduced with the same viral vectors). We injected 5×10^4 SCs in 1 μl of medium slowly into each end of the PN (total of 10^5 SCs per PN) [12]. Placement of the cell-injected PN sheaths on confluent or near-confluent beds of the same cells allowed for further cellular infiltration [45,46]. PN pieces were maintained in culture in D-10S containing PEX (20 μg/ml) and forskolin (2 μM) for another day before grafting.

Optic nerve surgery. SC-repopulated PNs were grafted onto the cut left ON of F344 rats. All host animals were anesthetized with halothane (see earlier) and the ON was exposed intraorbitally after removal of the extraocular muscles. The ON was completely transected about 1.5 mm behind the optic disc. Care was taken to avoid damaging orbital blood vessels and the internal ophthalmic artery lying beneath the ON. We regularly verified the vascular integrity of the retina immediately after this procedure by fundoscopic examination [49]. Immediately after transection a PN graft was sutured with 10-0 suture onto the proximal stump of the axotomized ON [17,35,49]. The distal part of the PN was placed over the skull, and the free end was tied with 6-0 suture and secured to connective tissue.

Experimental groups. Transplanted animals were divided into two experimental groups for RGC survival and axonal regeneration studies. The first group (n = 7) received PN grafts reconstituted with LV-GFP-transduced SCs. The second group (n = 9) received PN grafts reconstituted with LV-CNTF-transduced SCs. Additional reconstituted grafts from the two groups were used for: (i) immunostaining for CNTF (LV-GFP, n = 4; LV-CNTF, n = 5) to examine CNTF expression 4 weeks after transplantation, (ii) RT-PCR (n = 5 each group) and ELISA (n = 5 each group) to measure CNTF mRNA and protein levels, and (iii) electron microscopy (n = 3 each group) to investigate the ultrastructure of the regrowing axons in the reconstructed PN grafts. One rat receiving an LV-GFP-manipulated PN was allowed to survive for 12 weeks in an attempt to see whether there was long-term transgene expression in this transplant model.

Retrograde labeling of regenerating retinal ganglion cells. In adult rodents, the fastest regenerating RGC axons grow in PN grafts at a rate of about 2 mm/day after an initial delay period of 4–5 days [50], and the number of regenerating RGCs reaches a peak level at 3–4 weeks post-PN–ON transplantation [51]. We therefore counted the number of adult axotomized RGCs regrowing axons into reconstructed PN conduits 4 weeks after the PN–ON surgery. To label regenerating RGC axons retrogradely, the graft lying on the skull was exposed and a small volume (0.2 μl) of 4% fluorogold (Fluorochrome, Denver, CO, USA) was injected into the distal end of the graft. Injection of a small volume of FG was essential to avoid diffusion of dye toward the optic disc and consequent staining of viable but nonregenerating RGCs. Cryostat sections of FG-injected PN grafts confirmed consistent FG labeling in the distal end of the PN bridges with limited diffusion.

Animals were kept for 2 days to maximize retrograde transport of the dye. They were then deeply anesthetized (Nembutal, ip) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Retinas and PN grafts were dissected out and postfixed in the same fixative for 1 or 2 h, respectively. Retinas were flat-mounted and coverslipped in Citifluor and the total number of FG-labeled RGCs was determined [35,49].

Immunohistochemical staining of viable RGCs. After the FG counts were made, whole retinas were immunostained with TUJ1 antibody (anti-βIII tubulin; BabCO, Richmond, CA, USA) and the number of TUJ1-positive cells per retina was determined (see supplementary information). TUJ1 immunostaining has recently been shown to be a reliable method for labeling viable RGCs in retinal wholemounts [35,49].

Cryosectioning and immunostaining of PN grafts. After removal of the retina, in each animal the PN graft was detached from the back of the operated eye and was cryoprotected in 30% sucrose overnight. Frozen longitudinal cryostat sections (16 μm thick) were cut. GFP expression was examined directly under a fluorescence microscope. To identify CNTF-positive and regenerating axons in the reconstituted PN grafts, immunohistochemistry was carried out (see supplementary information for details). Sections were immunostained with antibodies to CNTF, neurofilaments, S-100 (Dako, Glostrup, Denmark), or laminin (ICN, Aurora, IL, USA). Control IgG immunoglobulin and omission of primary antibody were used as negative controls.

Counts of axons that had regrown into PN grafts. On average, five or six sections were stained for each nerve, and in each of these sections the number of panneurofilament-positive regenerating axons was counted at increments of 625 μm from the proximal to the distal end of the PN graft [27]. The average number of axons per section was determined for each 625 μm and for each group; we then obtained an estimate of the mean

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number of regenerating axons per section at different distances along the PN grafts.

Reverse transcription PCR analysis. To test for continued CNTF transgene expression in SCs, RT-PCR was used to examine the levels of CNTF mRNA in LV-GFP- and LV-CNTF-manipulated SCs *in vitro* and PN grafts 4 weeks after *in vivo* transplantation (see supplementary information). Cycling conditions were 94°C for 60 s, 50°C for 60 s, 72°C for 60 s. Based on previous CNTF PCR studies in SCs [24], 35 cycles were used in this study. Controls (with and without RT enzyme) were used to check for genomic DNA amplification.

CNTF ELISA. CNTF levels in the conditioned media and protein extracts from transduced SCs *in vitro* and engineered PN grafts 4 weeks after transplantation were determined using quantitative sandwich ELISA (see supplementary information for details).

Semithin and ultrathin microscopy. To assess the viability of adult SCs in reconstructed PN grafts and to determine whether regenerating RGC axons were myelinated by these cells, three PN grafts from each treatment group were processed for semithin and EM analysis [12]. Transverse sections were taken from the proximal to the central part (about 1.5–3.5 mm from the PN–ON interface) of the reconstituted PN grafts. Semithin sections were stained with toluidine blue. Ultrathin sections were poststained with uranyl acetate for 15 min and lead acetate for 3 min and observed under a Philips transmission EM. For comparison, lengths of intact normal peroneal nerve were also removed and processed for EM.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data for this article may be found on ScienceDirect.

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