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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 (Bertelli and Ghizoni, 2003; Gilbert et al., 2006; Millesi, 1992). 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; Eggers et al., 2010; Nógrádi and Vrbová, 1996, 2001).

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 (Bonner et al., 2010; Hell et al., 2009; Su et al., 2009). Although satisfying survival of

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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 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; Novikov et al., 1995, Novikova et al., 1997; Wiese et al., 1999; Boyd and Gordon, 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 anesthesia 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 400 *g* at room temperature, the supernatant was discarded and the pellets were washed by re-suspension and centrifugation (400 g, RT) in 1× PBS. The rASCs were re-suspended in 5 ml rASC-medium (DMEM high glucose, 1% Penicillin/Streptomycin, 1% glutamine, 10% fetal calf serum [FCS]) and brought into culture in 75 cm^2 flasks. Aliquots of 1×10^6 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 400 *g*. Cells were resuspended in fresh medium and sewn in 175 ml flasks. After 24 h the medium was changed for further cultivation.

Cell staining with Cellbrite

In order to track rASCs after transplantation, the cells were labeled using the Cellbrite™ Red Cytoplasmic Membrane Staining Kit (Biotium CA, USA). The overall labeling efficacy was approximately 95%. Cells were trypsinized and suspended at a density of 1×10^6/ml in serum-free DMEM. After adding 5 μl of cell-labeling solution per 1 ml of cell suspension, the cells were incubated at 37 °C for 20 min. After a centrifugation step for 5 min at 1500 rpm at 37 °C, the supernatant was removed and cells were gently resuspended in warm (37 °C) medium. Following two more washing steps (centrifugation and resuspension), cells were resuspended in serum free DMEM at a concentration of 1×10^6/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 h 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 h prior to sampling and 500 μl 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 anesthesia and rinsed