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CHAPTER 9

Directing regenerating motor axons to the peroneal division of the transected rat sciatic nerve by selective lentiviral vector-mediated overexpression of GDNF

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Background Misdirection of regenerating motor axons has a negative impact on the functional outcome following repair of peripheral nerves. The aim of this study was to direct regenerating motor axons using a lentiviral vector encoding for glial cell line-derived neurotrophic factor (LV-GDNF).
Methods LV-GDNF was selectively injected into the peroneal nerve in the rat sciatic nerve model, directly after transection and direct coaptation repair at the tibial-peroneal bifurcation. Results were evaluated after four weeks with simultaneous retrograde tracing of the tibial branch with fast blue (FB) and peroneal nerve branch with diamidino yellow (DY). Control groups consisted of LV-sGFP injection and direct coaptation repair without viral vector injection. In addition, nerve segments taken distally from the tracer application site were analyzed qualitatively for the presence of motor axons with ChAT immunohistochemistry.
Results After LV-GDNF injection, there was a doubling of the number of DY labelled motoneurons (from which axons had regenerated to the peroneal branch) compared to the control groups, with also a slight decrease in the number of FB labelled motoneurons (from which axons had regenerated to the tibial branch), although differences were only significant for the latter. Qualitative analysis of the nerves showed an increased presence of motor axons in the peroneal branch after LV-GDNF injection ($P<0.05$).
Discussion This study provides a first indication that LV-GDNF injection can be used to direct regenerating motor axons. Technical aspects of lentiviral vector injection and potential use in clinical nerve repair are discussed.

INTRODUCTION

Misdirection or misrouting of regenerating axons is one of the factors that can explain the disappointing functional recovery observed after nerve injury and repair [1]. For example, following repair of a motor nerve that is injured proximally to a branch point, regenerating motor axons may be directed to the wrong target branch and as a result reinnervate the inappropriate target muscle. This leads to co-contraction, because motoneurons that originate from the same moroneuron pool may have different target muscles. Co-contraction of different muscles may result in synkinesis (after facial nerve repair), mass movements (for example of shoulder and biceps muscles after repair of the upper trunk in brachial plexus injuries) or reduced motion (in case of reinnervation of antagonistic muscles).
In some patients it is possible to separately reconstruct nerve fascicles with different functions, but frequently, especially in more proximal injuries, this fascicular orientation may not be as well defined. In these cases misdirection at the coaptation site does occur, because axons do not always regenerate in a straight course to the distal nerve, but often first travel laterally at the coaptation site, before entering a distal endoneurial tube [2].

Little is still known about the impact of misdirection of regenerating motor axons on functional recovery, especially in motor nerve repair. Most research on specificity of regeneration has been performed in the femoral nerve that distally divides into the saphenous branch (SB), that is purely sensory, and a motor branch (MB) to the quadriceps muscle [3]. Experimental studies using this model have shown that motor axons preferentially regenerate towards the MB, which was termed *preferential motor reinnervation* [4, 5]. Although this phenomenon may be partially explained by *pruning* of misdirected collaterals [4] (that retract in favour of correctly directed axons), selective targeting of motor axons to the motor branch may be regulated by the expression of specific guidance molecules (e.g. L2 and HNK-1) [6, 7], by the expression of adhesion molecules (e.g. PSA-NCAM) by the regenerating motor axons [8], and/or the production of different growth factors by the Schwann cells in the MB [9].

In motor nerves innervating different distal target muscles, there are probably no such guiding cues or differences in growth factor expression between the different motor branches, for as many studies have shown limited specificity of regenerating axons for the different motor branches or target muscles [1, 10-15]. Most of these experiments on specificity of motor axon regeneration have been performed in the rat sciatic nerve model that distally branches into the tibial nerve (innervating muscles involved in plantar flexion) and peroneal nerve (innervating muscle involved in dorsiflexion). Recently, it was shown that following transection and surgical repair of the rat sciatic nerve only 42% of the peroneal motoneurons were correctly directed towards the peroneal nerve branch [1] (**Chapter 4**). Ankle motion analysis demonstrated that dorsiflexion function did not recover and that there were even signs for active plantar flexion during the swing phase (normally the moment of maximum dorsiflexion). This is probably due to the fact that the largest portion of peroneal motoneurons had regenerated towards the tibial nerve.

The goal of this study was to direct regenerating motor axons from the proximal stump selectively towards the peroneal nerve branch after transection of the sciatic nerve at the tibial-peroneal bifurcation by injection of a lentiviral vector encoding for GDNF into the peroneal nerve. Gene therapy is a new upcoming method in peripheral nerve regeneration that can also be applied to selectively guide regenerating axons by overexpression of certain growth factors [16-18]. In this study, GDNF was chosen because it is a potent neurotrophic factor for motor axons [19, 20]. The peroneal nerve was chosen, because of its relatively smaller size compared with the tibial nerve [1], which makes it is easier to detect a potential directing effect of LV-GDNF compared with injection into the larger tibial nerve. In addition, if possible to increase regeneration towards the peroneal branch, one of our future aims would be to investigate the impact on the recovery of dorsiflexion function). In the present study we first investigated the viral spread after LV-GDNF injection into the peroneal nerve using an ELISA set to detect the expression of GDNF at different levels in the sciatic, tibial and peroneal nerves. Second, the directing effect of LV-GDNF was analyzed four weeks after sciatic nerve injury and direct coaptation

repair at the bifurcation using simultaneous retrograde tracing with the tracers fast blue (FB) and diamidino yellow (DY) that were applied to respectively the tibial and peroneal nerve branches. ChAT immunohistochemistry was also performed to analyze the effect on the presence of motor axon branches in the distal nerves.

METHODS

Experimental groups

In all experiments adult female Wistar rats (Harlan, Horst, The Netherlands), weighing between 220 and 250g, were used. Animals were housed under standard conditions at a 12:12 h light/dark cycle with food and water ad libitum. All the experimental procedures were performed in accordance to the European directive for the care and use of laboratory animals (86/609/EEC) and were approved by the animal experimental committee of the Royal Netherlands Academy of Sciences. A total of 37 animals were used in this study. In 8 animals, the viral spread after LV-GDNF injection into the peroneal nerve was tested: in 4 animals the sciatic nerve was intact, in the other 4 animals the nerve was first transected and repaired at the tibial-peroneal bifurcation, after which the viral vector was injected. Simultaneous retrograde tracing and ChAT immunohistochemistry was performed in 8 animals 4 weeks after transection injury and repair, 8 animals after repair with LV-GDNF injection, and 8 animals after repair with LV-GFP injection. Five animals, in which the sciatic nerve was not transected, were used to obtain control values for simultaneous tracing.

Lentiviral vector preparation

The LV vectors encoding GDNF and stealth GFP have been described previously [21, 22]. The titers of the LV vector stocks were calculated by determining the p24 content (ng/μl) with an Elisa (zeptometrix, Buffalo, USA). Both vectors were titer matched to a viral titer of 6 ng/μl of p24 particles. All experiments were performed with the same viral stocks.

Surgical technique and animal care

Animals were deeply anesthetized with isoflurane (Isoflo, Abbotth, Hoofddorp, the Netherlands). The left sciatic nerve was exposed through a dorsal glutteal splitting approach with the aid of an operating microscope (OpMi-1, Zeiss, Sliedrecht, the Netherlands). The sciatic nerve was cut at the bifurcation into the tibial and peroneal nerve branches and these nerves were separately re-attached to the proximal sciatic nerve stump with 2-3 epineurial 10-0 nylon sutures for each branch (Ehtilon, Johnson& Johnson, Amersfoort, The Netherlands), while maintaining the correct fascicular orientation. In the viral vector groups subsequently, a 2 μl solution of 0.1M sodium buffered saline (pH 7.4) containing LV-GDNF or LV-GFP (total of 12 ng p24 particles) was injected into the peroneal nerve 5mm distal to the coaptation

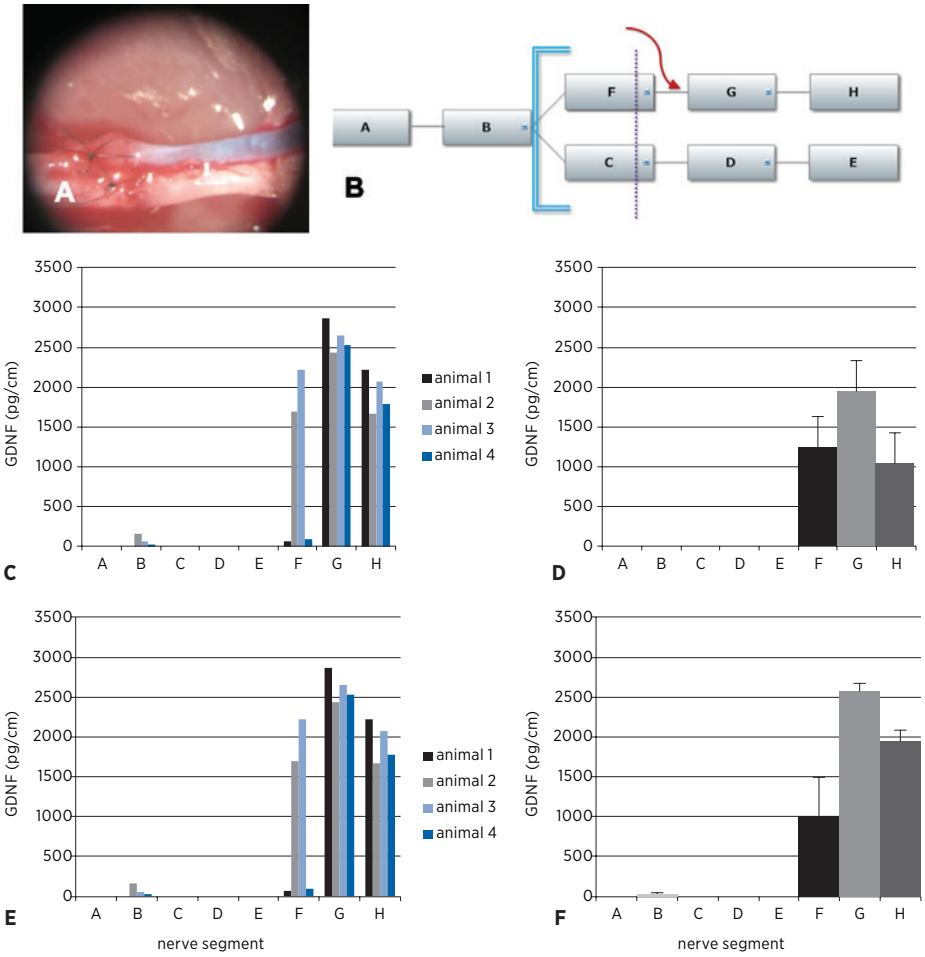

Figure 1

A: Example of LV-GDNF injection with viral spread (blue) in the peroneal nerve up to the repaired bifurcation. B: Schematic model of nerve segments (A-H) analyzed in ELISA experiment; blue line represents transection and repair site, purple line represents sites of tracer application, and red arrow site of viral vector injection. C: separate values for GDNF concentrations in different segments in unlesioned group, D: mean values (and SEM) for GDNF concentrations in different segments in unlesioned group, E: separate values for GDNF concentrations in different segments in the transection-coaptation repair group, D: mean values for GDNF concentrations in different segments in the transection-coaptation group.

site using a glass capillary with an 80 µm diameter tip attached to a 10 µl Hamilton syringe (Hamilton Company, Reno, USA) was added 0.1% Fast Green (Sigma, Zwijndrecht, the Netherlands) to the vector solution to visualize vector spread during injection. The needle was inserted through the epineurium into the peroneal fascicle and then was further inserted (1-2 mms) in a distal direction, parallel to

the nerve, before injection. Subsequently 2 μl of viral vector solution was slowly injected. Pictures of the viral spread were taken in all cases through the objective of the microscope. The needle was carefully removed to prevent leakage of vector solution. The skin was closed. Animals received buprenorphine (Schering-Plough B.V., Maarssen, the Netherlands) for postoperatieve analgesia and were kept at 37°C until recovery.

ELISA

Four weeks after injection of LV-GDNF, all animals in both groups (with and without transection injury and repair) were euthanized using Nembutal (sodium pentobarbital; 0.11ml/100g, Sanofi Sante, Maassluis, the Netherlands). The sciatic nerve was dissected into 0.5 cm segments starting proximal to the bifurcation (segment A and B) up to 1.5 distal to the bifurcation of the peroneal (segments F, G, and H) and tibial branch (segment C, D, and E) (Figure 1B). These segments were snap frozen on dry-ice. To quantify the amount of GDNF the nerve segments were homogenized in a mortar containing liquid nitrogen and resuspended in 250 μl lysis buffer (137 mM NaCl, 20 mM Tris/HCL, pH 8.0, Nonidet P40, 10% glycerol, 0.1% Polysorbate 20, 0.5 mM sodium othonovadate and 1 tablet / 50 ml of Roche total protease inhibitor).

The concentration of GDNF was measured with an ELISA kit (Emax #g7620, Promega, Madison, Wisconsin, USA) on high binding ELISA plates (Nunc-Immuno Maxisrop #439454). The procedure was performed following the manufacturer's instructions and the final GDNF concentration was expresses in pg/cm of nerve segment (A to H).

Simultaneous retrograde tracing

After four weeks simultaneous retrograde tracing was performed with FB and DY (both from EMS-Chemie, Mannedorf, Switzerland). First, the peroneal nerve was transected about 2 to 3mm from the coaptation site (proximal to the previous site of injection). The proximal end of the nerve was placed in a cup containing 1.5 μl of 5% DY solution for 30 minutes. After that the nerve end was cleaned with 0.9% saline and sutured in surrounding tissue to prevent tracer leakage and cross-contamination. Subsequently the same procedure was performed for the tibial nerve branch (also transection 2-3mm from the bifurcation site) except the nerve was placed in a cup containing 1.5 μl of 5% FB tracer. After one week of survival for retrograde transport of the tracer, the animals were perfused with phosphate-buffered saline and a solution containing 4% paraformaldehyde and 10% sucrose. Lumbar sections of the spinal cords were removed, postfixed overnight, and then cryoprotected for one day in 25% sucrose in o.1M sodium phosphate-buffered saline pH 7.4 (PBS). After that the tissue was embedded in tissue-freezing medium (OCT Compound 4583, Tissue-Tek, Sakura, Zoeterwoude, the Netherlands) by snap-freezing in 2-methylbutane and stored at -80°C until sectioning. Saggital longitudinal 30-μm-thick sections were cut on a cryostat at -20°C. Slides were immediately evaluated

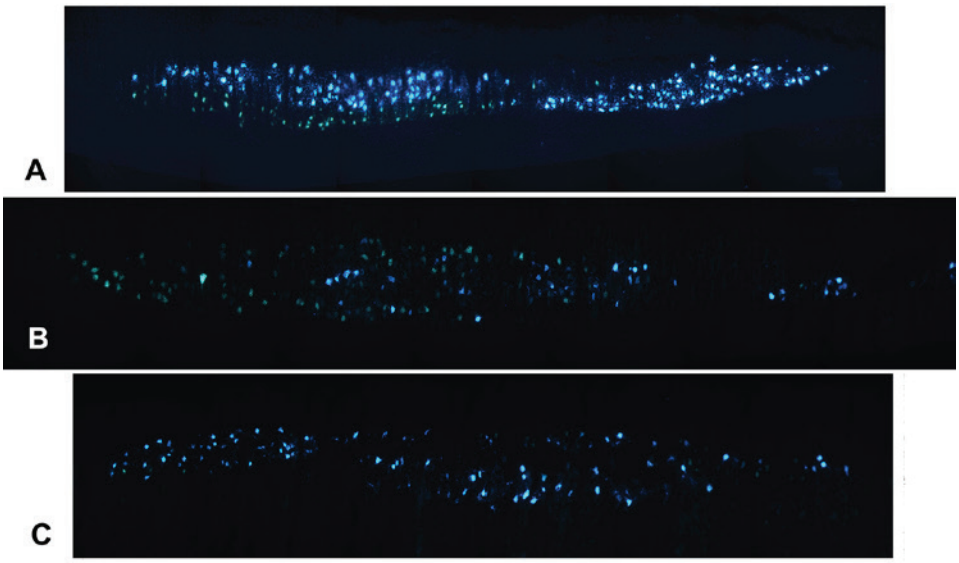

Figure 2

Microscopic images of examples of the distribution of FB- and DY-labeled motoneurons in the anterior horn of the spinal cord in a normal animal (A), and in animals after LV-GDNF (B) and LV-sGFP injection (C). FB-labeled motoneurons have blue cytoplasm. DY-labeled motoneurons have yellow nuclei. In the normal animal the DY- and FB-labeled motoneurons are grouped in two separate pools, respectively the peroneal and tibial motoneuronpool. In the case of LV-GDNF injection (B) there are clearly more DY-labeled motoneurons present than in the case of LV-sGFP injection (C). The organisation of profiles is lost and DY-labelled motoneurons are also present in the area normally exclusively occupied by tibial motoneurons.

at x10 magnification with a fluorescent microscope (Axioplan 2 Zeiss, Sliedrecht, the Netherlands). Profiles with a yellow nucleus were counted as DY labelled, with blue cytoplasm and a dark nucleus as FB labelled, and with blue cytoplasm and yellow nucleus as double FB-DY labelled (see figure). All sections were counted by one observer (GCdR), who was blinded for the experimental groups. No correction were made for the possibility of counting split motoneurons.

ChAT immunohistochemistry

Right after transection of the peroneal and tibial branches for tracer application, a 2mm nerve segment distal to the transection sites of these nerves were removed in the 6 of LV-GDNF and 7 of the LV-sGFP injected animals and fixed in 4% paraformaldehyde (in the other animals there was not 2 mm of tibial and/or peroneal nerve left distal to the tracer application sites). Immunohistochemistry was performed for choline acetyl transferase (ChAT). Briefly, sections were submitted to antigen retrieval (0.01 mg/ml proteinase K, 0.1% Triton X-100 in PBS) for 7 min followed by 3 washes in PBS. Endogenous peroxidase was blocked for 30 min (5% H2O2, 10%

methanol in PBS). Subsequently, the tissue was blocked using blocking buffer for 30 min (5% fetal bovine serum, 0.3% Triton X-100 in PBS). Sections were incubated at 4°C overnight in blocking buffer containing the primary antibody 1:200 (ChAT, Ab144p, Chemican, Hampshire, UK). After 3 washes the tissue was incubated for 2 hours with blocking buffer containing the seconday antibody 1:200)biotinylated horse anti-goat, Vector Laboratories, Burlingame, US). The sections were washed 3 times and incubated for one hour with avidin-biotin-peroxidase complex 1:800 (Vectastain Elite AC kit, Vector, Laboratories, Burlingame, USA). After washing in PBS sections were stained with 3.3'-Diamidinobenzidine (DAB) in TBS containing o.o1%H2O2 and 0.2 mg/ml NiSO4(NH4)2SO4 (nickel ammonium sulphate) resulting in a dark precipitate. Sections were dehydrated and embedded in Entalian.
Qualitative analysis of the slides for the presence of ChAT positive fibers was performed by 5 blinded observers who scored each slide as – (no ChAT positive fibers), +, ++, or +++, for increasing presence of ChaT fibers (Figure 2) . The scores of all observers for the same slide were added and mean scores per group were calculated.

Statistics

For all comparisons of values for different experimental groups an ANOVA test was used with posthoc Bonferroni test. For the qualitative analysis of motor axons branches (ChAT immunohistochemistry) by 5 blinded observers first the kappa value was calculated. *P*-values <0.05 were considered significant.

RESULTS

Transgene expression

Results of ELISA performed on 0.5 cm sections showed that the mean highest concentration GDNF was reached in the second segment (G) from the bifurcation (Figure 1D). The concentration of GDNF in the segment directly distal to the bifurcation (F) was slightly lower. Results were not significantly different after nerve transection and repair (Figure 1F), suggesting that transection and repair does not negatively influence the transfection of Schwann cells in the peroneal nerve branch. However, there were animal-to-animal variations (Figure 1C and E): in the group without lesion, there was one animal with a low concentration GDNF in segment F (<500 pg/cm) and in the group with transection injury and repair, there were 2 animals with concentrations <100 pg/cm. These animals did have increased levels of GDNF in next segment (G), but thus not in the segment adjacent to the transection and repair site. Further, in one animal in the unlesioned group and 3 animals in the transection and repair group, there was a slightly increased level of GDNF in the sciatic nerve segment proximal to the bifurcation (B). In the tibial nerve segments increase in GDNF concentrations did not occur, demonstrating

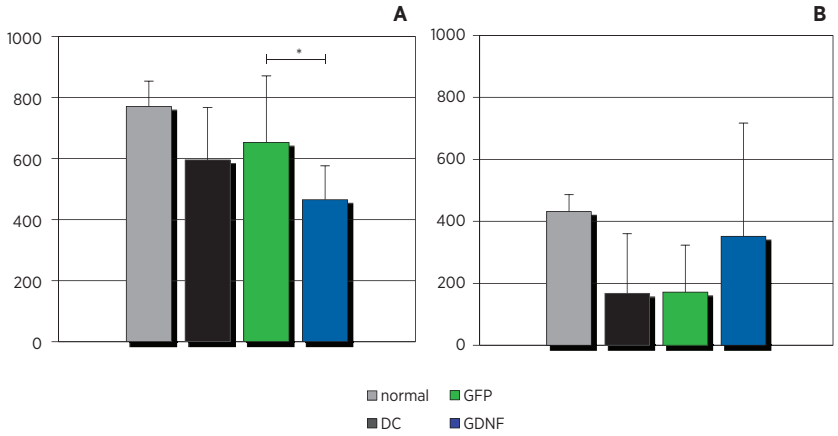

Figure 3

(A) FB and DY (B) profile counts (mean and SEM) for normal animals, direct coaptation repair (DC), and LV-GDNF and LV-sGFP injection groups. After LV-GDNF injection there is an increase in the number of DY-labeled profiles compared with the DC and LV-sGFP groups, although not significantly. The number of FB-labeled profiles is significantly decreased in the LV-GDNF group, compared with the LV-sGFP group (*).

that it is possible to selectively transduce the peroneal branch of the rat sciatic nerve using an LV-vector without cross-over to the tibial branch.

Simultaneous retrograde tracing

In all cases of direct coaptation repair (with or without viral vector injection) the distribution of DY- and FB-labelled profiles had shifted compared with normal animals. Examples are provided in Figure 2. Profiles counts (Figure 3) showed an increased number of DY labelled motoneurons (from which axons had regenerated to the peroneal branch) after LV-GDNF injection (352 ± 366) compared to the control groups (LV-GFP injection: 171 ± 152 and DC: 168 ± 192), although the difference in numbers of DY between the groups was not statistically significant (P = 0.15). There were variations in the numbers of DY-labeled motoneurons between the different animals especially in the LV-GDNF group (spread in number of DY-labelled profiles in the LV-GDNF group: 5 - 916, in the LV-GFP group: 3 - 448, and in the DC group: 9 - 424), with 2 animals in the LV-GDNF group that had respectively 916 and 876 DY-labelled profiles, and 486 and 409 FB-labelled profiles, resulting in a 2:1 DY to FB ratio, which is normally 1: 2 (normal animals: FB-labelled profiles 770 ± 83 and DY-labelled profiles 431 ± 55) (Figure 3). The number of FB-labelled profiles was slightly decreased after LV-GDNF injection (465 ± 111) compared with LV-GFP injection (653 ± 217) (P = 0.047), but not significantly compared with DC repair (595 ± 172) (P = 0.1102). Numbers of double-labelled (FB-DY) profiles were negligible (≤ 10 in all groups). The total numbers of regenerated motoneurons (FB and

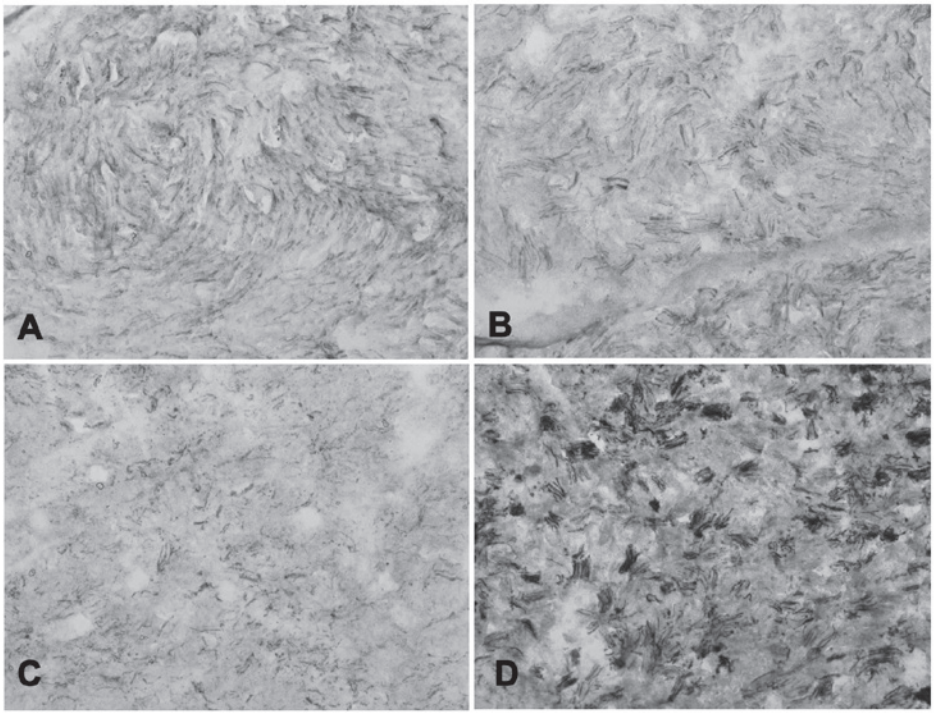

Figure 4

Microscopic images (x20 magnification) of examples of sections taken through the tibial (A and C) and peroneal nerve (B and D) after LV-sGFP (A and B) and LV-GDNF (C and D) injection analyzed with Chat immunohistochemistry. In Figure 4D there are clearly more motor axons than in the other sections (A-C). The motor axons are grouped, which could be explained by grouped/contained regeneration of axonal branches inside basal lamina tubes.

DY) were significantly decreased compared with normal (1202 ± 116) after DC (763 ± 196), LV-GDNF (817 ± 383) and LV-GFP (824 ± 234) (P = 0.0493).

Presence of motor axon branches in distal nerves

The cross-sectional area of the peroneal nerve after LV-GDNF injection was significantly enlarged compared with the LV-GFP control group (Figure 5B). ChAT immunohistochemistry also demonstrated an increased presence of motor axons in the peroneal nerve after LV-GDNF injection (Figure 4D) compared with the contralateral tibial nerve and compared with peroneal nerves in the LV-GFP group (Figure 4A-C); motor axons were grouped, and therefore difficult to quantify. Because of the latter, a semi-quantitative estimate of the slides by 5 blinded observers was performed, which showed a significantly increased presence of motor branches in the peroneal nerve in the LV-GDNF group compared with the LV-GFP group (P<0.05) (Figure 5C). There was a good correlation for the scoring between the

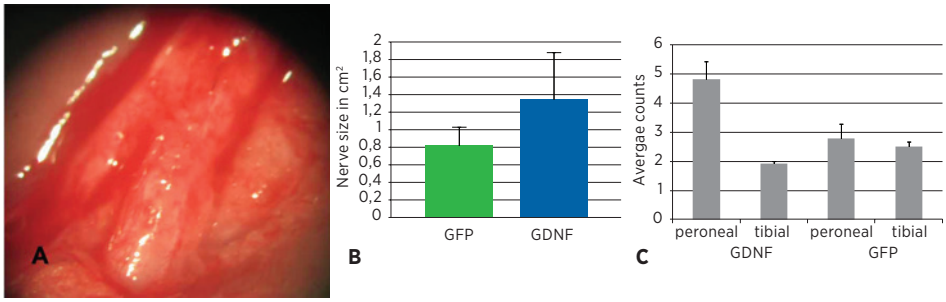

Figure 5

A: Example of image taken through the objective of the microscope at re-exploration after 4 weeks before tracer application. The peroneal nerve (on the right) is clearly enlarged compared with the tibial nerve and hyperaemic. B: Sizes of the tibial and peroneal nerves for sections obtained from the nerve segments taken distally from the tracer application sites in the LV-sGFP and LV-GDNF groups. C: Results for qualitative analysis of the sections of the tibial and peroneal nerves in the LV-sGFP and LV-GDNF groups.

different observers (kappa 0.64). Abundant fibers (as demonstrated in Figure 4D) were observed in 4 out of 6 LV-GDNF injection cases. Interestingly there was no correlation between the successful cases for simultaneous tracing (2) and ChAT immunohistochemistry (4); in only one case there was both an increase in number of DY-labeled profiles and number of ChAT positive fibers.

DISCUSSION

In this study we demonstrate that lentiviral vector-mediated gene transfer can be used to increase the expression of a neurotrophic factor to a specific branch of an intact and a transected and surgically repaired peripheral nerve. We used this approach to investigate whether it is possible to guide motor axons to a nerve branch that is expressing high levels of transgenic GDNF. Although the variability was high retrograde tracing revealed that there were on average more motoneurons from which axons had regenerated towards the peroneal branch that overexpressed GDNF than in the group with the control vector. In two animals an unusual high number of DY-labelled profiles was observed even reversing the normal peroneal : tibial motoneuron distribution (from 1:2 to 2:1), something that has not been reported before in other studies that have used the same simultaneous retrograde tracing technique to evaluate results after direct coaptation repair in the rat sciatic nerve model [1, 23] (**Chapter 4**). Moreover, in the animals that received an LV-GDNF injection into the peroneal branch significantly less motoneurons had projections towards the tibial nerve branch. Semi-quantitative estimate of the distal nerves with ChAT immunohistochemistry also showed an increased presence of motor axon branches in the distal peroneal nerve compared with the tibial nerve after

LV-GDNF injection. Overall these results thus indicate that viral vector mediated neurotrophic factor expression may be used to direct regenerating motor axons. However, the model used in this study was still limited by several technical aspects that are discussed below.

Technical aspects of the model

Different factors may explain the variation that was found in this study for the number of DY-labelled profiles in all groups. First of all, repair of the nerve at the bifurcation might have varied from animal to animal. We determined the branching point under the operating microscope, transected the nerve and subsequently sutured the tibial and peroneal nerves back to the proximal nerve, while maintaining correct fascicular alignment. The sciatic nerve, although optically divided at this point however may still consists of two large fascicles. In some cases therefore the tibial and peroneal nerves might have been repaired in continuity, and direction of tibial motoneurons towards the peroneal branch in these cases might have been limited (although cross-over in these cases may also occur [24]). A solution for this problem would be to use a Y-shaped conduit with proximal insertion of the sciatic nerve proximally and tibial and peroneal nerves distally, as has been used in earlier experiments on neurotropism [14, 25-28].

A second explanation for the variation in DY-labelled profiles, might be that the site of maximum number of motor axons may have varied relative to the site of tracer application due to difference in site of the so-called '*candy store*'. This site, at which motor axons are trapped due to a relatively higher concentration of neurotrophic factor compared to more distally in the nerve, may have varied from animal to animal, as can be concluded from the variation in concentration of GDNF at different levels in the peroneal nerve (Figure 1D and F). The site of the candy store may thus have varied relative to the site of retrograde tracing; in some cases the tracer may have been applied distal, and in others proximal to the candy store. From this we can also conclude that in the future in order to limit the effect of the candy store and to observe possible functional effects of directing regenerating motor axons the creation of a regulatable lentiviral construct will be essential.

Another explanation, for the observed variations, as can also be concluded from the ELISA results, is that there might have been variation in Schwann cell transduction efficiency after LV-GDNF injection. In two out four animals in the ELISA experiment we observed a very low concentration in the peroneal nerve segment just distally to the tibial-peroneal bifurcation (segment F). It could be that a certain treshold of GDNF had to be exceeded before motor axons were preferentially directed towards the peroneal nerve branch. This variation in GDNF expression could have been caused by a surgical variation in viral vector injection. When injecting, the needle has to be placed directly into a nerve fascicle and not into the perifascicular space. If not injected correctly, the viral solution may leak out of the nerve through the opening created in the perineurium. A solution for this potential problem of tracer leakage, might be to perform multiple injections at multiple

sites (as was done in a study by Hu et al [16], who injected the nerve 18 times at 3 different sites). However, we believe that for future potential clinical application, the number of injections should be preferably as low as possible, because injection may harm the architecture of the nerve by disrupting the orientation of basal lamina tubes or even by creating a separate dead-end channel inside the fascicle caused by the trajectory of the needle. More research is also needed on the spread of virus after injection into the nerve. Recently, White et al for example reported that the distribution of lentiviral vector after injection in the striatum may vary due to the size of the vector relative to the perivascular space [29]. We have found that our vector spreads more easily in the nerve after injection in proximal direction than in distal direction (unpublished observations). Therefore, in this experiment we injected into a distal direction, because in case of injection into a proximal direction the vector may have leaked out of the nerve at the coaptation site or even may have crossed over into the tibial nerve. This spread into proximal direction could explain the slight increase in GDNF concentration that was noted in the sciatic nerve proximal to the bifurcation site (B), although this might also be explained by the migration of transfected Schwann cells.

Finally, viral vector injection into the peroneal nerve might have interfered with retrograde tracing with DY. As illustrated in Figure 5A, at re-exploration the peroneal nerve in most cases had a swollen, hyperaemic aspect. During the 30 minutes of tracer application sometimes accumulation of blood inside the cup was observed, which might have limited DY tracer uptake. This could also explain the fact that abundant motor axons were observed in 4 out 6 cases with ChAT immunohistochemistry. Nevertheless, retrograde tracing in the analysis of the directing effect of viral vector injection on regenerating axons still is the most valuable evaluation method, because an increase in number of motor axons can also be explained by excessive branching inside the distal basal lamina tubes rather than increased regeneration towards the peroneal nerve.

Future potential clinical applications of lentiviral vector injection

Although some biosafety issues remain [30, 31], lentiviral vectors are already being used in clinical trials for Parkinson's disease, β-thalassemia, X-linked adrenoleukodystrophy (ALD), and AIDS [32] and many more clinical applications are currently being investigated. Also, in future clinical nerve repair lentiviral vector injections may play a role. Possible applications include: preventing atrophy and death of axotomised motoneurons; increasing the number and velocity of regenerating axons crossing coaptation sites and/or nerve grafts; preventing denervated muscle atrophy; to upgrade the results for autograft repair by the expression of different growth factors; and finally to direct/guide regenerating axons, which was the aim of the present study. The latter may be applied in the repair of mixed nerves, for example in median nerve repair at the wrist a viral vector encoding for a neurotrophic factor selectively enhancing motor axon regeneration (as for example GDNF) could be injected into the recurrent branch (innervating the muscles of

the thenar compartment) and a viral vector encoding for a neurotrophic factor selectively enhancing sensory axon regeneration (as for example NGF) into the digital cutaneous branches. For sensory regeneration Hu et al. [16] recently demonstrated using an adenoviral vector encoding for NGF that also sensory axons can be directed. In their study Ad-NGF injection into the saphenous branch 1 week after transection and direct coaptation repair of rat femoral nerve resulted a significantly increased ratio of DRG neurons regenerated towards the saphenous branch (SB/MB ~2) compared with injection of Ad-GFP (SB/MB~1) determined with simultaneous tracing 3 weeks after injury and repair. Most studies using adenoviral vectors in the peripheral nervous system however have been limited to the relatively short term, because of high immunogenicity of adenoviral vectors and the rapid humoral destruction of transduced cells [33].

To our knowledge the present study provides the first indication that viral vectors may be applied to direct regenerating motor axons. In this study we used a lentiviral vector encoding for GDNF, because this neurotrophic factor has been shown to improve motoneuron survival and regeneration after prolonged axotomy [34], and because motor neurons express receptors for GDNF (RET and GFRα-1) and upregulate these receptors after axotomy [35]. However, other molecules involved in guidance may also be used in gene therapy to either attract regenerating motor axons or divert them (for example by injecting a vector encoding for semaphoring 3A) [36-38]. The latter may be applied to prevent loss of axons towards a nerve branch that is of less interest (as for example the lateral antebrachial cutaneous nerve in repair of the musculocutaneous nerve).

CONCLUSIONS

Viral vectors may be applied in the future to direct regenerating motor axons to improve the results of motor nerve repair. More research is needed to improve the technique of viral vector injection and new vectors are currently also being developed (regulatable vectors and vectors expressing other molecules involved in axonal guidance). The results of this study can be used as basis for future research.

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