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Differentiated Mesenchymal Stem Cells for Sciatic Nerve Injury

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Abstract Sciatic nerve injury is common and may cause neurological deficits. Previous studies showed that administration of neurotrophic factors (NTFs), naturally occurring proteins that support the development and survival of neurons, preserved and protected damaged motor neuron in the injured sciatic nerve. We have been successful in converting bone marrow-derived mesenchymal stem cells into astrocyte-like cells that produce and secrete NTFs (NTF^+ cells). These cells demonstrate typical astrocyte morphology, express characteristic astrocyte markers and secrete high levels of NTFs. We have already shown that these cells and their conditioned media can protect neurons in culture and in animal models of neurodegenerative diseases. In the current study we examined whether NTF^+ cells are capable of rescuing motor neurons in a rat sciatic nerve injury model, where the right hind limb sciatic nerve was crushed. Rats were transplanted with NTF^+ cells, MSCs or PBS into the lesion site. In rats injected with the NTF^+ cells motor function was markedly preserved. Moreover, NTF^+ cells significantly inhibited the degeneration of the neuromuscular junctions and preserved the myelinated motor axons. Our findings suggest that autologous therapeutic approach can alleviate signs of sciatic nerve injury and probably other neurological disorders.

Keywords Sciatic nerve injury · Motor neuron · Mesenchymal stem cells · Neurotrophic factors

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Introduction

One of the most common peripheral neuropathy is sciatica, damage to the sciatic nerve, with reported prevalence of 43%. Sciatica is characterized by muscle weakness, reflex changes and numbness. Sciatica can be caused by tumors, cysts or other extraspinal insults. The majority of sciatica patients suffer from persistent and severe type of pain, motor dysfunctions and prolonged disability [1].

Stem cell-based therapy for peripheral nerve injury has been suggested to exert favorable effects on nerve regeneration including axonal regrowth and myelin formation. These effects have shown to improve motor function, as a result of motor neuron regeneration, as well as sensory recovery [2–5]. The possible effect of cell implantation might be the induction of an improved environment for the damaged tissues afforded by the stem cells and the protection of the peripheral neurons. A reason for this protective effect might be that the cells secrete neurotrophic factors (NTFs) which are naturally occurring polypeptides that support the development and survival of neurons.

Several studies concerning peripheral nerve injury have demonstrated NTFs play an important role in the development, maintenance and regeneration of the nervous system. The brain derived neurotrophic factor (BDNF) was shown

to prevent the loss of motor units and to contribute to the maintenance of muscle mass when administered to the hind limb muscles of mice after peripheral nerve injury [6–9]. The glial derived neurotrophic factor (GDNF) and the insulin growth factor 1 (IGF-1) are two of the most potent survival factors known for peripheral neurons. Several studies have shown that GDNF and IGF-1 can prevent neuronal degeneration in mice and rats after axotomy, as well as the programmed cell death of motor neurons during development [6, 9–12]. In the $SOD1^{G93A}$ transgenic mice model for amyotrophic lateral sclerosis (ALS), overexpression of GDNF and/or IGF-1 in muscles, resulted in hyperinnervation of the muscles by motor neurons [6, 9–16]. Peripheral neurons bind, internalize, and retrogradely transport GDNF and IGF-1 from muscles in a receptor-dependent manner [6, 9–12]. Moreover, GDNF is important for neuron branching at the neuromuscular junction (NMJ) and for modulating synaptic plasticity [17]. Increased expression of GDNF in the muscles of $SOD1^{G93A}$ transgenic mice delays disease onset, improves locomotor performance, and increases their lifespan. In addition, the survival of motor neurons is increased when GDNF levels in the muscles of $SOD1^{G93A}$ transgenic mice are high. The vascular endothelial growth factor (VEGF) is another factor contributing to the pathogenesis of ALS and the increased expression of VEGF in motor neuron of $SOD1^{G93A}$ transgenic mice, augmented their survival and enhanced motor performance [18, 19]. Moreover, intracerebroventricular administration of VEGF in a rat model of ALS, dramatically increased motor neuron survival and an intraperitoneal injection of VEGF led to the preservation of NMJs [20, 21].

Mesenchymal stem cells (MSCs) are a distinct population of cells in the bone marrow that can be easily obtained and used for autotransplantation. These cells have the potential to differentiate into bone, cartilage, and fat tissues [9]. They also express neural markers [22] and have been shown to be beneficial in various animal models of neurodegenerative disorders, such as Parkinson's disease [23], multiple sclerosis [24], and stroke [25].

In our previous studies we developed a two-step medium based differentiating protocol for inducing MSCs into neurotrophic factors secreting cells (NTF^+). The induced cells demonstrated astrocyte-like morphology and expressed characteristic astrocyte markers such as glial fibrillary acidic protein (GFAP) and glutamine synthetase. These cells produced and released high amounts of NTFs, such as BDNF and GDNF. In vitro studies have demonstrated that the cell-conditioned medium protected neurons against various neurotoxins. Moreover, in animal models of Parkinson's disease, multiple sclerosis and Huntington's disease, the transplanted cells showed marked improvements [26, 27].

Sciatic nerve crush is the most commonly used model for nerve injury to test numerous neurodegenerative therapeutic modalities [1–5].

In the current study, employing the sciatic nerve crush model, we demonstrated that intramuscular injection of the NTF^+ cells markedly inhibited the degeneration of the NMJs and preserved motor function.

Materials and Methods

The Sciatic Nerve Crush Model in Rats

The sciatic nerve crush model was applied on male Sprague–Dawley rats (n=14; Harlan, Jerusalem) weighing 230–250 g. Rats were placed under 12-hour-light/12-hour-dark conditions and grown in individually ventilated cages (IVC) with ad libitum access to food and water. All experimental protocols were approved by the Tel Aviv University Committee of Animal Use for Research and Education. Every effort was made to reduce the number of animals used and to minimize their suffering. Rats were anesthetized with Chloral hydrate 7 mg/ml (Sigma-Aldrich, St. Louis). The right sciatic nerve was exposed and a vessel clamp was applied 10 mm above the first branching of the nerve, for 30 s.

The Protocol for Neurotrophic Factor-Secreting Cell Induction

MSCs were isolated from the femurs and tibias of adult male wild-type Sprague–Dawley rats by adhesion to non coated tissue culture plastic. MSCs were propagated in vitro for 2 passages and characterized, as we have previously described [28]. MSCs were replaced with the step 1 medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, 12.5 units/ml nystatin (SPN, Biological Industries, Beit Haemek, Israel), 2 mML- glutamine (Biological Industries, Beit Haemek, Israel), 20 ng/ml human epidermal growth factor (R&D Systems Inc., Minneapolis), 20 ng/ml human basic fibroblast growth factor (hbFGF) (R&D Systems), and 10 μl/ml N2 supplement (5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium, 100 μg/ml transferrin (Invitrogen, Carlsbad, CA). After 72 h in the step 1 medium, cells were placed in the step 2 medium, which consisted of DMEM supplemented with SPN, 2 mM L-lutamine, 1 mM Dibutyryl cyclic AMP (Sigma- Aldrich, 0.5 mM 3-isobutyl-1-methylxanthine, 20 ng/ml hbFGF, 50 ng/ml human neuregulin1- β1 (R&D Systems), and 5 ng/ml platelet-derived growth factor-AA (PDGF; Peprotech, Rocky Hill, NJ). As a control we used untreated MSCs that were grown

for 3 days in a serum-free medium containing DMEM, glutamine, and SPN [27].

Cell Transplantation

In order to enable us to trace the surviving cells after transplantation, cells were labeled with superparamagnetic iron oxide (SPIO) (5 g/ml; Feridex; Bayer HealthCare, Leverkusen, Germany) as previously described [27]. Cells were incubated with poly-L-lysine (1 g/ml medium; 70–150 kDa; Sigma-Aldrich) for 1 h before adding SPIOs to the medium on the last day of the first differentiation step (day 3). Cultures were washed with the step 2 medium after 24 h. On the end of the differentiation induction (day 6), the treated cells were trypsinized, counted, and resuspended in PBS. One day after surgery, the control group was injected with 100 μl PBS into the lesion site (n=14). The second group was injected with 10^6 MSCs suspended in 100 μL PBS into the lesion site (n=14) and the third group was injected with 10^6 NTF^+ cells suspended in 100 μl PBS. The injection was through the muscles into the nerve surrounding above the first branching of the nerve.

Motor Function Measurements by Rotarod

Rats were examined for motor functioning twice a week, 1 week before injury and 3 weeks after. Motor activity was measured by the rotarod (San Diego instruments, USA) test. In this test, following a brief training period, adult wild-type rats are able to remain balanced on a rotating rod in accelerated speed, from 0 to 25 RPM for up to 4 min. After a sciatic nerve crush, the rat's ability of balancing is damaged and the animal falls off the rod after shorter periods of time. The machine has a laser beam that detects the fall [10, 11]. The average of three consecutive runs from each session on the rotarod were assessed and the groups' performance was compared.

Lateral Reflex Measurements

The lateral reflex of the rats was tested blind, 11 days after the crush. A withdrawal reflex to pinch of the fifth toe was measured using serrated forceps. Force and duration of this pinch was held as constant as possible The reflex was estimated compared to the uninjured hind limb and scored as 0 for no reflex, 1 for partial reflex, and 2 for full reflex. Since the test suffers from high variability we took many repeated measurements and the data represented the average [29].

Electrophysiological Study

Compound muscle action potential (CMAP) amplitudes were recorded from the sciatic innervated cranial tibial muscles following electric stimulation of the sciatic nerve. An active monopolar needle electrode was placed over the sciatic nerve at the sciatic notch and a supramaximal intensity electric stimulus of 0.1 ms duration was applied. Conduction latency and CMAP were recorded in both the injured and intact hind limbs. An average of ten consecutive runs from each measurement was documented. Both the CMAP amplitude and the conduction latency were converted to the ratios of the measurements taken at the injured side, divided by those of the normal side to adjust for the effect of the anesthesia, muscle masses and other physical variations between rats [30, 31].

Immunohistochemistry

To analyze cell survival, hind limb muscles of rats were removed and frozen in liquid nitrogen, 15 days after transplantation. Muscles were sectioned at 20 μm using a cryostat (Leica CM1850) and placed on glass slides for staining. The sections were fixed with 4% PFA-PBS and dyed with Prussian blue staining to detect the SPIO-labeled transplanted cells. Rabbit α- BDNF (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used to visualize the BDNF expressing cells. After washing with PBS, sections were incubated with a biotinlated antibody (Goat anti- rabbit, Invitroigen, Carlsbad, CA, USA, ready to use) for 1 h followed by DAB staining using a Vector ABC kit (Vector, Peterborough, UK).

Assessment of Neuromuscular Junction Innervations

Endplate innervation was marked by alpha-bungarotoxin and synaptophysin as described previously [32]. Hind limb muscles were dissected and frozen in liquid nitrogen. Muscles were sectioned at 20 μm using a cryostat and placed on glass slides for staining. The sections were fixed with 4% PFA-PBS and labeled with alpha-bungarotoxin conjugated with fluorescence marker Alexa Fluor 594 (1:1000, Invitrogen, CA, USA) and anti-synaptophysin (rabbit polyclonal, 1:100, Santa Cruz Biotechnology, Santa Cruz, USA) antibodies overnight at 4°C. After washing with PBS, the sections were incubated with anti- rabbit Alexa Fluor 488-conjugated antibody (1:1,000, Invitrogen) for one hour at room temperature followed by washes, and covered with cover glasses using aqueous mounting medium (Invitrogen). We classified NMJs into two groups based on the degree of innervation of postsynaptic receptor plaques by nerve terminals [32]. Endplates were scored as "innervated" if there was overlap with the axon terminal, or "denervated" if the end-plate was not associated with an axon [32].

Statistical Analysis

The results are expressed as means ± SE. The *one way ANOVA* test was used to compare the three groups.

Statistical calculations were performed using SPSS, version 13 (SPSS, Chicago, USA).

Results

Improved Motor Function after Cell Transplantation

All rats suffered from a right hind limb limp after crush and their motor function deteriorated. All the rat groups performed equally well on the rotarod before the injury. However, immediately after the crush, the performance of rats markedly declined by 60%. Five days later, PBS treated rats demonstrated poor motor function (41% ± 4.6 on rotarod) while the MSCs treated rats showed a moderate improvement (57.8% ± 4.1, $p<0.05$) and NTF$^+$ cells treated rats demonstrated significant better motor performance (73.6% ± 5.3, $p<0.01$). The same trend was observed 11 days after the crush (67.5% ± 5.2, 73.9% ± 6.5 and 87.8%±3.4 in PBS, MSCs and NTF$^+$ respectively) (Fig. 1).

Lateral Reflex Recovery Following NTF$^+$ Transplantation

The lateral reflex of the hind limb is controlled by the sciatic nerve. After sciatic nerve crush, no reflex was detected in response to lateral tow pulling. PBS treated rats demonstrated poor reflex response (0), whereas MSCs treated rats showed modest response (0.67±0.67) and NTF$^+$ treated rats showed a full reflex response (2, $p<0.01$) (Fig. 2).

Electrophysiology Study Indicates Axonal Regeneration in NTF$^+$-Treated Rats

After the sciatic crush, nerve degeneration was accompanied by a decreased compound muscle action potential (CMAP) and an increase in nerve conduction latency as indicated by electromyography (EMG). These measures were calculated as the ratio between the injured and uninjured hind limbs. Eleven days after injury, impaired CMAP and conduction latency were restored in the NTF$^+$ treated group. The average ratio of CMAP in the PBS treated group was 0.037±0.016 while in the MSCs treated rats the average ratio was 0.17±0.007. The best ratio, however, was of the NTF$^+$ treated rats (0.73±0.15 $p<0.01$ (Fig. 3a). The same pattern of improvement in axonal regeration was also observed in the average ratio of conduction latency. While the latency ratio of PBS and MSCs treated rats was 2.44±0.26 and 2.22±0.18 respectively, the NTF$^+$ treated rats demonstrated normal latency, the ratio being 0.96±0.021, $p<0.01$ (Fig. 3b).

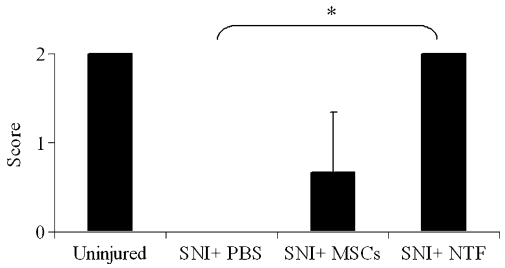

Fig. 2 Lateral reflex recovery after NTF$^+$ cells transplantation. One day after sciatic nerve crush, NTF$^+$ cells, MSCs or PBS were inoculated into the injury site. Lateral reflex was tested 12 days later and scored as 0 for no reflex, 1 for partial reflex and 2 for full reflex ($n=14$, means ± SEM, *$p<0.05$, as determined by ANOVA test)

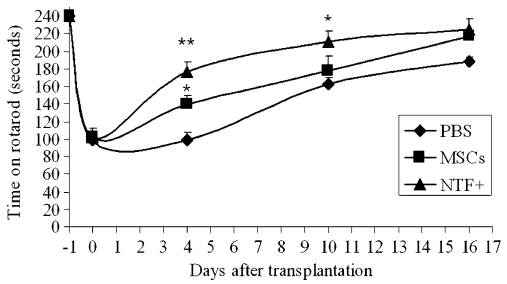

Fig. 1 NTF$^+$ cells inoculation after sciatic nerve injury (SNI) in rats, rescue the motor functioning. Day after mechanical crush of the right hind limb, rat's NTF$^+$ cells, MSC or PBS were inoculated into the injury site. Motor recovery was examined by rotarod test and presented by time spent on rod in seconds 1, 4, 10, and 16 days after transplantation ($n=14$, means + SEM, *$p<0.05$, **$p<0.01$, as determined by ANOVA test)

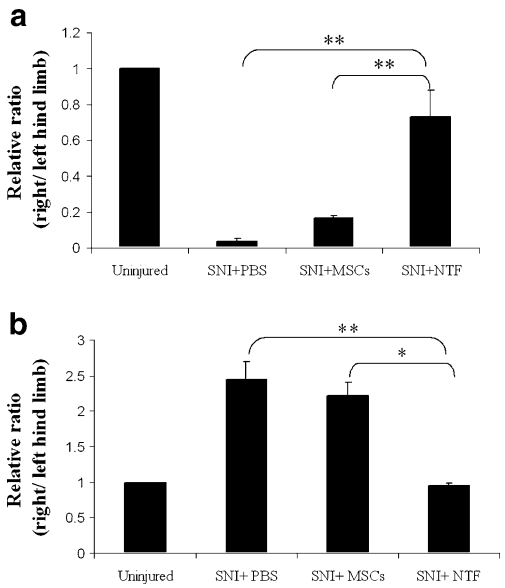

Fig. 3 Restoration of nerve conduction after NTF$^+$ cells inoculation. Twelve days after NTF$^+$ cells, MSCs or PBS transplantation into sciatic nerve crush site, nerve conduction was tested by electromyography. **a** Compound muscle action potential and **b** conduction latency, presented as a ratio between the injured and uninjured hind limbs ($n=14$, means ± SEM, *$p<0.05$, **$p<0.01$ as determined by ANOVA test)

NTF$^+$ Inhibited NMJs Denervation

Three weeks following sciatic nerve crush, NMJs were analyzed within the crush area and the gastocnemios and tibialis muscles. Double stained endplates, with acetylcholine receptor ligand, alpha bungarotoxin and antibodies against the post synaptic protein, synptophysin, were counted as innervated NMJ (Fig. 4a). After examining over 100 stained slides, we observed 72% preservation of innervated NMJs in the injured gastocnemios and tibialis muscles, as compared to the uninjured hind limb. In contrast, the MSCs transplanted rats demonstrated a moderate reduction to 90% ($p<0.05$) in innervated NMJ while the NTF$^+$ transplanted rats showed almost full preservation (99%) of the innervated NMJ compared to the uninjured limbs (Fig. 4b).

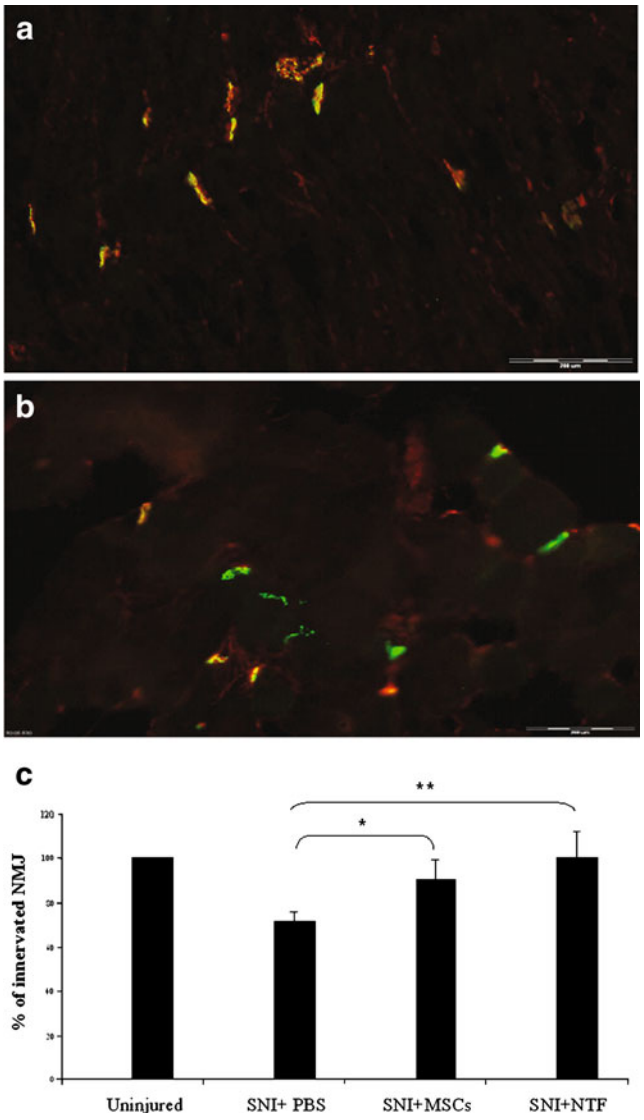

Fig. 4 NTF$^+$ cells transplantation preserve innervated neuromuscular junctions. Three weeks after sciatic nerve crush and transplantation of NTF$^+$ cells, MSC or PBS, rats were sacrificed. Hind limbs muscles were double stained using alpha bungarotoxin (*green*) and anti-synaptophysin antibodies (*red*). **a** Presentative image of hind limb muscle section transplanted with NTF$^+$ cells. **b** Presentative image of hind limb muscle section transplanted with PBS. **c** Quantification of integrated NMJs ($n=5$, means + SEM, *$p<0.05$, **$p<0.01$ as determined by ANOVA test)

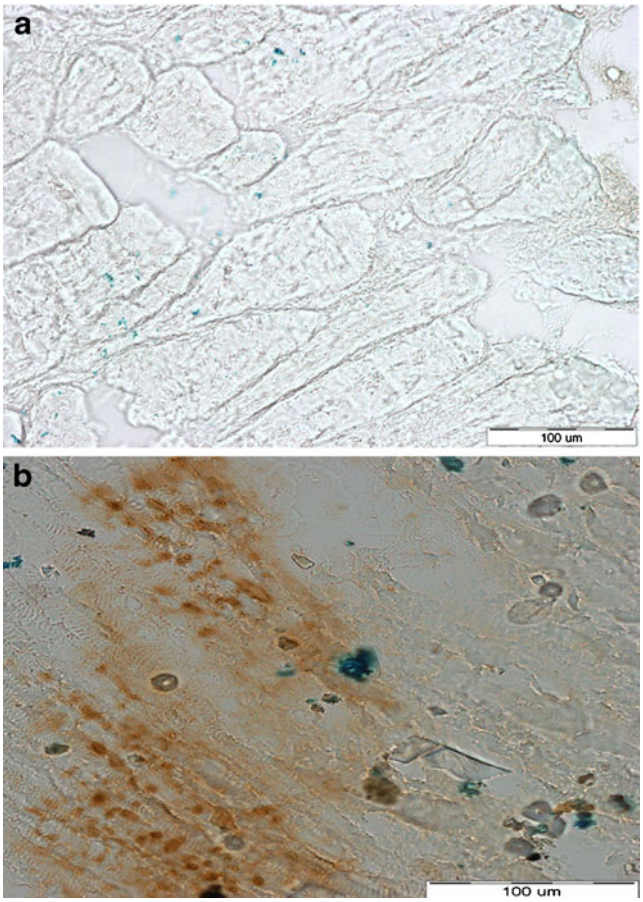

Fig. 5 NTF$^+$ cells survived and secreted BDNF in the transplanted muscles. Three weeks after sciatic nerve crush and transplantation of NTF$^+$ cells, MSC or PBS, rats were sacrificed. **a** Iron positive MSCs in the transplanted hind limb muscles (*blue*). **b** Iron positive NTF$^+$ in the transplanted hind limb muscles and BDNF positive staining (*brown*)

NTF$^+$ Cells Survive Function in Transplanted Tissue Three Weeks After Transplantation

Histology of the rats' hind limb muscles 3 weeks after transplantation using Prussian blue staining reveled significant amount of SPIO-labeled cells in transplantation site. Moreover, immunostaining with specific antibodies reveled high levels of BDNF in NTF$^+$ cells and their surrounding muscles tissue. BDNF couldn't be detected in and surround the MSCs. (Fig. 5). There was no sign of tumor formation in the transplanted area.

Discussion

In the current study, we found that intramuscular injection of NTF^+ cells markedly inhibited the degeneration of the NMJs and preserved the motor function in a unilateral sciatic nerve injury model, induced by crushing rats' right sciatic nerve. The transplanted cells survived, expressed and secreted BDNF in the injured tissue for at least 3 weeks after transplantation.

Many studies have tried treating the various models of motor neuron disorders with the administration of neurotrophic factors and investigated the regenerated axons and functional recovery [6–9, 13–21]. Although there are some indications of restoration and recovery of the motor function, clinical trials of systemic or intrathecal administration of recombinant NTFs to patients with motor neuron disorders did not show significant efficacy [10, 11].

It was speculated that the NTF^+ ineffectiveness was due to their short half-life, poor delivery and low concentrations at target sites [10, 11, 33]. The use of cellular transplants to deliver NTFs, either through the normal release from the transplanted cells or after in vitro manipulations of cells for the overexpression of certain NTFs, could provide improved sustained delivery.

Various studies have demonstrated the beneficial effect of transplanting NTFs into motor neurons disorder models. Cheng et al. (2009) reported that GDNF-modified human amniotic fluid-derived MSCs promoted nerve regeneration in a rat sciatic nerve crush model [34]. In another study, human MSCs engineered to secrete GDNF were transplanted into a rat model of ALS. They found that these cells significantly increased the number of neuromuscular connections and motor neuron cell bodies in the spinal cord at mid stages of the disease, delayed disease progression and increased lifespan [35].

Our group has previously shown that after a two-step medium based protocol MSCs can be induced to NTF-secreting cells [36]. We have shown that after intracerebroventricular transplantation of these cells, clinical symptoms are attenuated in a mouse model of multiple sclerosis [37]. The NTF^+ cells also provide protective effects in a rat model of Parkinson's disease. The cells reduced the amphetamine-induced rotations and persevered the dopaminergic terminals in the striatum [38, 39]. Our data indicated that the NTF^+ cells provided protective agents and supplied an environment that nursed the damaged nerve tissue. In the present work, we used several measurements to analyze the regenerated motor axons and the integrity of the hind limb NMJs after sciatic nerve crush. One day after injury, we observed a marked decrease in motor function as indicated by the rotarod test. NTF^+ cells were transplanted into the lesion site 24 h after injury. Four days later, we observed a dramatic beneficial effect on the motor function in the NTF^+ treated rats.

The high CMAP and low latency indices recorded in the hind limb muscles of NTF^+ cells-treated rats, as compared to MSCs and PBS-treated rats, provided evidence that NTF^+ cells preserved the myelinated motor axons and innervated peripheral muscles.

From prior experiments, we learned that control rats that went through an opening of the muscle tissue and exposure of the sciatic nerve without crushing it, did not show any deficits in their motor behavior. These rats acted exactly like uninjured rats, as indicated by rotarod test. From these experiments we conclude that during the sciatic nerve crush procedure, the muscles remain undamaged and that the motor dysfunction is caused directly and only from the nerve injury. From the above we can conclude that the cells improve the nerve injury rather than any muscular problem.

One of the major caveats of stem cell transplantations is the fate of the transplanted cells. Here we show that transplanted MSCs and NTF^+ can integrate and survive at the host muscles of rats after sciatic nerve crush, for at least 3 weeks. In addition, we found that the integrated NTF^+ cells secreted BDNF in the hind limbs muscles, near the injury site. Together, our results clearly indicate that our novel cell-based therapy preserved the number of myelinated axons and muscle innervation and accelerate motor recovery.

As muscle is an accessible tissue, the present study suggests a very straightforward approach to the treatment of peripheral nerve injury. Indeed, we believe that our NTF^+ cells facilitate the cessation of the process of damage, accelerate the regeneration of the crushed nerve, improving reinnervation of denervated peripheral muscles and restoring motor function.

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Conflict of Interest statement The authors declare no potential conflicts of interest.

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