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Lentiviral-mediated transfer of CDNF promotes nerve regeneration and functional recovery after sciatic nerve injury in adult rats

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ABSTRACT

Peripheral nerve injury is often followed by incomplete and unsatisfactory functional recovery and may be associated with sensory and motor impairment of the affected limb. Therefore, a novel method is needed to improve the speed of recovery and the final functional outcome after peripheral nerve injuries. This report investigates the effect of lentiviral-mediated transfer of conserved dopamine neurotrophic factor (CDNF) on regeneration of the rat peripheral nerve in a transection model in vivo. We observed notable overexpression of CDNF protein in the distal sciatic nerve after recombinant CDNF lentiviral vector application. We evaluated sciatic nerve regeneration after surgery using light and electron microscopy and the functional recovery using the sciatic functional index and target muscle weight. HE staining revealed better ordered structured in the CDNF-treated group at 8 weeks post-surgery. Quantitative analysis of immunohistochemistry of NF200 and S-100 in the CDNF group revealed significant improvement of axonal and Schwann cell regeneration compared with the control groups at 4 weeks and 8 weeks after injury. The thickness of the myelination around the axons in the CDNF group was significantly higher than in the control groups at 8 weeks post-surgery. The CDNF group displayed higher muscle weights and significantly increased sciatic nerve index values. Our findings suggest that CDNF gene therapy could provide durable and stable CDNF protein concentration and has the potential to enhance peripheral nerve regeneration, morphological and functional recovery following nerve injury, which suggests a promising strategy for peripheral nerve repair.

1. Introduction

Peripheral nerve injury is a common clinical injury, which is usually caused by transportation accidents, natural disasters and other traumatic or the iatrogenic side effects of surgery. The peripheral nerve system (PNS) displays more significant regenerative potential than the central nervous system (CNS) after injury, but in many cases, the regeneration is far from perfect, leading to sensory and motor impairment of the affected limb. In various types of peripheral nerve injury, nerve transection, especially injury causing large nerve gaps, may have disastrous influence on the patient's quality of life. However, at present, the only treatment strategy for severe nerve lesions are microsurgical techniques using either autologous nerve grafts or direct nerve suture [1,2]. Autologous nerve grafting remains a golden standard for bridging an extended gap in transected nerves. The interposition of a nerve graft that minimises the hazardous tension has a certain therapeutic effect but does not provide full recovery [3]. Because of the limited availability of autologous nerve grafts and the associated donor site morbidity, new therapeutic approaches have been sought.

Previous animal experiments and clinical studies have confirmed the performance of peripheral nerve regeneration can be improved by the application of neurotrophic factors, Schwann cells or stem cells, and nerve conduits [4–6]. NTFs play an irreplaceable role in neuronal survival, differentiation and maintenance, which is a necessary prerequisite for functional reinnervation of transected peripheral nerves [7,8]. NTFs enhance nerve regeneration but cannot improve abnormal axon growth, which results in a mismatch of connections between nerve cells and the surrounding target [9]. These conditions seriously affect the normal nerve function recovery, whereas reducing the therapeutic effect of the growth factor. Therefore, the search for novel neurotrophic factors for peripheral nerve injury, which could be used in novel therapeutic approaches, is highly wanted.

In 2007, a potent NTF, called a conserved dopamine neurotrophic factor (CDNF) was identified as a novel NTF with strong trophic activity on dopaminergic neurons trophic [10]. CDNF has been demonstrated to be one of the most potent factors protecting and repairing the dopaminergic neurons in Parkinson's disease models [11]. In addition, in our previous studies, the overexpression of CDNF in astrocytes displayed the potential to alleviate cell

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damage and proinflammatory cytokine secretion, which may represent a promising strategy for neuroprotection in peripheral nerve injury [12]. However, the role of CDNF in peripheral nervous regeneration is unknown. Therefore, we applied overexpression of CDNF in a rat sciatic nerve transection model to investigate the effect of CDNF on sciatic nerve regeneration.

2. Materials and methods

2.1. Recombinant CDNF lentiviral vector design and production

Recombinant CDNF lentiviral vector design and production were performed as previously described [12]. Viral multiplicity of infection (MOI) was determined by Lenti-X GoStix (Clontech, TaKaRa, CA, USA).

2.2. Animals

Wistar rats were obtained from the Laboratory Animal Centre of Shandong University. A total of 45 male Wistar rats weighing 230–250 g were randomly assigned to CDNF (animals that received recombinant CDNF lentiviral vector, $n = 15$), LV (animals that received empty lentiviral vector, $n = 15$), and PBS (animals that received phosphate buffer, $n = 15$) groups. Animals were kept under controlled light/dark conditions (12/12 h), temperature (22 °C), and humidity (60%). Food and water were available ad libitum. All efforts were made to minimise suffering and animal numbers by using appropriate protocols. The protocol was approved and monitored by the International Guiding Principles for Animal Research, as stipulated by the World Health Organisation and as adopted by the Laboratory Animal Centre of Shandong University.

2.3. Surgical procedures

A rat sciatic nerve transection model was used in this study. Surgical procedures were performed under deep anaesthesia induced by i.p. injection of pentobarbital sodium (20 mg/kg). Surgical sites were shaved and prepared with 75% surgical alcohol. The left sciatic nerve was exposed through a gluteal muscle-splitting incision and transected using microsurgery scissors. For animals in the CDNF group, 6 μL (containing LV-CDNF 5 MOI) of the vector solution was then injected into the segment of transected sciatic nerve immediately using a 10-μL syringe (Shanghai Gaoge Industrial and Trading Co., Ltd, Shanghai, China). The LV group received the vector without the transgenes, and the PBS group received PBS as a negative control. All animals received an immediate entubulation repair within a silicone tube (Shandong Institute of Medical Instruments, Shandong, China), with an inner diameter of 1.5 mm and secured with 7-0 microsutures at each end, leaving a 3-mm gap between the proximal and distal stumps. Then, the musculature was replaced and the skin was sutured, after which the animals were returned to their cages.

2.4. Western blot analysis

Transgene expression was evaluated at 4 weeks after surgery. One centimetre-long sciatic nerve segments immediately distal to the graft were excised and frozen in liquid nitrogen at 4 weeks after injury. Protein was extracted and determined using a BCA Protein Assay Kit according to the manufacturer's instructions. Equivalent amounts of protein (10 μg) for each sample were separated by 10% acrylamide–SDS–PAGE, using 5% stacking and 12% separating gels. The samples were subsequently transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA). Primary antibodies (goat anti-CDNF, 1:1000, R&D Systems, Inc., Minneapolis, MN, USA) and a rabbit anti-goat immunoglobulin (IgG)–horseradish peroxidase (HRP) secondary antibody (1:30,000, Cell Signalling Technology, Danvers, MA) were applied. Equal amounts of protein loading were confirmed by reprobing the membranes with the mouse anti-GAPDH-HRP antibody (1:10,000, Abcam). Protein bands were detected using a FluorChem E Chemiluminescent Western Blot Imaging System (Cell Biosciences, Santa Clara, CA) and quantified by densitometry analysis using Image J software (National Institutes of Health, USA).

2.5. Histomorphological analysis

After the animals were euthanised, the sciatic nerve was harvested from the animals, and the nerve tissue was fixed in 4% formaldehyde on a plastic plate by stay sutures to keep the nerve straight. The nerve was embedded, cut longitudinally into 5-μm thick sections and stained with haematoxylin–eosin (H&E) and for immunohistochemistry using primary antibodies to neurofilament 200 (1:200; Beijing Biosynthesis Biotechnology Co., Ltd, Beijing, China) and S-100 (1:200; Beijing Biosynthesis Biotechnology Co., Ltd, Beijing, China). A goat anti-rabbit immunoglobulin (IgG)–horseradish peroxidase (HRP) secondary antibody (1:200; Beijing Golden Bridge Biotechnology Co., Ltd, Beijing, China) was applied. Images were captured using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Image-Pro Plus software (Media Cybernetics) was used to quantify the NF200-positive and S-100-positive areas at 400× magnification. Ultra-thin 70-nm sections were observed using transmission electron microscopy (performed by the Department of Electron Microscopy, Shandong University School of Medicine). A JEM-1200EX (JOEL, Tokyo, Japan) was used to capture the images. Image-Pro Plus software was also used to measure the thickness of the myelin sheath.

2.6. Target muscle weight

At 4 weeks and 8 weeks, as a simple and direct measure of CDNF effect on target muscles, after the animal were euthanised, the gastrocnemius muscle from the operated limbs of six rats in each group were exposed, dissected, removed and weighed immediately for comparison.

2.7. Functional analysis

The degree of recovery was monitored by the assessment of walking patterns in the hind limb to obtain a sciatic function index (SFI), according to the method described by Bain et al. [13]. At weeks 2, 4, 6, 8 after surgery, five animals in each group were submitted to a walking track analysis and measurement of the SFI based on the previously described protocol. Paw length and toe spreads were measured and used to calculate the SFI using the following formula: $SFI = 109.5(ETS - NTS)/NTS - 38.3(EPL - NPL)/NPL + 13.3(EIT - NIT)/NIT - 8.8$. In this expression, EPL indicates the operated experimental paw length; NPL, the normal paw length; ETS, the operated experimental toe spread, which represents the distance between the first and fifth toes; NTS, the normal toe spread; EIT, the operated experimental intermediary toe spread, which represents the distance between the second and fourth toes; and NIT, the normal intermediary toe spread. A value of −100 indicates total impairment, and 0 indicates normal or complete recovery.

2.8. Statistical analysis

The data are presented as the means ± SEM. Statistical analysis of the data were performed using a one-way analysis of variance (ANOVA), with post hoc Student LSD (Least Significant Differences)

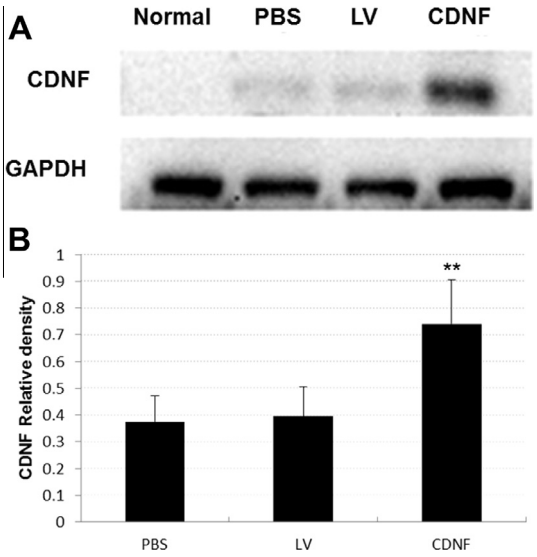

Fig. 1. Identified CDNF production in the distal sciatic nerve at 4 weeks after injury. (A) Western blot of CDNF protein. (B) The levels of CDNF were expressed as a relative ratio of the target protein to GAPDH. The overexpression of CDNF protein in the distal sciatic nerve after recombinant CDNF lentiviral vector application was considerably higher than in the nerves injected with LV and PBS.

pairwise comparisons applied as appropriate. Differences were considered to be statistically significant if the value of p was <0.05 (*) or 0.01 (**).

3. Results

3.1. CDNF production in the distal sciatic nerve

Extensive recent studies suggest that lentiviral vectors are highly efficient in many nerve regeneration models [6,14–16]. Our previous studies have reported that lentiviruses encoding CDNF could efficiently transfer and express CDNF in vitro [12]. We tested the CDNF content in the distal sciatic nerve from each group and the normal nerve using Western blotting analysis (Fig. 1A). At 4 weeks after injection, the expression of CDNF protein in the CDNF group was considerably higher than in nerve injected with LV and PBS, whereas the CDNF content in the normal rat sciatic nerve was particularly low (Fig. 1). The CDNF level in the control repair groups that received PBS injections or empty lentiviral vectors did not differ significantly between each other at the week 4 time point.

3.2. Histological analysis

The graft repairs remained intact and a newborn nerve formed in all groups. After harvesting the newborn nerve at week 4 and week 8, we tested the longitudinal section of the newborn and normal nerve using haematoxylin and eosin staining (HE) (Fig. 2A). The regenerated nerve in the CDNF group exhibited good ordered structured compared with the nerves of the other two groups.

We investigated the axonal regeneration by staining NF200-positive axons in the regeneration cables and normal nerves (Fig. 2B). The CDNF group exhibited significantly better axonal regeneration compared with the other two groups at 4 weeks and 8 weeks after injury (Fig. 2D) ($n = 5$, $p < 0.01$). Longitudinal sections of the sciatic nerve from the regeneration cables and normal nerves were stained for S-100, a Schwann cell marker (Fig. 2C), and the percentage of S-100 positive stained area was also statistically analysed (Fig. 2E). The group treated with recombinant CDNF lentiviral vectors displayed significant improvement of S-100 expression and Schwann cell regeneration compared with the LV and PBS groups ($n = 5$, $p < 0.01$) at 4 weeks and 8 weeks after the injury.

3.3. Remyelination of the regenerating sciatic nerve

Ultra-thin sections were also observed under a transmission electron microscope. As shown in Fig. 3, the electron micrograph

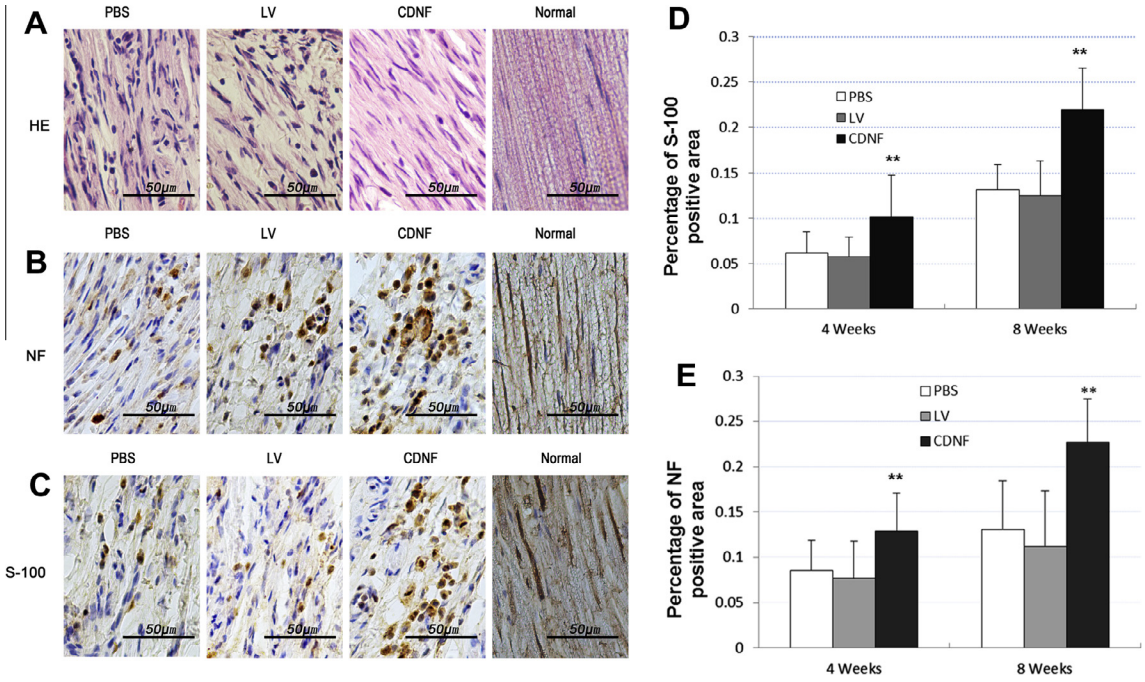

Fig. 2. Histological analysis. (A) At 8 weeks after injury, the longitudinal sections of the PBS, LV and CDNF groups were compared with that of normal nerves as analysed by HE staining. (B) At 8 weeks after injury, immunostaining with anti-NF200 antibody of the longitudinal sections of the PBS, LV and CDNF groups were compared with similarly analysed normal nerves. (C) At 8 weeks after surgery, immunostaining of the Schwann cell marker S-100 in the longitudinal sections of PBS, LV, PBS group was compared with that of normal nerves. (D) Quantitative analysis of NF200-positive area in longitudinal section of each group. (E) Quantitative analysis of S100-positive area in longitudinal section of each group. $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$.

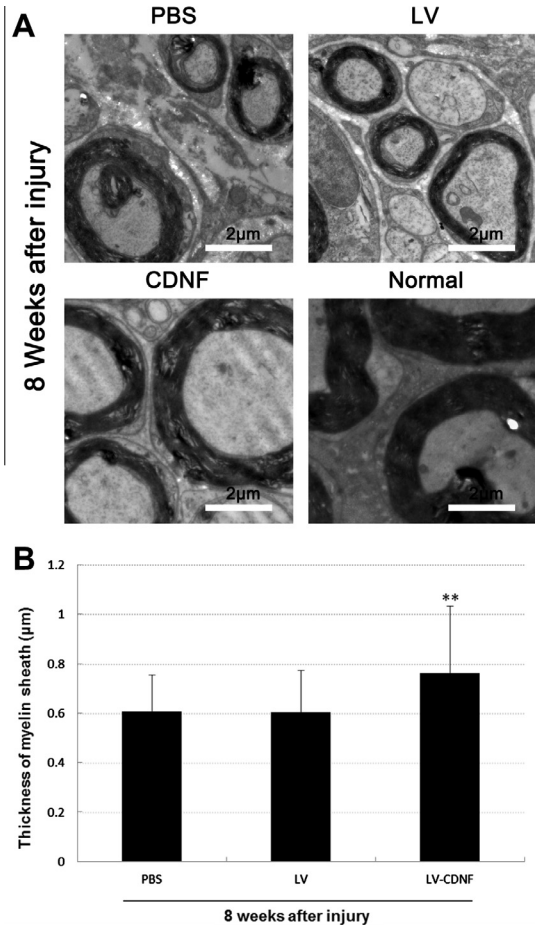

Fig. 3. Remyelination of sciatic nerves. (A) Transmission electron micrographs (TEMs). At 8 weeks after injury, ultra-thin cross-sections of regenerating sciatic nerves from the CDNF, LV and PBS groups were compared with that of the normal nerve group using transmission electron microscopy. (B) The statistical analysis of thickness of the myelin sheath around the axons. The thickness in the PBS group was 0.60 ± 0.17 μm, and in the LV group, it was 0.60 ± 0.14 μm, whereas the thickness in the CDNF group was significantly greater than that of the two control groups (0.75 ± 0.27 μm). $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$.

of the regenerating sciatic nerve 8 weeks after injury also indicated an important difference between the groups. The thickness in the PBS group was 0.60 ± 0.17 μm, and in the LV group it was 0.60 ± 0.14 μm, whereas the thickness in the CDNF group was significantly greater than that two former groups (0.75 ± 0.27 μm) ($n = 5$, $p < 0.01$) (Fig. 3B).

3.4. Changes in gastrocnemius muscle weight and walking track analysis

As a simple and direct measure of CDNF effect on the reinnervation of target muscles, we weighed the gastrocnemius muscles at 4 weeks and 8 weeks after injury in all groups (Fig. 4A). In the PBS and LV groups, the weights of gastrocnemius muscles did not differ significantly, whereas the CDNF group muscles (1.00 ± 0.10 g) were, on average, 1.18-times heavier than that of the PBS group muscles (0.84 ± 0.15; $p < 0.01$). We estimated the functional nerve recovery of the rats using walking track evaluation. As shown in Fig. 4B, several weeks after injury, the positive functional outcomes of axon regeneration from walking track analyses were observed. Although in the first few weeks, the sciatic nerve index value of all the groups was low, the SFI value was significantly increased in the CDNF group compared with the controls

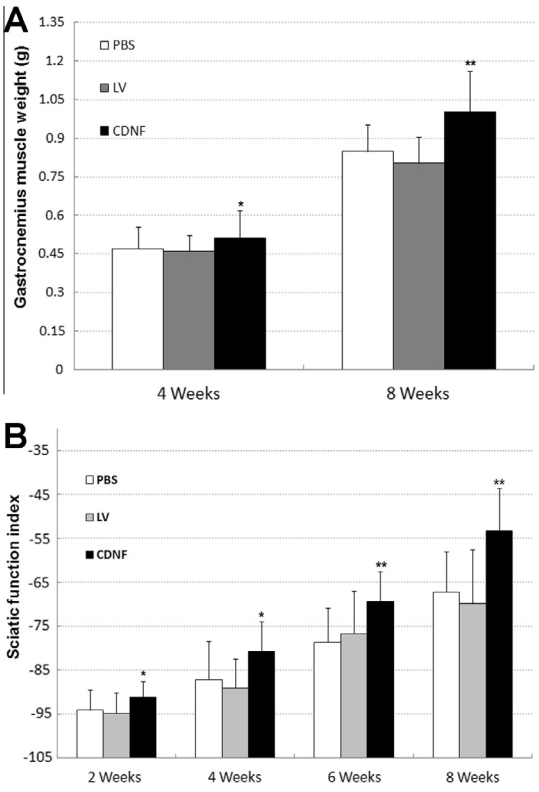

Fig. 4. Changes in gastrocnemius muscle weight and functional recovery of the sciatic nerve. (A) Myological assessment results suggested animals in the CDNF group displayed significantly higher muscle weight than animals in the two other groups at 8 weeks post-surgery. (B) Although at 2 weeks and 4 weeks after surgery, the sciatic nerve index values of all the groups were low, the SFI value was significantly increased in the CDNF group compared with the controls at 6 weeks and 8 weeks after surgery. $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$.

at 6 weeks and 8 weeks after surgery ($n = 5$, $p < 0.01$), but there were no significant differences between the PBS and LV groups ($n = 5$, $p > 0.05$).

4. Discussion

The regeneration potential of the peripheral nervous system has become the focus of interest in recent years. Although the PNS has notable regenerative potential, there is a lack of effective methods to facilitate regeneration. Many studies indicate that the favourable environment of the PNS plays an important role in its regenerative potential. To create a better environment to enhance the therapeutic effects of nerve growth factors, we combined nerve conduits with gene therapy in this study. Nerve conduits have yielded promising results in bridging short nerve gaps [17,18]. Here, a silicone tube was used to connect the distal and proximal nerve. In addition, we injected recombinant CDNF lentiviral vector into transected sciatic nerves to perform gene therapy in this model. Without damaging the sound nerves, the silicone tube offers a very promising alternative pathway for nerve repair. Silicone tube can effectively prevent the invasion of fibrous tissue and allow transected stump sprouting in the lumen, providing a relaxed nerve regeneration chamber.

Neurons upregulate endogenous NTFs within the first few weeks following a peripheral nerve injury. The upregulation is normally slow, implying that the initial basal level could be a limiting factor. However, early tests demonstrated the application of a variety of exogenous neurotrophic factors in nerve regeneration model, but the treatment effects were poor and variable [19,20].

Sustained and effective application of neurotrophic factors is difficult because these proteins are not easily spread to the nervous tissue and have a short half-life. In this study, we observed that endogenous CDNF in the transected sciatic nerve increases at the 4-week point, indicating that the initial basal level of CDNF could be a limiting factor, and recombinant CDNF overexpression could have a beneficial effect on regeneration of injured peripheral axons. Here, we used a lentiviral-based system to overexpress CDNF, which provided a durable and stable protein concentration to produce the optimal therapeutic effect.

Lindholm and colleagues identified "conserved dopamine neurotrophic factor" (CDNF) as a homologue of the recently identified mesencephalic-astrocyte-derived neurotrophic factor (MANF) [10,21]. These two proteins form a distinct family of secreted factors that contain eight conserved cysteine residues that form 3–4 intramolecular disulphide bonds that may constitute a unique protein fold. Mammals have CDNF and MANF, whereas invertebrate animals including *Caenorhabditis elegans* and *Drosophila melanogaster* also have a homologous gene for MANF/CDNF [22]. CDNF is expressed throughout the nervous system including the cortex, hippocampus, midbrain, cerebellum, striatum and substantia nigra, and, in adulthood, it is also expressed in other tissues, such as the heart, skeletal muscle and testes [10].

Both MANF and CDNF have been demonstrated to protect and repair midbrain dopaminergic neurons in vivo [10,23]. Therefore, MANF and CDNF may be potential therapeutic agents for the treatment of Parkinson's disease (PD). Similarly, chronic infusion of CDNF reduced amphetamine-induced turning behaviour and prevented the loss of TH-immunoreactive cells in a 6-OHDA lesioned nigro-striatal system [24]. In a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model, striatal CDNF administration is both neuroprotective and neurorestorative for the TH-positive cells in the nigrostriatal dopamine system [25]. MANF mRNA is increased after brain ischemia and epileptic insults in the hippocampus and in the cerebral cortex [26]. Similarly, recent studies indicate that MANF has neuroprotective effects, can reduce ischemic brain injury and promote behavioural recovery in rats [27]. MANF also efficiently rescued cortical neurons in a rat stroke model and protected microglial cells against inflammatory injury [28,29]. Our previous studies revealed that overexpression of CDNF in astrocytes provides cells the potential to resist cell injury and proinflammatory cytokine secretion [12]. Though their neuroprotective mechanisms and receptors are still unknown, all of these results indicate that CDNF and MANF play crucial roles in neuronal protection and restoration in the central nervous system.

In this study, we tested the hypothesis that CDNF gene therapy enhances axonal regeneration after transection and tubulisation repair of the rat sciatic nerve. Our results demonstrated that this therapy was able to improve nerve regeneration across a 3-mm gap inside a silicone tube. In agreement with our previous studies, recombinant CDNF lentiviral vector injection resulted in dramatically increased levels of CDNF around the transected/injury site. As shown by HE staining, the CDNF-treated nerves display better tissue organisation. Our study indicates a significant general increase in regeneration, as proved by inspection of axonal marker NF200 and Schwann cell marker S-100. Quantitative analysis of the sciatic nerve remyelination data also indicates that the thickness in the CDNF group was significantly greater than in the two control groups. All morphological analysis indicated that the addition of CDNF could improve nerve regeneration.

Functional recovery after a transection injury is very limited and usually accomplished by the development of muscle contractures and atrophy. Thus, we evaluated the target muscle and tested the SFI value. In this study, the CDNF group displayed higher muscle weights, and these results are in agreement with the better histomorphological analysis results observed. In addition to these findings, the SFI value also indicated a greater improvement in motor recovery in the CDNF group. These results can be explained by the increased number of regenerating axons.

In summary, this study has demonstrated that CDNF promotes sciatic nerve regeneration in a rat transection model within a silicone nerve conduit, as implemented using direct injection lentiviral vector therapy. The recombinant CDNF lentiviral vector provided a durable and stable protein concentration to produce the optimal therapeutic effect. We conclude that CDNF gene therapy could promote axonal regeneration and functional recovery. These findings suggest that CDNF gene therapy could be a promising tool for application in human peripheral nerve injury. The receptors and mechanism behind these functions are still unknown, and subsequent studies will explore the related molecular interactions and signalling pathways.

Acknowledgments

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