Spatiotemporally limited BDNF and GDNF overexpression rescues motoneurons destined to die and induces elongative axon growth

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Abstract

Axonal injury close to cell bodies of motoneurons induces the death of the vast majority of affected cells. Neurotrophic factors, such as brain derived neurotrophic factor (BDNF) and glial cell derived neurotrophic factor (GDNF), delivered close to the damaged motor pool in a non-regulated manner induce good survival of injured motoneurons and sprouting of their axons but fail to induce functional reinnervation.

To avoid these drawbacks of high levels of neurotrophic expression, we devised an *ex vivo* gene therapy system to induce transient expression of BDNF/GDNF in transfected rat adipose tissue-derived stem cells (rASCs) which were grafted around the reimplanted ventral root, embedded in collagen gel.

Strong BDNF/GDNF expression was induced *in vitro* in the first days after transfection with a significant decline in expression 10-14 days following transfection. Numerous axons of injured motoneurons were able to enter the reimplanted root following reimplantation and BDNF or GDNF treatment (192 ± 17 SEM vs 187 ± 12 SEM, respectively) and produce morphological and functional reinnervation. Treatment with a combined cell population (BDNF+GDNF-transfected rASCs) induced slightly improved reinnervation (247 ± 24 SEM). In contrast, only few motoneurons regenerated their axons in control animals (63 ± 4 SEM) which received untransfected cells. The axons of surviving motoneurons showed elongative growth typical of regenerative axons, without aberrant growth or coil formation of sprouting axons.

These findings provide evidence that damaged motoneurons require limited and spatially directed amounts of BDNF and GDNF to support their survival and regeneration. Moreover, neurotrophic support appears to be needed only for a critical period of time not longer than for two weeks after injury.

Introduction

Plexus injuries in humans usually involve the avulsion of one or more ventral roots (Millesi 1992, Bertelli and Ghizoni 2003, Gilbert et al. 2006). Experimental ventral root avulsion in animals mimics the features of proximal plexus lesions in humans, resulting in degeneration and later loss of the affected motoneurons in the ventral horn and atrophy of the target muscles (Koliatsos et al., 1994). Reimplantation of the avulsed ventral root in the spinal cord increases the survival rate of the affected motoneurons, but promotes only limited reinnervation by the motor axons (Carlstedt et al. 1986, 1993, Nógrádi and Vrbová 1996, 2001, Eggers et al. 2010).

Several attempts have been made to improve the extent of reinnervation by surviving motoneurons after ventral root avulsion and reimplantation. The use of riluzole (a compound that blocks voltage-gated sodium and calcium channels and inhibits presynaptic glutamate release) after experimental root avulsion and reimplantion of the L4 ventral root, dramatically improved survival of injured motoneurons, accompanied by an extensive axonal regeneration resulting in solid reinnervation (up to 65%) of the target denervated muscles (Nógrádi and Vrbová 2001, Nógrádi et al. 2007). Local application of recombinant GDNF, a member of the transforming growth factor beta family induced rescue of the injured motoneurons and satisfactory reinnervation of the target muscle when applied in co-treatment with riluzole (Bergerot et al. 2004). Other strategies involved the use of progenitor and stem cells in the vicinity of the reimplanted ventral root (Hell et al. 2009, Su et al. 2009, Bonner et al. 2010). Although satisfying survival of the injured motoneurons could be achieved, all these strategies failed to induce functional regeneration of the target muscles.

Neurotrophic factors mediate survival, differentiation, neurite outgrowth and functional plasticity in the central and peripheral nervous system. *In vitro* and *in vivo* experiments confirm the promoting effects of neurotrophic factors (BDNF, NT3, NT4/5) on motoneuron

survival (Henderson et al. 1993, Li et al 1994). The presence of messenger RNA coding for neurotrophic factors at appropriate stages in spinal cord and limb bud development and mRNA for their receptors on the motoneurons emphasize the high impact of neurotrophins in a certain time windows of embryonic development of the spinal motoneurons (Henderson et al. 1993).

GDNF and and other neurotrophic factors (BDNF, NT-3,NT-4/5) support motoneuron survival under several experimental conditions (Oppenheim et al. 1992, Henderson et al. 1994, Oppenheim et al. 1995, Wu et al. 1995, Vejsada et al. 1995, 1998; Kishino et al. 1997, Novikova et al. 1995, 1997, Wiese et al. 1999, Boyd, JG. T. Gordon T. 2001). On the other hand, their use failed in the cases of promoting motoneuron axonal regeneration after avulsion injuries. Blits et al. (2004) injected GDNF-inducing Adeno-associated Virus (AAV) into the injured spinal cord segment. Although this treatment resulted in good motoneuron survival, no functional reinnervation was achieved. Similarly, the same group (Eggers et al. 2008) tried to induce reinnervation of reimplanted ventral root and peripheral targets with direct injection of lentivirus inducing the expression of GDNF in the reimplanted ventral root, without success to induce elongative growth of the regenerating motor axons. The side effect of this uncontrolled and persistent virus-based production of neurotrophic factors at the site of injury was formation of irregular coils of sprouting axons, likely due to the so called "candy store" trapping effect of neurotrophic factors on the injured axons. Recent efforts to establish a gradient of neurotrophins along the injured sciatic nerve to force axons to grow along the whole length of the nerve have proven unsuccessful. The likely reason for this could be that even the lowest dose of viral constructs applied prohibited axonal growth and myelination of the coil-forming axons (Eggers et al. 2013).

Based on the above data it can be hypothesized that by reducing the amount, duration and location of neurotrophic factor overexpression after ventral root avulsion, the axons of injured

motoneurons will be attracted to the site of neurotrophic factor application but will not be trapped there. The aim of the present study is to establish conditions by applying non-viral gene therapy, enabling regenerating motor axons of surviving motoneurons to follow an elongative growth pattern and reinnervate the denervated hind limb muscles.

Material and Methods

Isolation and culturing of rat adipose derived stem cells

Epididymal fat pads were isolated aseptically from male Sprague-Dawley rats under terminal anaesthesia and transferred into 60 ml sterile PBS for further dissection. All subsequent steps were carried out in a vertical laminar flow box to avoid infection.

The fat pads were cut into small pieces and blood vessels were removed. Tissue pieces were washed twice with PBS followed by digestion in 10 ml of 1.5 mg/ml collagenase 1 solution (Worthington Biochemical Co., Lakewood, NJ, USA) for 30 min at 37°C and 180 U/min shaking. The digested tissue was filtered through a 100 µm cell strainer and transferred into 50 ml Falcon tubes. Following centrifugation for 7 min at 400g at room temperature, the supernatant was discarded and the pellets were washed by re-suspension and centrifugation (400g, RT) in 1x PBS. The rASCs were re-suspended in 5ml rASC-medium (DMEM high glucose, 1% Penicillin/Streptomycin, 1% glutamine, 10% fetal calf serum [FCS]) and brought into culture in 75cm^2 flasks. Aliquots of 1x10^6 cells/ml in DMEM (5% FCS) with 10% dimethylsulfoxide (DMSO) were made and stored in liquid nitrogen.

Frozen aliquots were thawed on 37°C before re-culturing and centrifuged for 5 min at 400g. Cells were resuspended in fresh medium and sewn in 175ml flasks. After 24h the medium was changed for further cultivation.

Cell staining with Cellbrite

In order to track rASCs after transplantation, the cells were labelled using the Cellbrite™ Red Cytoplasmic Membrane Staining Kit (Biotium CA, USA). The overall labelling efficacy was approximately 95%. Cells were trypsinized and suspended at a density of $1x10^6$/ml in serum-free DMEM. After adding 5µl of cell-labeling solution per 1ml of cell suspension, the cells were incubated at 37° for 20 minutes. After a centrifugation step for 5 min at 1500 rpm at 37°, the supernatant was removed and cells were gently resuspended in warm (37°) medium. Following two more washing steps (centrifugation and resuspension), cells were resuspended in serum free DMEM at a concentration of $1x10^6$/ml for further *in vivo* use.

Design of pVAX1-rBDNF and pVAX1-mGDNF vectors

cDNA sequences for rat BDNF and mouse GDNF were purchased from Open Biosystems/Thermo Scientific (Pittsburgh, PA, USA). Both genes were amplified by using Taq-polymerase. Primers were designed to introduce a Kozak translation initiation sequence as well as specific restriction sites for further cloning into pVAX1 (Life Technologies, Paisley, UK). pVAX1 is a DNA vaccination plasmid vector designed for high-level transient expression. The resulting pVAX1-rBDNF and pVAX1-mGDNF plasmids (Fig. 1A-B) were verified by sequencing and restriction digests.

Lipofectamine-based transfection of rASCs with pVAX1-rBDNF and pVAX1-mGDNF

Lipofectamine 2000 (Life Technologies, Paisley, UK), a liposomal transfection agent was used for transient transfection in this study. Rat ASCs were plated at a density of approximately 28,000 cells/cm^2 in 24 well plates and transfected at a density of approx. 80% with pVAX1_mGDNF or pVAX1_rBDNF. Formation of micro particles took place in serum-free DMEM high glucose to exclude interference of FCS with Lipofectamine 2000. Medium was changed 4 hours after transfection to reduce cytotoxic effects of cationic liposomes. The

DNA: Lipofectamine 2000 ratio of 1:1 was chosen in order to achieve maximum transfection rate at low cytotoxicity, based on our previous experience (data not shown).

Evaluation of transgene expression: Enzyme linked immuno-sorbent assay (ELISA)

The expression kinetics of therapeutic transgenes were evaluated by the ELISA technique (Boster Biological Technology Ltd, Fremont, CA, USA). BDNF or GDNF polyclonal antibodies were used to pre-coat 96-well plates and biotinylated monoclonal secondary antibodies were applied. Antibody binding was visualized by using avidin-biotin-peroxidase complexes by subsequent adding 3,3´,5,5´-Tetramethylbenzidine (TMB). Medium was removed 24 hours prior to sampling and 500µl of fresh rASC-medium was added. According to the manufacturer's instructions supernatants were sampled and stored at -20°C for further analysis with ELISA. Assuming a low target protein concentration 1:2 dilutions were prepared and for each ELISA an individual standard curve was prepared. Number of experiments = 4, number of replicates = 6.

Biological activity assay

Rat embryos (12.5 days old) were removed from pregnant females (n=2) under deep terminal ketamine-xylasine anaesthesia and rinsed in fresh Hanks' medium. The thoracic and lumbar dorsal root ganglia (DRG) were carefully dissected and explanted onto Petri dishes filled with medium and incubated with conditioned supernatants of transfected and untransfected rASCs (n=4 for each group), respectively. The supernatants were taken 24 h after transfection of rASCs with pVAX1-rBDNF and pVAX1-mGDNF and stored at -70 °C until further use. The amount of neurotrophic factors in the supernatant was determined by using the ELISA technique and the working concentrations were set to 5 ng/ml. After 24 h incubation with the supernatants the DRGs were carefully washed and photographed under the microscope by

using an Olympus DP70 digital camera (Olympus Ltd, Tokyo, Japan). The extent of longitudinal neurite outgrowth was determined in each experimental group through the use of the Image J (NIH) image analysis program.

Ventral root avulsion-reimplantation and transplantation of rASC cells

All together 48 female Sprague-Dawley rats (Animal Research Laboratories, Himberg, Austria, and Animal Facilities at the Faculty of Medicine, University of Szeged, weighing 180-220 g body weight, average age 6 weeks) were used. Twenty-four animals were used for short-term survival studies (5 and 10 days of survival, 4 animals in each group, except control group), 15 rats survived for 3 months following the original operation (n=5 in each group) and 4 intact animals were used to determine the number of motoneurons in the intact L4 segment. All the operations were carried out under deep ketamine-xylazine anaesthesia (ketamine hydrochloride: 90 mg/kg body weight, Ketavet, Pharmacia & Upjohn Co.; xylazine: 5 mg/kg body weight, Rompun, Bayer Co.) and sterile precautions. To maintain the body temperature at 37.0±0.5°C, the rats were kept on a heating pad during the surgery. Laminectomy was performed at the level of T13–L1 vertebrae, the dura was opened and the left L4 ventral root was pulled out leaving the dorsal roots intact. Then the cut end of the ventral root was inserted into the lateral part of the spinal cord. To avoid damage to the cord, a small hole was created on the lateral surface of the cord, and the avulsed root was inserted into the hole using a watchmaker's forceps (Dumont, Switzerland, No. 5). Special care was taken to avoid damage to the cord, including its motoneuron pool, or to the reimplanted root (Fig. 1C). The reimplanted root was surrounded by stem cells (3×10^5) mixed in 10µl of 2.5mg/ml collagen solution along a 3 mm length of the root (type 1, Sigma Aldrich). Animals in group 1 served as controls: their L4 ventral root was avulsed and reimplanted and they received untransfected rASCs. In group 2 animals the reimplanted root was surrounded by 3×10^5 stem cells

transfected with pVAX1_mGDNF (GDNF-rASC). Group 3 animals received stem cells transfected with pVAX1_rBDNF (BDNF-rASC) while group 4 animals received a mixed stem cell population composed of equal numbers of BDNF- and GDNF-rASCs (GDNF+BDNF-rASC; total number of cells was 3 x 10^5). The spinal cord was covered with the remaining dura, the wound was closed and the animals were allowed to recover (Pajenda et al. 2013). Animals were sacrificed 10 days or 3 months after the primary operation. The experiments were carried out with the approval of the Animal Protocol Review Board of City Government of Vienna and with that of the Committee for Animal Experiments, University of Szeged and rules regarding the care and use of animals for experimental procedures were followed. All the procedures were carried out according to the Helsinki Declaration on Animal Rights. Adequate care was taken to minimize pain and discomfort.

Retrograde labelling

Operated animals that survived for 3 months (n=5 in each group) and intact animals (n=4) were deeply reanaesthetized as described above. On the left side the ventral ramus of the L4 spinal nerve was sectioned and the proximal stump of the nerve covered (Fig. 1D) with few crystals of Fast Blue (FB, Illing Plastics GmbH, Breuberg, Germany). Five days after the application of the fluorescent dye the animals were reanaesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4).

Immunohistochemistry

Twenty-five μm thick transverse spinal cord sections and twenty μm thick longitudinal sections of the operated roots were cut on a cryostat (Leica CM1850, Leica GmbH, Germany) and mounted onto gelatin-coated glass slides. Nonspecific binding sites were subsequently

blocked with 1% milk powder solution. Primary antibodies were incubated overnight at 4 °C, washed, and then incubated with fluorescent-conjugated secondary antibodies for 1 h at room temperature. The following primary antibodies were used: chicken polyclonal anti-green fluorescent protein, (GFP, Merck-Millipore, 1:2000) BDNF and GDNF, respectively, (both from Abcam Ltd, Cambridge, UK, 1:100), anti-neurofilament 200kD (NF200, Abcam Ltd, Cambridge, UK, 1:200). Secondary antibodies were used as follows: Alexa Fluor 488 goat anti-chicken, Alexa Fluor 594 donkey anti-rat, Alexa Fluor 546 rabbit anti-mouse, Alexa Fluor 405 goat anti-rabbit (all from Life Technologies Corporation, 1:400). Sections processed for choline-acetyltransferase (ChAT) immunohistochemistry were preincubated in 3% normal goat serum for 1 h, then treated with a polyclonal goat anti-ChAT antibody (Millipore-Chemicon, Hofheim, Germany, 1:100) overnight at 4◦C. The immune reaction was completed by using the avidin-biotin technique (reagents were purchased from Vector Laboratories, Burlingame, CA), and finally were tyramide-amplified with the Alexa Fluor 546 TSA kit (Tyramide Signal Amplification; Life Technologies). Fluorescent signals were detected in an Olympus FX51 epifluorescence microscope equipped with a DP70 digital camera (Olympus Ltd, Tokyo, Japan). Confocal microscopic images were obtained by using an Olympus FluoView® FV10i compact confocal microscope. Digital images were resized and their contrast and brightness were adjusted.

Cell counts

The number of retrogradely labelled and ChAT positive cells was determined (Fig. 1E). To avoid double counting of the same neuron present in two consecutive sections, the retrogradely labelled neurons were mapped with the aid of an Olympus (Olympus Ltd, Tokyo, Japan) drawing tube, and their locations were compared to those of labelled neurones in the previous section (Nógrádi et al. 2007, Pintér et al. 2010). The proportion of FB+/ChAT+ cells

was determined in each experimental group. All sections from the L4 motoneurone pool were used.

Analysis of locomotion pattern – CatWalk gait analysis

To determine and analyze the parameters of the hind limb movement pattern, the 'CatWalk' automated quantitative gait analysis system was used (Noldus Ltd, version 7.1; Wageningen, The Netherlands). Animals were pre-trained once one week before surgery and the cumulative pre-training values representing the parameters of intact animals are included in the diagrams. This computer-assisted method of locomotor analysis made it possible to quantify several gait parameters, including duration and speed of different phases of the step cycle and print areas detected during locomotion length (Hamers et al., 2001, 2006). The following parameters were taken into account during the analysis:

max. contact area (expressed in mm2): The maximal contact area describes the paw area contacted at the moment of maximal paw-floor contact during the stance phase.

the print area (expressed in mm2): total floor area contacted by the paw during the stance phase.

the mean print intensity (expressed in arbitrary units): the mean pressure exerted by one individual paw during the floor contact.

the stance duration (expressed in s): the stance duration is the time of the stance phase.

the step cycle duration (expressed in s): It represents the time between two consecutive paw placements (stance + swing duration).

Statistical analysis

The t-test and the two-way measurement analysis of variance (ANOVA) computed by using Tukey's all pair-wise multiple comparison procedures or Bonferroni's post hoc test, were used to compare the group data. Non-parametric data were analysed through the use of Mann-Whitney U test. The various tests were used according to the nature of the data.

Results

In vitro analysis of gene expression

The rASCs transfected with either pVAX-rBDNF or pVAX-mGDNF were kept in culture and supernatants were analyzed through the use of ELISA for their BDNF/GDNF content. Figure 2 shows that the plasmids were able to induce the required time course pattern of protein production: both neurotrophin levels peaked few days after transfection (72h for BDNF and 48h for GDNF, Fig. 2) and expression dropped below the 24h levels by 9 days in culture. These data may suggest that both neurotrophic factors are produced in considerable amounts for approximately 7 days, i.e. during the critical period of motoneuron survival after avulsion injury (Koliatsos 1994, Nógrádi et al. 2007). The neurotrophic factors expression levels, however, displayed differing time course: while BDNF was shown to have a peak expression level at 400% relative to the 24h values and declined by 9 days, GDNF levels reached approximately 250% and dropped back considerably by 9 days, this level of protein expression was maintained till the end of the time-course study (Fig. 2). Considering absolute values, GDNF expression was always considerably greater than that of BDNF. Untransfected cells produced negligible amounts of GDNF or BDNF.

Analysis of *in vitro* biological activity of transfected rASCs

In order to prove that the transfected cells produced biologically potent neurotrophic factors, we performed an *in vitro* essay. Embryonic dorsal root ganglia were treated with supernatants of BDNF- or GDNF-producing rASCs and their effects was investigated after 24h. Both BDNF and GDNF induced an approximately twofold longitudinal neurite outgrowth compared with the control ganglia that received supernatant of untransfected rASCs. Interestingly, co-treatment with both BDNF and GDNF, but with identical cumulative

neurotrophic factor levels as in the cases of individual treatments did not result in improved neurite outgrowth (Fig. 3).

General observations, functional improvement, CatWalk gait analysis system

Behavioral analysis was started 2 weeks after surgery and the first signs of functional recovery were observed 4-5 weeks after reimplantation of the ventral root. Control (group 1, treatment with untransfected rASCs) showed minimal improvement, i.e. their affected hind limb was placed laterally with minimal dorsiflexion of the ankle joint and the spreading reflex was negligible or completely missing. In contrast, animals that received transfected rASC grafts around the reimplanted L4 ventral root (groups 2-4) developed an improving movement pattern similar to that of the intact hind limb. Movements of the ankle joint, especially dorsiflexion was extensive and toe spreading was present. Quantitative gait analysis obtained from the CatWalk automated gait analysis system showed that the earliest differences in functional recovery between these groups appear from week 6 and these differences became significant by week 8 (Fig. 4). Improved footprint parameters (*max. contact area, print area, mean intensity*) and dynamic parameters characteristic of the step cycle (*stance duration and step cycle duration*) showed improved movement pattern in groups 2-4 animals (Fig 4).
Interestingly, animals that received combined rASC (BDNF+GDNF) grafts (group 4) displayed only slightly, but not significantly better footprint (max. contact area and print area) parameters than the animals treated with either GDNF or BDNF (groups 2 and 3).

Localization of BDNF- and GDNF-transfected cells around the reimplanted ventral root

Rats that received transfected rASCs embedded in collagen matrix placed along the reimplanted ventral root, formed a well-defined tissue cuff around the reimplanted ventral root 10 days after grafting (Fig. 5). The majority of the grafted rASCs were labelled with CellBrite (Fig. 5A-B and F-G) and expressed GDNF (Fig. 5D-E) or BDNF (Fig. 5I-J),

respectively. None of the grafted cells was found to have migrated into the ventral root and a small gap, thought to be due to fixation, was found between the ventral root and the cuff of grafted rASCs. The Schwann cells within the ventral root also moderately expressed GDNF and BDNF (Fig. 5A and F).

Retrograde labelling studies, number of reinnervating motoneurons

The number of retrogradely labelled motoneurons, i.e those cells that were able to send their axons into the reimplanted ventral root correlated with the functional data. Control animals that had their ventral root avulsed and reimplanted and received untransfected rASC grafts (group 1) had few reinnervating motoneurons (63 ± 4 SEM, n=5). In contrast, in the spinal cords of group 2 and 3 animals which received a GDNF-rASC or BDNF-rASC grafts, respectively, much more retrogradely labelled motoneurons were found (192 ± 17 SEM vs 187 ± 12 SEM, n=5 each), without significant difference between these two groups (Fig. 6). On the other hand, grafting of mixed populations of GDNF- and BDNF-rASCs around the reimplanted root (group 4, n=5) produced slightly improved results: The numbers of reinnervating cells were 247 ± 24 SEM (Fig. 6).

It is expected that reinnervating motoneurons regenerate their axons in an elongative growth pattern following a moderate growth-promoting stimulus. Indeed, axons of reinnervating motoneurons were present in the reimplanted ventral roots in both control and treatment groups, although in differing numbers. Ventral roots of control (group 1) animals contained only few thin neurofilaments-stained fibres compared with the thicker and numerous axons of the intact L4 ventral roots (Fig. 7A-B) while much more thin axons were found in the spinal roots of group 2-4 animals that received transfected rASCs (Fig. 7C-E). We found no evidence of collateral sprouting of nerve fiber coil formation within the reimplanted roots suggesting that the temporarily limited release of neurotrophins by the rASCs induced only

elongative growth of regenerating axons (Fig. 7C-E). Again, combined treatment with both GDNF- and BDNF-rASCs induced no observable difference to treatments with these factors alone.

Analysis of choline acetyltransferase (ChAT) expression in injured and reinnervating motoneurons

Next the localization of ChAT-expressing surviving and Fast Blue-labelled reinnervating cells was compared. In control rats (group 1) the few retrogradely labelled motoneurons in the ventral horn were ChAT immunoreactive, and many ChAT immunoreactive motoneurons were found unlabelled with Fast Blue (Fig. 8A-C). Stronger colocalization was found in spinal cords of group 2-4 animals, who received transfected rASCs along their reimplanted ventral root. However, in these spinal cords there were still numerous ChAT immunoreactive cells which were not retrogradely labelled (Fig. 8D-L).

Accordingly, in control animals the proportions of Fast Blue-labelled motoneurons on the operated side compared with that of the ipsilateral ChAT immunoreactive motoneurons were 17% ± 3 (SEM, group 1; Fig. 9) and only 5% ± 1 (SEM) of the total L4 motor pool was able to reinnervate the target muscles. In contrast, in animals that received neurotrophic factor-releasing rASCs (group 2-4) 36- to 50% of the total motoneuron pool survived (ChAT+ motoneurons) and 15- 20% of that was able to reinnervate (FB-labelled motoneurons, Fig. 9). None of these three treatment groups were significantly different from each other in respect of the proportion of FB+ and ChAT+ motoneuron numbers / total motoneuron pool, but they were statistically different form the control group.

It is noted, that the reinnervating cells in these 3 groups comprised 15, 16, 20% of the intact L4 motoneuron pool (100% ± 14 SEM, n=4), suggesting a moderate reinnervating mechanism. On the other hand, treatment with transfected rASCs improved the ability of surviving motoneurons to reinnervate peripheral targets. While in control animals only

approx. 30% of the ChAT+ cells could send their axons into the reimplanted ventral root, in group 2-4 animals this ratio was approximately 40% (Fig. 9).

Discussion

Here we have provided evidence that a spatiotemporally controlled expression of the neurotrophic factors BDNF/ GDNF after ventral root avulsion and subsequent reimplantion of the root promotes motoneuron survival and induces elongative axonal growth of the axons with functional reinnervation of the target muscles.

These effects were achieved by **a**) local application of the transgene-expressing mesenchymal stem cells around the root, where only limited amounts of neurotrophic factors could reach the growing axons and by **b**) a temporally regulated expression pattern. This is due to the intrinsic episomal nature of the plasmid resulting in a transient neurotrophic factor expression pattern.

It has been well established that some neurotrophic factors, especially BDNF and GDNF have a profound effect on motoneuron survival and axonal outgrowth. However, reinnervation of the peripheral targets, e.g. skeletal muscle failed in the studies where virus-based BDNF and/or GDNF expression was induced in the spinal cord, ventral root or the peripheral nerve pathways (Blits et al. 2004, Eggers et al. 2008, 2010). Eggers et al. (2013) combined neurosurgical reimplantation of avulsed ventral spinal roots with lentiviral vector-mediated expression of GDNF in the reimplanted roots to create a neurotrophic factor gradient from the reimplanted roots to the ventral spinal cord that would attract motor axons toward the root. Lentiviral vector mediated over expression of GDNF prevents motoneuron death and enhances axonal outgrowth into reimplanted nerve roots. High locally applied concentrations of GDNF caused trapping of growing axons and lead to coil formation (Eggers et al. 2008). The same group has recently made efforts to create a gradient of GDNF in the sciatic nerve after ventral root avulsion and reimplantion using lentiviral vector-mediated expression of GDNF. Sprouting and coil formation of the axons was observed throughout the nerve provided with the GDNF-gradient, even at relatively low concentrations of the lentivirus

applied. The size and number of coils increased with the level of GDNF expressed in the peripheral nerve. Moreover, the number of Schwann cells was increased, but the myelination of the axons was severely impaired. The total number of regenerated and surviving motoneurons was not enhanced (Eggers et al. 2013).

On the other hand, intrathecal administration of recombinant GDNF after avulsion and reimplantation of the L4 ventral root into the spinal cord enhanced the survival rate of the injured motoneurons, especially when it was applied in combination with riluzole (Bergerot et al. 2004).

There are several hypotheses why potent neurotrophic factors, like GDNF and BDNF rescued the injured motoneurons but failed to promote the elongative growth of regenerating axons.

According to the widely accepted view on the mechanism of action of neurotrophic factors, they provide a directional guidance for regenerating injured axons, which is readily observed in *in vitro* experiments (Tessier-Lavigne and Goodman, 1996). The failure of in vivo experiments in supporting elongative axonal growth was thought to be due to the high and sustained neurotrophic factor concentrations around the axons.

The possibility that axon trapping is caused by a different mechanism unrelated to the neurotrophic effect of GDNF has been suggested, too. It is argued, that GDNF could have direct effects on Schwann cells and on the interactions of Schwann cells with axons or on myelination (Hase 2005). These results, however have clearly shown that abundant and sustained application/overexpression of these neurotrophic factors may rather prevent axonal regeneration after a successful initial phase of promoting of axon growth.

Several in vivo studies, mainly focusing on the regenerative effects induced by neurotrophic factors in the injured spinal cord have shown that transient expression of BDNF or GDNF

significantly promoted the regenerative growth of injured axons (Blesch and Tuszynski 2007). Boyd and Gordon (2002) have shown that low dose of exogenous BDNF (0.5±2 mg/day for 28 days) promoted axonal regeneration of motoneurons whose regenerative capacity was reduced by chronic axotomy, while high doses of BDNF (12±20 mg/day for 28 days) significantly inhibited motor axonal regeneration, after both immediate nerve repair and coaptation following chronic axotomy. The dose-dependent facilitation and inhibition of peripheral nerve regeneration with exogenous BDNF could be reversed by the functional blockade of BDNF receptors (Gordon and Boyd 2001, 2002). Taken together, these findings suggested that injured neurons are very sensitive to the neurotrophic effect and the level, gradient, duration and localization of neurotrophic factor expression all have a high impact on regenerating injured axons (Anton et al. 1994, Bonner et al. 2010).

The question can be raised whether the relatively good functional recovery based on moderate morphological reinnervation in the present study could be further improved. In our earlier studies we have shown that administration of riluzole, a compound capable to block presynaptic release of glutamate and to inhibit voltage gated Na^+ and Ca^{2+} channels induced reinnervation of denervated hind limb muscles up to an extent of 67% after a ventral root avulsion injury (Nógrádi and Vrbová 2001, Nógrádi et al. 2007). In vitro evidence has also been provided that riluzole is able to promote neurite growth of sensory ganglion neurons (Shortland et al. 2006) further strengthening the neuroprotective effects of this compound. Moreover, intraspinal or intraradicular transplantation of the neuroectodermal stem cell line NE-FP-4C (ATCC No.: CRL-2936) resulted in similarly extensive reinnervation of the hind limb muscles after the avulsion and reimplantation of the L4 ventral root (Pajenda et al. 2013). It is possible that slightly more GDNF or BDNF secreted at the site of injury could further improve the reinnervation by the surviving motoneurones. On the other hand, it could be argued, that increased release of these neurotrophic factors would already induce a trapping

effect on the regenerating axons, rather than supporting their elongative growth as seen in the recent study by Eggers et al. (2013).

It was interesting to find that the combined action of GDNF and BDNF did not induce significantly improved regeneration, suggesting that the combined application of these neurotrophic factors does not generate a synergistic effect in this model.

Further studies are required to be performed in order to elucidate the mechanisms of neurotrophic factors on injured motoneurons and to determine the dose of individual neurotrophic factor that support survival and regeneration of injured motoneurons but do not cause trapping of their regenerating axons. Taking into consideration the extreme sensitivity of these injured neurons to neurotrophic factors it appears likely that only very low doses, applied to the indirect vicinity of the injured axons are able to fulfill these requirements.

References:

Anton, ES, Weskamp, G, Reichardt, LF, Matthew, WD, 1994. Nerve growth factor and its low-affinity receptor promote Schwann cell migration. Proc Natl Acad Sci U S A. 91, 2795-2799.

Bergerot, A., Shortland, P. J., Anand, P., Hunt, St. P.,and Carlstedt Th., 2004. Co-treatment with riluzole and GDNF is necessary for functional recovery after ventral root avulsion injury. Exp Neurol 187, 359-366.

Bertelli, J.A., Ghizoni, M.F., 2003. Brachial plexus avulsion injury repairs with nerve transfers and nerve grafts directly implanted into the spinal cord yield partial recovery of shoulder and elbow movements. Neurosurgery 52,1385-1389.

Blesch, A., Tuszynski, M H., 2007. Transient growth factor delivery sustains regenerated axons after spinal cord injury. J Neurosci 27,10535–10545

Blits, B., Carlstedt, T.P., Ruitenberg, M.J., De Winter, F., Hermens, W.J., Dijkhuizen, P.A., Classens, J.W.C., Eggers, R., van der Slius, R., Tenenbaum, L., Boer, G.J., & Verhaagen, J. 2004. Rescue and sprouting of motoneurons following ventral root avulsion and reimplantion combined with intraspinal adeno-associated viral vector mediated expression of glial cell line derived neurotrophic factor or brain derived neurotrophic factor. Exp Neurol. 189, 303-331.

Bonner, JF., Blesch, A., Neuhuber, B., Fischer, I., 2010. Promoting directional axon growth from neural progenitors grafted into the injured spinal cord. J Neurosci Res. 88, 1182–1192.

Boyd, JG. T. Gordon T 2001 The Neurotrophin Receptors, trkB and p75, Differentially Regulate Motor Axonal Regeneration, J Neurobiol. 49, 314-325.

Boyd, JG, Gordon, T., 2002. A dose-dependent facilitation and inhibition of peripheral nerve regeneration by brain-derived neurotrophic factor. Eur J Neurosci. 15, 613-626.

Carlstedt, T., Lindå, H., Cullheim, S., Risling, M., 1986. Reinnervation of hind limb muscles after ventral root avulsion and implantation in the lumbar spinal cord of the adult rat. Acta Physiol Scand. 128, 645-646.

Carlstedt, T., Hallin, R.G., Hedström, K.G., & Nilsson-Remahl, I.A., 1993. Functional recovery in primates with brachial plexus injury after spinal cord implantion of avulsed ventral roots. J Neurol Neurosurg & Psych., 56, 649-654.

Eggers R., Hendriks, W.T.J., Tannemaat, M.R, van Heerikhuize, J.J, Pool, C.W., et al. 2008. Neuroregenerative effects of lentiviral vector-mediated GDNF expression in reimplanted ventral roots. Mol Cell Neurosci 39, 105–117.

Eggers, R., Tannemaat, M.R., Ehlert, E.M., Verhaagen, J., 2010. Spatio-temporal analysis of motoneuron survival, axonal regeneration and neurotrophic factor expression after lumbar ventral root avulsion and implantation. Experimental Neurology 223, 207–220

Eggers, R., De Winter F., Hoyng, St. A., Roet, K.C.D., Ehlert, E.M., Malessy, M.J.A., Joost Verhaagen, J., Tannemaat, M.R., 2013. Lentiviral vector-mediated gradients of GDNF in the injured peripheral nerve: effects on nerve coil formation, Schwann cell maturation and myelination. PLOS ONE-8-8-e71076

Gilbert, A., Pivato, G., Kheiralla, T., 2006. Long-term results of primary repair of brachial plexus lesions in children. Microsurgery 26, 334–342.

Hamers, F.P.T., Lankhorst, A.J., van Laar, T.J., Veldhuis, W.B., & Gispen, W.H., 2001. Automated quantitative gait analysis during overground locomotion in the rat: Its application to spinal cord contusion and transection injuries. J Neurotrauma, 18, 187-201.

Hamers, F.P.T., Koopmans, G.C., Joosten, E.A.J., 2006. Catwalk assisted gait analysis in the assessment of spinal cord injury. J Neurotrauma, 23, 537-548.

Hase, A., Saito, F., Yamada, H., Arai, K., Shimizu, T., Matsumura, K., 2005. Characterization of glial cell line-derived neurotrophic factor family receptor alpha-1 in peripheral nerve Schwann cells. J Neurochem 95: 537–543.

Hell, R.C.R., Costa, M.M.S., Goes, A.M. Oliveira, A.L.R., 2009. Local injection of BDNF producing mesenchymal stem cells increases neuronal survival and synaptic stability following ventral root avulsion. Neurobiol Dis. 33, 290-300.

Henderson, C. E., W. Camu, C. Mettling, A. Gouin, K. Poulsen, M. Karihaloo, J. Rullamas, T. Evans, S. B. McMahon, M. P. Armanini and et al. 1993. Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature 363, 266-270.

Henderson, C. E., H. S. Phillips, R. A. Pollock, A. M. Davies, C. Lemeulle, M. Armanini, L. Simmons, B. Moffet, R. A. Vandlen, L. C. Simpson and et al. 1994. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266, 1062-1064.

Kishino, A, Ishige, Y., Tatsuno, T., Nakayama, C., Noguchi, H., 1997. BDNF prevents and reverses adult rat motor neuron degeneration and induces axonal outgrow. Exp Neurol. 144, 273-86.

Koliatsos, V.E., Price, W.L., Pardo, C.A., & Price, D.L., 1994. Ventral root avulsion: An experimental model of death of adult motor neurons. J Comp Neurol. 342, 35-44.

Li, L., R. W. Oppenheim, M. Lei and L. J. Houenou 1994. Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse." J Neurobiol 25, 759-766.

Millesi H, Chirurgie der peripheren Neven, 1992. Urban & Schwarzenberg Vienna

Nógrádi, A., Vrbová, G., 1996. Improved motor function of denervated rat hind limb muscles by embryonic spinal cord grafts. Eur J Neurosci. 8, 2198-2203.

Nógrádi, A., Vrbová, G., 2001. The effect of riluzole in rats on the survival of injured adult and grafted embryonic motoneurones. Eur J Neurosci. 13, 113-118.

Nógrádi, A., Szabó, A., Pintér, S., Vrbová, G., 2007. Delayed riluzole treatment is able to rescue injured spinal motoneurons. Neuroscience 144, 431-438.

Novikov, L., Novikova, L., Kellerth, J.O., 1995. Brain-derived neurotrophic factor promotes survival and blocks nitric oxide synthase expression in adult rat spinal motoneurons after ventral root avulsion. Neurosci Lett. 200, 45-48.

Novikova, L., Novikov, L., Kellerth JO. 1997. Effects of neurotransplants and BDNF on the survival and regeneration of injured adult spinal motoneurons. Eur J Neurosci. 9, 2774-2777.

Oppenheim, R. W., Q. W. Yin, D. Prevette and Q. Yan. 1992. Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature 360, 755-757.

Oppenheim, R. W.,L. J. Houenou, J. E. Johnson, L. F. Lin, L. Li, A. C. Lo, A. L. Newsome, D. M. Prevette and S. Wang 1995. Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature 373, 344-346.

Pajenda, G., Pajer, K., Márton, G., Hegyi, P., Redl, H., Nógrádi 2013. Rescue of injured motoneurones by grafted neuroectodermal stem cells: Effect of the location of graft. Restor Neurol Neurosci. 31, 263-274

Pintér, S., Gloviczki, B., Szabó, A., Márton, G., Nógrádi, A. 2010. Increased survival and reinnervation of cervical motoneurons by riluzole following avulsion of the C7 ventral root. J Neurotrauma, 27, 2273-2282.

Shortland, P.J., Leinster, V.H., White, W., Robson, L.G. 2006. Riluzole promotes cell survival and neurite outgrowth in rat sensory neurones in vitro. Eur J Neurosci 24, 3343-3353.

Su, H., Zhang,W., Guo,J., Guo,A., Yuan, Q., Q., Wu W., 2009. Neural Progenitor Cells Enhance the Survival and Axonal Regeneration of Injured Motoneurons after Transplantation into the avulsed ventral horn of adult rats. J Neurotrauma 26, 67–80.

Tessier-Lavigne, M., Goodman, CS., 1996. The molecular biology of axon guidance, Science 274, 1123-33

Vejsada, R., Sagot, Y., Kato, A.C., 1995. Quantitative comparison of the transient rescue effects of neurotrophic factors on axotomized motoneurons in vivo. Eur J Neurosci. 7, 108-115.

Wiese, S., Metzger, F., Botmann, B., Sendtner, M., 1999. The role of p75NTR in modulating neurotrophin survival effects in developing motoneurons. Eur J Neurosci. 11, 1668–1676.

Wu, W., Lin, L.F., Lei, M., Oppenheim R.W.,Houenou, L.J., 1995. Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor. Proc Natl Acad Sci USA. 92, 9771–9775.

Figure 1: Schematic diagrams show the structure of pVAX1 vectors and the surgical procedure

A-B: The structure of the pVAX1 vector backbone with the inserted therapeutic genes

C: Schematic drawing of the final result of the surgical procedure showing the reimplantation of the L4 root and the localization of the rASCs embedded in collagen around the reimplanted root. Note the injured motoneurons (red) located in the ventral horn of the spinal segment.

D: Schematic drawing showing the procedure of retrograde labelling of regenerated motoneurons with Fast Blue (blue) from the L4 spinal nerve three months after the avulsion injury.

E: Microphotograph taken from the ventral horn of a spinal cord section where retrogradely labelled (blue) and surviving (red) motoneurons are located. Arrowheads point to double labelled cells. Scale bar = 200 µm.

Figure 2: ELISA-evaluated production of GDNF and BDNF by transfected and untransfected cells *in vitro*

Bar charts show the time course of absolute gene expression (pg/ml) by the transfected cells *in vitro*. The untransfected cells produce no or negligible amounts of neurotrophic factors. The transfected cells have a peak of neurotrophic factor production at 72h for BDNF and at 48h for GDNF, with a considerable decline after 5 days. Note that the levels of neurotrophic factors do not reach the 24h values from day 9 onwards.

Figure 3: Biological activity of neurotrophic factors secreted by pVAX1_rBDNF- and pVAX1_mGDNF-transfected rASC cells.

A: Neurite outgrowth of explanted and cultured E12.5 ganglion cells stimulated by the supernatant of untransfected rASCs.

B-D: Examples of *in vitro* longitudinal neurite outgrowth of rat embryonic dorsal root ganglia after 24 h in conditioned medium derived from pVAX1_rBDNF- or pVAX1_mGDNF-transfected rASC cells. Scale bar = 250 μm.

E: Quantification of neurite outgrowth as fold change of the value of mock controls. Medium from untransfected cells induces limited outgrowth, whereas medium from pVAX1_rBDNF- and pVAX1_mGDNF-transfected rASCs increases neurite outgrowth twofold, respectively. Combined medium containing supernatants both from pVAX1_rBDNF- and pVAX1_mGDNF-transfected rASCs does not produce augmented longitudinal neurite outgrowth. Error bars: mean ± SEM, n = 6 per group. No significant difference was found among the groups treated with supernatants derived from transfected cells. $p < 0.05$; one-way ANOVA followed by Bonferroni's post-hoc testing.

Figure 4. Results of the CatWalk automated gait analysis

The gait analysis revealed significant differences between groups 1 (treatment with untransfected rASCs) and groups with various treatment strategies (groups 2-4). The earliest significant differences in functional recovery between control and treatment groups appeared from week 8 in case of most parameters. Both the parameters indicating improved stability of foot placing (*max. contact area, print area, mean intensity, stance duration*) and the improved *step cycle)* suggested significant functional reinnervation in groups with neurotrophic factor treatments. Note that the parameters of animals in groups 2-4 have significantly improved but still did not approach the pre-training levels. Asterisks indicate significant differences between groups 1 and 2-4 by using the two-way measurement analysis of variance (ANOVA) computed according to Tukey's all pair-wise multiple comparison procedures, $p < 0.05$. (Symbols in the figure: ♦ = untransfected rASC grafting, ▲ = GDNF-rASC grafting, ▼ = BDNF-rASC grafting, ► = BDNF+GDNF-rASC grafting, ■ = pre-training results)

Figure 5. Location and gene expression patterns of grafted rASCs.

A and **F** show GDNF- and BDNF-rASCs 10 days after transplantation around the reimplanted ventral root, respectively. The cells appear as a tissue mass outside the connective tissue sheath of the root (not labelled). There is some GDNF and BDNF expression detected in Schwann cells of the root itself. The rASCs labelled with CellBrite prior to grafting did not migrate into the reimplanted root. **B-E** and **G-J** (confocal images of the framed areas in **A** and **F**) indicate the protein expression pattern of grafted stem cells. Note that many, but not all of the grafted cells are labelled with CellBrite and again, not all of them express GDNF or BDNF. Arrows point to cells that are both CellBrite+ and GDNF/BDNF+. Note that the confocal images were obtained by the use of various optical thickness settings and they not necessarily depict the same cell population as photographs taken from a thick section with epifluorescence microscopy. Scale bar in **A,F** = 100 μm, in the rest of the figures = 20 μm.

Figure 6. Bar chart showing the results of retrograde labelling studies from the L4 spinal nerve. Transplantation of untransfected stem cells around the reimplanted ventral root (group 1) resulted in low number of retrogradely labelled motoneurons in the L4 segment. Transplantation of GDNF-rASCs, BDNF-rASCs or the combination of these two transfected cell types (groups 2-4) induced considerable reinnervation of the reimplanted root (no significant difference was found between these groups). * = significant difference between groups 1 and groups 2-4, values are shown as mean +/- SEM. $p < 0.01$, t-test.

Figure 7. Elongative growth of the regenerating axons in the reimplanted L4 ventral roots. The reimplanted ventral roots taken from intact (**A**), control (**B**) and neurotrophic factor-treated (**C-E**) L4 ventral roots were sectioned and processed for 200kD neurofilament immunohistochemistry. In all samples elongative growth patterns were found, without signs

for sprouting and coil formation. Note the small number of regenerating axons in the root treated with untransfected rASCs. Scale bar = 50 µm.

Figure 8: Representative images of reinnervating and surviving motoneurons in the L4 spinal segment of rats treated with rASCs, 3 months after grafting.

A-C: Transverse sections of spinal cord taken from L4 spinal cord segment of an animal in group 1 (untransfected rASC graft) with retrogradely labelled motoneurons and ChAT immunereactive neurons, respectively. **D-F**: Spinal cord sections taken from a group 2 animal (L4 ventral root avulsion and reimplantation and GDNF-rASC grafts). **G-I**: Spinal cord sections taken from a group 3 animal (L4 avulsion and reimplantation and BDNF-rASC graft). **J-L**: Retrogradely labelled and surviving ChAT-positive cells in the L4 segment of a group 4 animal (combined GDNF+BDNF-rASC treatment). Note the great numbers of surviving (ChAT-positive) cells in the ventral horn at the operated side in **D,G** and **J** and the relatively greater numbers of retrogradely labelled cells in the ventral horns of animals treated with transfected rASCs (**E,H** and **K**). There is a striking difference in the numbers of surviving and reinnervating motoneurons between group 1 and groups 2-4 animals (note **A-C** with few labelled motoneurons in the ventral horn). Surviving and reinnervating motoneurons were retrogradely labelled with FB and the same sections processed for ChAT immunohistochemistry. Arrowheads in C, F, I and L point to $ChAT^+/FB^-$ cells, while arrows indicate ChAT+ only cells. Note that due to the different objective depths used during taking the photographs, some FB-labelled cells appear to express only faint ChAT. Scale bars in A-B = 200 µm , in C-N: 100 µm.

Figure 9. Percentage of surviving motoneurons in the L4 ventral horn in various experimental paradigms. Cholinacetyltransferase immunohistochemistry was applied to detect and determine the numbers of surviving motoneurons after ventral root avulsion and

reimplantation. Note that in control animals only limited number of motoneurons survived and even fewer had the chance to regenerate into the ventral root. In groups 2-4 the higher surviving rate resulted in considerably better reinnervation rate. * and ** = significant difference between groups 1 and groups 2-4, values are shown as mean +/- SEM. $p < 0.01$, t-test.