Design and \textit{in vitro} Characterization of Therapeutic Expression Systems for \textit{ex vivo} Neuroprotective Gene Therapy

verfasst von

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angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, September 2013

Studienkennzahl lt. Studienblatt: A 490

Studienrichtung lt. Studienblatt: Diplomstudium Molekulare Biologie

Betreut von: O. Univ. Prof. Dr. Hans Lassmann
Dedication

This work is dedicated to my family, my friends and my partner, who came along with me all the way and are going to be there for the next chapters of my life.
Danksagungen

Diese Diplomarbeit wurde am Ludwig Boltzmann Institut für experimentelle und klinische Traumatologie unter der Leitung von Prof. Dr. Heinz Redl durchgeführt. An dieser Stelle möchte ich Ihm für die Ermöglichung dieser Arbeit und die Korrektur derselbigen danken.

Spezieller Dank gebührt meinem Betreuer Dr. Georg "der Architekt" Feichtinger und Mag. Ara Hacobian, die mir stets rat- und tatkräftig zur Seite gestanden sind, ohne Sie wäre diese Arbeit nicht möglich gewesen.


Großer Dank gilt weiters Hrn. o Univ.- Prof. Dr. Hans Lassmann, der mir durch seine Betreuung von Seiten der Universität Wien / Medizinischen Universität Wien die Fertigstellung dieser Arbeit ermöglicht hat.

Schließlich möchte ich meinen Eltern danken, die es mir nicht nur ermöglicht haben zu Studieren sondern mich auch stets moralisch in allem was ich tat bestärkt haben, meinen Geschwistern Christian und Deborah, meinen Freunden Georg Lora, Michael Ebner, Daniel Kiesehofer, Andreas Mangold, Stefan Geißler, Markus Grabher, Hans Metzler, Claudia Birnbaumer, Daniel Trojer, Tobias Köb, Sabine Hieble und Wolfgang Hörleinsberger, die stets Verständnis aber auch die notwendige Ablenkung während dieser intensiven Zeit aufgebracht haben und last but not least, meiner wunderbaren Carmen, die mir den nötigen Halt für alles im Leben gibt.
Abstract

The discovery of an easily accessible and abundant pool of stem cells in adult adipose tissue some years ago, established new opportunities for stem cell therapy. Suddenly it was possible to harvest adult stem cells in great numbers by mere liposuction. These adipose derived stem/stromal cells are regarded as multipotent as they can differentiate into various tissues and have been shown to be immunomodulatory. ASCs are easy to cultivate in vitro and are supposed to be safe if administered into the body afterwards. They enable autologous stem cell therapy in a way that was not possible using bone marrow derived stem cells. The growing knowledge of the in vivo behaviour of ASCs allowed new therapeutic approaches including ex vivo gene therapy using these cells, for a variety of diseases and injuries.

In this study we evaluated the use of ASCs as a delivery system for the local transient expression of the neurotrophic factors BDNF and GDNF. The use of a plasmid-based system enabled a beneficial transient expression profile, simultaneously avoiding negative effects of viral based gene therapy like a strong response of the immune system or the possible integration into the host genome.

It could be shown in vitro that transfected ASCs not only secreted biologically active BDNF and GDNF after plasmid delivery but also that this expression was temporally restricted. Furthermore it was possible to construct plasmids, which contain reporter gene cassettes in addition to therapeutic gene cassettes, in order to evaluate the fate of transfected cells in vitro and in vivo in the future in experimental models.

In the follow up in vivo study, ex vivo gene therapy was carried out in a ventral root avulsion model in the rat. Transfected rat ASCs were applied around the reimplanted ventral root. It could be shown that animals which received BDNF or GDNF producing ASCs, displayed increased survival of motoneurons alongside elongative axonal regeneration and significant functional improvement. This study demonstrated as proof of concept that a plasmid based ex vivo gene therapy using adipose tissue-derived stem/stromal cells as shuttle systems for the in situ production of neurotrophic factors is feasible. Nevertheless, besides a closer look at the long-term behaviour of the transplanted ASCs as well as the effects of delayed transplantation, a further increase in transfection efficiency under good manufacturing practise (GMP) conditions is necessary in order to enable clinical translation of this therapeutic approach in the future.
ABSTRACT ................................................................................................................................. 4

LIST OF ABBREVIATIONS ........................................................................................................... 8

1 INTRODUCTION .......................................................................................................................... 11

1.1 DIFFERENTIAL REACTIONS OF THE PNS AND CNS TO INJURIES ........................................ 12
1.2 VENTRAL ROOT AVULSION INJURY ...................................................................................... 17
  1.2.1 Motoneuron death in the ventral horn and the problem of reinnervation of the ventral root ...... 18
1.3 THERAPEUTIC APPROACHES FOR ROOT AVULSION INJURIES ........................................ 20
  1.3.1 Surgery ............................................................................................................................... 20
  1.3.2 Enhancement of survival of injured motoneurons by drugs .................................................. 21
  1.3.3 Stem cell therapy ............................................................................................................... 23
  1.3.3.1 Adipose-tissue Derived Stem / Stromal Cells (ASCs) ..................................................... 24
  1.3.4 Neurotrophic factors ........................................................................................................ 28
  1.3.4.1 Brain derived neurotrophic factor (BDNF) ...................................................................... 29
  1.3.4.2 Gial cell-line derived neurotrophic factor (GDNF) ......................................................... 31
  1.3.4.3 Direct administration of recombinant BDNF or GDNF ................................................... 33
  1.3.5 Viral gene therapy with BDNF / GDNF ............................................................................. 33
  1.3.6 Non viral gene therapy with BDNF / GDNF ..................................................................... 34
  1.4 Summary ............................................................................................................................... 34
  1.4 Aim of the study .................................................................................................................... 35

2. MATERIAL AND METHODS ...................................................................................................... 36

2.1 MATERIALS ............................................................................................................................ 36
  2.1.1 Primers ............................................................................................................................... 36
  2.1.2 Plasmids ............................................................................................................................ 37
  2.1.3 Bacterial Strains ............................................................................................................... 41
  2.1.4 DNA size markers ............................................................................................................ 43
  2.1.5 DNA polymerases ........................................................................................................... 43
  2.1.6 Restriction enzymes ........................................................................................................ 43
  2.1.7 Antibodies ........................................................................................................................ 44
  2.1.8 Recombinant growth factors ............................................................................................ 44
  2.1.9 Cell culture material ......................................................................................................... 44
  2.1.10 Cell lines and primary cells ............................................................................................ 45
  2.1.11 Microscopes .................................................................................................................... 46
  2.1.12 In silico sequence analysis ............................................................................................. 47

2.2 MOLECULAR BIOLOGY METHODS ...................................................................................... 47
  2.2.1 Polymerase chain reaction (PCR) ..................................................................................... 47
  2.2.2 Agarose gel electrophoresis ............................................................................................. 49
  2.2.3 DNA extraction from agarose gels .................................................................................. 50
  2.2.4 Restriction digests of DNA ............................................................................................. 50
3 RESULTS ................................................................................................................................. 66

3.1 DESIGN AND EVALUATION OF THERAPEUTIC VECTORS CARRYING REPORTER GENES............. 66
  3.1.1 pVAX1_mBDNF and pVAX1_rBDNF ................................................................. 66
  3.1.2 Cloning of dsRed cassette into pVAX1_rBDNF + pVAX1_mBDNF .................. 67
  3.1.3 Cloning of a TurboRFP cassette into pVAX1_rBDNF + pVAX1_mBDNF .......... 69
  3.1.4 Cloning of a Click Beetle Luciferase (CBR) cassette into pVAX1_mBDNF and pVAX1_rBDNF .... 72
  3.1.5 In vitro functionality testing of the constructed plasmids ................................. 75

3.2 DESIGN OF A TURBORFP-CBR FUSION PROTEIN EXPRESSING PLASMID ......................... 78
  3.2.1 In vitro functionality testing of the pTurboFP365-CBR_Fusion vector ............... 81

3.3 EVALUATION OF A TRANSFECTION PROTOCOL SUITABLE FOR PRIMARY CELLS (RAT ADIPOSE TISSUE- DERIVED STEM CELLS) ......................................................................................................................... 82

3.4 PROOF OF TEMPORALLY RESTRICTED EXPRESSION OF NEUROTROPHIC FACTORS IN VITRO USING ELISA... 85
  3.4.1 Expression kinetics of pVAX1_mBDNF transfected rASCs ................................ 85

3.5 ESTABLISHMENT OF AN IN VITRO BIOLOGICAL ACTIVITY ASSAY ................................. 86
  3.5.1 Establishment of a cell culture protocol for primary dorsal root ganglia ............ 86
  3.5.2 Alternative biological activity assay using PC12 cells ..................................... 89

3.6 IN VIVO RESULTS ................................................................................................................... 92
  3.6.1 Outline of the study ......................................................................................... 92
  3.6.2 In vivo fate of implanted cells and evaluation of expression of therapeutic protein ................................................................................................................................. 93
  3.6.3 Survival of motoneurons and reinnervation of the ventral root .................................... 94
  3.6.4 Functional analysis (automated gait analysis) .................................................... 96
4 DISCUSSION ................................................................................................................................... 97
4.1 DESIGN AND EVALUATION OF THERAPEUTIC VECTORS CARRYING REPORTER GENES .................. 97
4.2 DESIGN OF A TURBO-RFP-CBR FUSION PROTEIN EXPRESSING PLASMID ......................................... 98
4.3 EVALUATION OF A TRANSFECTION PROTOCOL SUITABLE FOR PRIMARY CELLS (RAT ADIPOSE TISSUE-DERIVED STEM CELLS) ................................................................................................................................. 99
4.4 PROOF OF TEMPORALLY RESTRICTED EXPRESSION OF NEUROTROPHIC FACTORS IN VITRO USING ELISA .................................................. 100
4.5 ESTABLISHMENT OF AN IN VITRO BIOLOGICAL ACTIVITY ASSAY ......................................................... 101
4.6 IN VIVO RESULTS .................................................................................................................................. 101

5 REFERENCES ........................................................................................................................................ 104

6 APPENDIX .............................................................................................................................................. 114
6.1 SUMMARY ........................................................................................................................................... 114
6.2 ZUSAMMENFASSUNG .......................................................................................................................... 115
6.3 CURRICULUM VITAE ............................................................................................................................ 117
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionate</td>
</tr>
<tr>
<td>ART</td>
<td>Artemin</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose tissue-derived stem cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BGH</td>
<td>Bovine growth hormone</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone marrow-derived mesenchymal stem cell</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BPB</td>
<td>Bromphenol blue</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumine</td>
</tr>
<tr>
<td>BSF-3</td>
<td>B-cell stimulating factor-3</td>
</tr>
<tr>
<td>CBR</td>
<td>Click beetle luciferase red</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>ChAT</td>
<td>Cholin acetyltransferase</td>
</tr>
<tr>
<td>CIAP</td>
<td>Calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CT-1</td>
<td>Cardiotrophin-1</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Chemokine (C-X-C motif) ligand 12</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindol</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>2′-deoxynucleoside-5′triphosphate</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immuno-sorbent assay</td>
</tr>
<tr>
<td>enh</td>
<td>Enhancer</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FB</td>
<td>Fast Blue</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GFRα-1</td>
<td>Glial cell line-derived neurotrophic factor receptor alpha-1</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>HB-GAM</td>
<td>Heparin binding growth associated molecule</td>
</tr>
<tr>
<td>HF-PCR</td>
<td>High fidelity polymerase chain reaction</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>LB broth</td>
<td>Luria Bertani broth</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitor factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin associated glycoprotein</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MMP7</td>
<td>Matrix metalloproteinase 7</td>
</tr>
<tr>
<td>MN</td>
<td>Motoneuron</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NG2</td>
<td>Neural /Glial antigen 2</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NNT-1</td>
<td>Novel neurotrophin-1</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NT-4/5</td>
<td>Neurotrophin-4/5</td>
</tr>
<tr>
<td>NTN</td>
<td>Neurturin</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin-M</td>
</tr>
<tr>
<td>p75NTR</td>
<td>Low-affinity nerve growth factor receptor</td>
</tr>
<tr>
<td>PACE 4</td>
<td>Paired basic amino acid cleaving enzyme</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Proprotein convertase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
</tbody>
</table>
pg  pico gram
PGE2  Prostaglandin E2
PGF  Placental growth factor
PI3K  Phosphoinositide 3-kinase
PKC  Protein kinase C
PLC  Phospholipase C-γ
PNS  Peripheral nervous system
PP  Polypropylene
PSP  Persephin
rER  Rough endoplasmic reticulum
RFP  Red fluorescent protein
RNA  Ribonucleic acid
RT  Room temperature
SB buffer  Sodium borate buffer
SCI  Spinal cord injury
SD  Standard deviation
shc  Src homologous and collagen like adaptor protein
SOC  Sodium optimal Broth + glucose
SV40  Simian virus 40
T  Temperature
TGF-β  Transforming growth factor-β
Tm  Melting temperature
TMB  3,3’,5,5´-Tetramethylbenzidine
TNFα  Tumor necrosis factor-α
TRAF  TNF receptor associated factor
TrkB  Tyrosine-related kinase B
UV  Ultraviolet
VEGF  Vascular endothelial growth factor
XCFF  Xylene cyanol FF
1 Introduction

A highly developed nervous system is crucial for every form of higher physiological activity; it controls a wide range of organ functions, including vital ones. It enables us to sense the environment, integrate this information and to set actions in order to adapt and survive.

The human nervous system is divided into two major parts, the peripheral and the central nervous system.

The central nervous system (CNS) consists of the cerebrum, the cerebellum and the brain stem (taken together referred as brain), and the spinal cord. Therefore, as Galen (129-199 AD) proposed, all remaining parts of the nervous system, which are not part of the brain and the spinal cord, form the peripheral nervous system.

The peripheral nervous system (PNS) is further divided into A: the somatic PNS, which consists of the peripheral nerves and dorsal root ganglia - that innervate skin, joints and muscles, the latter being under voluntary control - and B: the visceral PNS, consisting of efferent postganglionic neurons, which innervate smooth muscles as well as glands[1].

The PNS conveys information from the CNS to the muscles via motor (efferent) neurons and transmits information from the periphery via sensory (afferent) neurons, residing in the ganglia along the spinal cord and the brain stem.

The neuron is the functional unit in the nervous system. It consists of the cell body (perikaryon) and one or more processes referred to as dendrites and axons. The neuron receives inputs through the dendrites, integrates these informations in the perikaryon and then forwards a corresponding impulse (positive or negative) via the axon.
The following sections give an overview of the mechanisms, challenges and possible treatments of injuries to the nervous system, with special regards to spinal root avulsion as a specific case of traumatic nerve injury.

1.1 Differential reactions of the PNS and CNS to injuries

PNS injuries are common injuries with an incidence in Europe of approximately 1 : 1000 (approximately 300,000 cases) annually [3], mainly caused by injury to the upper extremities [4]. Approximately 100,000 patients require peripheral nerve surgery in Europe every year [5]. Crush, ischemia, traction and penetrating injuries cause the majority of nerve injuries [6, 7]. Furthermore 73.5% of all injuries occur in the upper extremities [8]. In comparison to peripheral nerve injuries are spinal cord injuries (SCI) rather seldom, with an incidence of 10.4 :1.000.000 up to 25.4: 1.000.000 or approx. 9000 new cases per year in Europe [9, 10]. Similar to PNS trauma the most common cause of SCI are traffic accidents most commonly affecting 15-35 year-old males [11]. This data indicates that injury to the nervous system is a common cause of hospitalization and leads to common need of rehabilitation or even lifelong care and thereby causing a major emotional and economic burden to patients, the society and family members [12]. Despite recent advancements in the knowledge of neuron tissue regeneration and increasing microsurgical skills of the surgeons, the functional outcome, especially after SCI, is still unsatisfactory. Injury to the nervous system may occur at several stages as depicted in Figure 2.

![Figure 2: Lesions to the nervous system:](image)

(1) central nervous system
(2) ventral horn, motor neurons
(3) roots and spinal nerves
(4) peripheral nerves
(5) peripheral sensory nerve
(6) peripheral motor nerve
(7) motor endplates

*Taken from Mumenthaler, Stöhr and Müller-Vahl: Läsionen peripherer Nerven und radikuläre Syndrome [2]*
There are several well defined and marked differences regarding the regeneration of the CNS or the PNS after injury. One major difference is the type of myelinating cells: in the PNS one Schwann cell myelinates only one internode on one axon, whereas in the CNS one oligodendrocyte may facilitate the wrapping of up to 40 internodes on different axons. Schwann cell myelin is surrounded by a basal lamina while oligodendrocyte myelin in the CNS does not have a basal lamina. Denervated Schwann cells dedifferentiate, proliferate and switch from a myelinating to a non-myelinating growth-supportive phenotype [13], thereby losing inhibitory factors like myelin associated glycoprotein (MAG). Furthermore, they start the production of neurotrophic factors and form axon guidance structures within the basal lamina proximal to the lesion site called the bands of Büngner. Finally they contribute to the phagocytosis of myelin debris. Oligodendrocytes do not dedifferentiate thereby still express MAG in addition to other inhibitory factors like Nogo [14]. 50% of them undergo apoptotic cell death after CNS injury and they do not proliferate, so no bands of Büngner are formed, which makes it rather impossible for axons to show directed regrowth. Additionally oligodendrocytes do not participate in the removal of debris.

Another important difference between PNS and CNS regeneration is the response of cells of the immune system, particularly macrophages and their CNS equivalent microglia, to injury. While the macrophage reaction after injuries to the PNS is effective and aided by the phagocytic action of Schwann cells, the microglia/macrophage action after trauma of the CNS is slower and less controlled. Especially the rapid removal of debris seems to be an important step in axonal regeneration response [15].

Due to secondary damage involving edema, hemorrhage, acidosis, free radicals and excitotoxicity as well as massive inflammation, reactive astroglia and scar formation, the final permanent lesion is much larger than the primary lesion.

The most severe complication in peripheral nerve regeneration is the formation of a neuroma. Following a complete transection of a nerve (neurotmesis), connective tissue may grow into the gap between distal and proximal part of the nerve, forming an impenetrable barrier for the regrowing axonal sprouts. This leads to aberrant axonal growth as well as uncontrolled proliferation of fibroblasts and Schwann cells [2].
<table>
<thead>
<tr>
<th></th>
<th>PNS</th>
<th>CNS</th>
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<tbody>
<tr>
<td>myelinating cells</td>
<td>Schwann cells</td>
<td>Oligodendrocytes</td>
</tr>
<tr>
<td>scar formation</td>
<td>only after neurotmesis</td>
<td>astroglial scar with cyst formation</td>
</tr>
<tr>
<td>axon guiding structures</td>
<td>bands of Büngner</td>
<td>absent</td>
</tr>
<tr>
<td>regenerative capacity</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>inhibitory molecules</td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td>debris removal</td>
<td>fast</td>
<td>slow</td>
</tr>
</tbody>
</table>

**Table 1: Main differences between PNS and CNS regarding axonal regeneration**

**Figure 3: Schematic Illustration of the degeneration and regeneration of a cut motor axon:**

1. perikaryon  
2. proximal segment of the axon  
3. Schwann cell  
4. basal lamina  
5. dividing Schwann cells  
6. myelin sheath  
7. myelin debris  
8. macrophage  
9. bands of Büngner  

**Taken from Mumenthaler, Stöhr and Müller-Vahl: Läsionen peripherer Nerven und radikuläre Syndrome [2]**
If the distal and proximal stumps of a transected nerve are sutured together in order to exclude scar formation, regeneration of the peripheral nerve occurs in a manner as depicted in figure 3:

a.) Acute phase (1-4 days after neurotmesis): the distal part of the axon undergoes Wallerian degeneration and resident Schwann cells start to proliferate. Proximal to the injury Wallerian degeneration only takes place up to the first internode. Initial chromatolysis of rough endoplasmic reticulum (rER) in the perikaryon occurs.

b.) Subacute phase (10-21 days): atrophy of the target muscle can be seen and the bands of Büngner are formed by proliferating Schwann cells. Removal of myelin and axonal debris is achieved by Schwann cells and recruited macrophages. The proximal stump of the axon already shows sprouting and chromatolysis of the rER reaches its maximum.

c.) Later stages of axonal regeneration: elongative growth of the regenerating axon along the bands of Büngner and atrophy of the muscle advances. The chromatolysis of the rER is regressing.

d.) Re-innervation of the target muscle by the fastest growing axon sprout: Alongside with reinnervation the atrophy of the muscle reverses and axon sprouts that have not reached their target are retrieving. The new axon is already myelinated by differentiated Schwann cells. [2]
Injury of the spinal cord results in rather different events as shown in Figure 4.

a.) In the acute early phase of spinal cord injury metabolic disturbances occur and a strong inflammatory response mediated by the complement cascade and infiltrating neutrophils takes place. Ischemia and edema occur as an effect of the disrupted blood brain barrier (BBB).

b.) The subacute phase is characterized by recruitment of monocytes, macrophages and T-lymphocytes which contribute to necrotic events and the development of the secondary injury. Free radicals lead to cell death and demyelination of surviving oligodendrocytes. Furthermore, extracellular glutamate triggers cell death by excitotoxicity. Invading meningeal fibroblasts and reactive astrocytes form a growth-inhibitory scar.

c.) In the chronic phase, Wallerian degeneration continues and the loss of tissue in combination with its clearance leads to formation of one or several cysts surrounded by astrocyte rich scar tissue.

Taken from Bareyre, F.M. and M.E. Schwab, Inflammation, degeneration and regeneration in the injured spinal cord: insights from DNA microarrays.[16]

In conclusion it can be summarized that the functional outcome after injury to the peripheral nervous system is more promising than after CNS injury due to its higher regenerative capacity.
1.2 Ventral Root Avulsion Injury

Dorsal and ventral roots connect the spinal cord with the peripheral nervous system as depicted in Figure 5. While dorsal roots carry afferent, sensory fibers, the ventral roots contain axons of the motoneurons projecting to their target muscles [17].

![Figure 5: Motor neurons residing in the ventral horn of the spinal cord project their axons (depicted in blue = efferent fibers) through the ventral roots into the spinal nerve and innervate their target muscle. Sensory (afferent) fibers are depicted in red.[1] Taken from Bear, Connors and Paradiso: Neuroscience-Exploring the Brain[1]](image)

The majority of root avulsion injuries are caused by traffic accidents followed by sport accidents [18] or complicated births [19], and affect the brachial plexus. The cervical roots (C4-C8) as well as part of thoracic root Th1 form the brachial plexus innervating the upper extremity. Up to 70% of all traumatic brachial plexus injuries include avulsion of one or several roots. Avulsion of roots close to the spinal cord results in substantial loss of the injured motoneurons if no treatment is applied. Again, young men represent the vast majority of affected patients.

Root avulsion is one of the most severe injuries to the peripheral nervous system leading to massive impairment of the quality of life of patients [20-22]. In the majority of cases the ventral as well as the dorsal roots are affected thereby motor and sensory dysfunction occurs, in addition to unbearable extreme pain [18]. The pain following spinal root avulsions (C6, C7 and C8) has been described by a patient of Frazier and Skillern, 1911: “The pain is continuous, it does not stop a minute either day or night. It is burning or compressing… In addition, there is, every few minutes, a jerking sensation similar to that obtained by touching… a Leyden jar. It is a zigzag made in the skies by a stroke of lightning. The upper
part of the arm is mostly free of pain; the lower part from a little above the elbow to the tips of
the fingers, never.” [23].
On one hand, spinal root lesions are comparable to lesions of the spinal cord itself since
regeneration after avulsion requires axon growth in central nervous tissue [24]. On the other
hand, the close proximity to a growth-supporting peripheral nerve stump, the unique
composition of the glial scar and unique properties of motoneurons concerning their ability to
survive and regenerate after root avulsion, distinguish this injuries from spinal cord lesions. It
should be noted here that there are only two types of neurons in the spinal cord which have
almost the full length of their axon outside of the spinal cord: preganglionic autonomic
neurons and motoneurons.

1.2.1 Motoneuron death in the ventral horn and the problem of
reinnervation of the ventral root
Somatic motoneurons (MNs) command voluntary muscle contraction. Their dendritic trees as
well as their cell bodies, which are among the largest in the CNS [25], and the most proximal
parts of their axons lie within the ventral horn of the spinal cord. The dendritic tree of an α-
motoneuron carries up to 100 000 synapses [26, 27], mediating inhibitory, excitatory and
cholinergic input. The greater part of the axon is found outside of the CNS in ventral roots
and peripheral nerves [25].
After root avulsion motoneurons show shrinkage of the cell body and dendrites, resulting in a
round instead of a usually multipolar shape of the cell body [28]. Motoneurons attempt to
survive and regenerate which is emphasized by upregulation of neurotrophins and receptors
for neurotrophins. Low-affinity neurotrophin receptor p75 [29], glial-derived neurotrophic
factor (GDNF) receptor (GFRα-1) [30], brain derived neurotrophic factor (BDNF) and its
high-affinity receptor trkB [31] are amongst them. Furthermore, synaptic covering is
decreased by up to 70% after ventral root avulsion [32, 33]. Preferentially excitatory synaptic
inputs are reduced [33, 34], indicated by the downregulation of N-methyl-D-aspartate
(NMDA) receptors which may be an attempt to attenuate excitotoxicity [35]. Finally,
upregulation of several Laminin-binding integrin receptors has been observed after injury
[36], indicating the importance of presence of Laminins for axon regrowth [25].
The intrinsic properties of the motoneurons and the long lasting defect of the blood spinal
cord barrier, which allows favorable blood-born molecules to enter the lesion site [24], result
in a remarkable regenerative capacity of motoneurons even after injuries very proximal to the
cell body [24], if proper strategies are applied to rescue them. The central scar tissue may be aiding regenerative events by a rapid elimination of inhibitory oligodendrocytes [24] and the presence of axon growth-supporting extracellular matrix molecules such as Laminin and various types of collagen [37, 38].

Motoneurons are able to survive and regenerate their axons after injury at some distance from the cell body but, despite the switch to a regenerative / surviving mode in gene expression of motoneurons [28], injuries very proximal to the perikaryon such as ventral root avulsion induce cell death of up to 80-90% of the motoneurons [39-44].

One major cause of this massive loss of motoneurons following root avulsion is thought to be an overexposure to the excitatory neurotransmitter glutamate and subsequent glutamate excitotoxicity [45]. Activation of Ca\(^{2+}\)-permeable glutamate receptors NMDA and \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) lead to the depolarization of the neuron and excessive influx of Ca\(^{2+}\) [46]. Depolarization of the neuronal membrane further induces influx of Ca\(^{2+}\) via voltage-gated Ca\(^{2+}\) channels and reverses action of the Na\(^+\)/Ca\(^{2+}\) exchanger [47]. Increased intracellular Ca\(^{2+}\) causes apoptotic and necrotic cell death through a variety of downstream mechanisms including production of nitric oxide, energy depletion, DNA damage and autophagy [48, 49].

Lucas and Newhouse described the toxic effects of glutamate for the first time 1957 [50]. Later on, excitotoxicity was linked to ischemic neuronal cell death [51, 52]. Due to the high levels of intracellular glutamate (10µmol/L), disruption of neurons by traumatic injury can lead to a massive elevation of extracellular glutamate. A concentration of 2-5µmol/L of extracellular glutamate already leads to excitotoxic injury [53].

A newer concept states that excitotoxicity is the underlying cause of neuronal cell death after ischemia, traumatic injury as well as in several neurodegenerative diseases like Huntington’s disease, Parkinson’s disease, Alzheimer’s disease and amyotrophic lateral sclerosis [45].

The inflammation following injury exacerbates the excitotoxic situation. Activated microglia release ATP and proinflammatory cytokines like tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) to recruit reactive astrocytes. Reactive astrocytes release excitatory glutamate into the extracellular space [54]. It should be noted that infiltrating T cells play a key role in modulating the neuroinflammatory reaction [33, 55]. Invading T cells and natural killer (NK) cells have a positive influence on the survival of injured motoneurons [56]. Neurotrophic factors produced by these cells may counteract the negative impact of, for example, interferon gamma [25].
Retraction of the ventral root represents another major problem after ventral root avulsion [42]. Neurotrophic and neurotopic support by denervated Schwann cells is not provided anymore and motoneurons are missing the essential cues to survive and regenerate their axons. Furthermore, the denervated distal stump is an important guidance structure for reinnervating motor axons [57]. The finding that regeneration of the axon and reinnervation of the target muscle strongly depends on an available guidance structure (and molecules which derive from such a structure) led to several therapeutic approaches.

In conclusion it can be stated, that although motoneurons attempt to survive and regenerate after root avulsion, a progressive loss of motoneurons due to glutamatergic excitotoxicity [58] and deprivation of neurotrophic factors (produced by Schwann cells in the ventral root) can be observed. The maximal rate of loss takes place between the second and the third week after injury [28]. This massive loss of motoneurons accounts for the poor functional outcome after ventral root avulsions.

1.3 Therapeutic approaches for root avulsion injuries

1.3.1 Surgery

The conventional approaches to treat ventral root avulsions are mostly palliative methods, like nerve transfer [59] or implantation of a peripheral nerve graft [60, 61]. In order to reestablish the function of muscles, nerve transfer can be examined. By intentionally dividing an intact nerve and transferring it to a more important irreparable injured nerve, the surgeon may achieve basic functionality of the paralyzed muscle [59, 62]. However in case of multiple root avulsions, not enough donor nerves are available in order to repair all injured nerves. These techniques are not curative but only palliative therefore alternative strategies are desirable.

More recent studies have shown that direct reimplantation of the avulsed root may promote survival and regeneration of spinal motoneurons and is not causing further injury to the spinal cord in the process [24, 39, 42, 63]. Reimplantation may be performed after multiple root avulsions and results in a functional outcome comparable to nerve transfer [64]. It has been shown that motor axons preferentially grow into the Schwann cell sheaths of motor nerves rather than endoneurial tubes of sensory nerves [13, 65, 66]. Reimplantation of the avulsed root(s) has the advantage of exposing the regenerating motor axons to their former environment. Neurotrophic factors produced by the reimplanted root may be available to
injured motoneurons in the ventral horn, but only for a limited period of time [67]. This is consistent with the finding that surgical reimplantation alone is not sufficient to significantly improve motoneuron survival and locomotor function three month after surgery [67]. Contradicting these data, it should be noted that Carlstedt et al showed functional recovery in 3 of 10 patients that underwent reimplantation surgery [68].

Although injured motoneurons do not die immediately after injury and some are able to survive after reimplantation surgery [69-71], not all surviving motoneurons manage to regenerate their axons into the avulsed ventral root [72]. The extent of damage, the site of implantation [73] and the timepoint seem to be crucial for the positive outcome of the surgery [74]. It can be stated that an early timepoint for surgery is always recommended for two reasons, firstly, motoneurons are only able to survive for a limited period of time and secondly, reinnervation of a long-time denervated atrophic muscle rarely results in a functional outcome [2].

1.3.2 Enhancement of survival of injured motoneurons by drugs

In order to reduce excitotoxic cell death of motoneurons following root avulsion many different targets in the chain of events of excitotoxicity were investigated. Efforts in modulating excitotoxicity include [45]:
- blocking the NMDA receptor,
- providing free radical scavengers and antioxidants,
- nitric oxide synthase inhibitors,
- glutamate release blockers.

The latter being the only one with clinical relevant results [75, 76].

Riluzole (2-amino-6-trifluoromethoxbenzothiazole), a drug that inhibits presynaptic glutamate release [77], blocks voltage-activated Na\(^+\) and Ca\(^{2+}\) channels [78] and enhances neuroprotective intracellular heat shock proteins (HSPs) [76, 79]. The main effects of riluzole on glutamatergic transmission and motoneuron excitability are depicted in figure 6.
Figure 6: Schematic diagram showing the main effects of riluzole.

(A) In low concentrations, enhancement of glutamate uptake by presynaptic neurons and astrocytes and

(B) Reduction of glutamate release. Riluzole limits thereby the absolute level of extracellular glutamate.

(C) Riluzole inhibits persistent Na$^+$ currents, therefore limiting the excitability of neurons.[76]

(Taken from Cifra et al 2012: Riluzole: What it does to spinal and brainstem neurons and how it does it)

Since surgery may not be feasible directly after injury, due to problems in diagnosis and severe accompanying injuries, most of the time delayed treatment is performed. Administration of riluzole may result in a sustained survival of motoneurons in the time period between injury and surgery, therefore allowing more motoneurons to regenerate their axons into a reimplanted ventral root.
1.3.3 Stem cell therapy

Besides surgery and drug administration, cellular replacement/ neurotrophic support after nerve injury has been contemplated. Stem cells provide the capability of self-renewal and differentiation into multiple lineages. These attributes make them an ideal candidate for regenerative medicine in order to repair damaged tissue/organs.

Early attempts using embryonic spinal cord grafts [80, 81] and embryonic motoneurons [82] have been effective in increasing survival and axonal outgrowth in experimental ventral root avulsion models. Due to safety and ethical reasons, the search for alternatives to embryonic stem cells has become a focus of investigation in the last decade.

Stem cell populations are present in adult tissue besides bone marrow and epithelia. Multipotent adult stem cells are also present in neural tissue and adipose tissue and are capable of trans-differentiation into cells of other lineages [83]. Adult stem cells may offer cellular replacement and trophic support for injured/dying neurons. Endogenous neuronal progenitor cells have been shown to undergo proliferation and migration to the ventral horn followed by neuronal differentiation in an amyotrophic lateral sclerosis model, a severe disease which is characterized by progressive loss of motor neurons [84, 85].

Adult mesenchymal stem cells (MSCs) derived from either bone marrow (BMSCs) or adipose tissue (ASCs) show significant potential in models of nerve injury also. Transplantation of undifferentiated mesenchymal stem cells into nerve conduits improved axonal regeneration and motor function in nerve gap models [86-89]. Schwann cell-like cells, differentiated from bone marrow stromal cells or adipose derived stem cells, show functional, morphological and molecular characteristics of genuine Schwann cells [90-92]. Local injection of BDNF producing BMSCs into the rat spinal cord increased the survival and synaptic stability after ventral root avulsion [93].

Gimble et al proposed a list of criteria for stem cells in order to be feasible for regenerative medicine:

1. abundant quantities (millions to billions of cells) present in donor tissue
2. minimally invasive harvesting procedure possible
3. differentiation potential into multiple cell lineage pathways in a regulatable and reproducible manner
4. safe and effective transplantation to autologous / allogeneic host
5. manufacturing in accordance with current good manufacturing practice guidelines possible [94, 95]

Adipose-tissue derived stem cells (ASCs) may fulfill these criteria for an “ideal” stem cell.
1.3.3.1 Adipose-tissue Derived Stem / Stromal Cells (ASCs)

Observation of several pathological conditions, such as obesity and progressive osseous heteroplasia, brought up the idea of the existence of multipotent stem cells in the adipose tissue [96-98]. There is an annual turn-over of 10% of all mature adipocytes, which is facilitated by ASCs [99]. ASCs are an easily, minimally invasively accessible, abundant source of stem cells [100], which makes them an ideal candidate for cell therapies tailored for the treatment of nerve injuries [101]. Adipose-derived stem cells are characterized by a fibroblast-like morphology, rapid plastic adherence, cell proliferation over long culture periods and multilineage differentiation potential as well as several surface antigens similar to bone marrow derived mesenchymal stem cells [95-97, 102].

There is evidence, that adipose-tissue derived stem cells have an immunomodulatory capacity in vitro as well as in vivo, respectively [103-109]. The underlying mechanisms of this modulatory function are not fully understood but may imply direct cell contact [110] as well as cytokines and chemokines, released by ASCs respectively [106, 108]. A study by Cui et al. [106] indicates that soluble factors and cytokines released by ASCs are responsible for their immunosuppressive effect, especially prostaglandin E2 (PGE2).

Furthermore it has been shown that ASCs are able to survive for up to 12 weeks after transplantation into for example an injured peripheral nerve [101], therefore they may influence the inflammation reaction and regeneration not only in the acute phase but also at later stages after injury.
Several groups investigated the secretome of ASCs.

<table>
<thead>
<tr>
<th>Secreted Proteins</th>
<th>Source</th>
<th>Passage</th>
<th>Samples</th>
<th>Identification Technique</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL12 adiponectin, angiotensin, cathepsin D, pentraxin, leptin</td>
<td>Human liposuction aspirates</td>
<td>P1</td>
<td>collected in serum free medium</td>
<td>2D Page, tandem mass spectrometry, Western blot</td>
<td>Zvonic et al. [111]</td>
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<tr>
<td>HGF, GM-CSF, ILs 6,7,8,11, TNF-α</td>
<td>Human liposuction aspirates</td>
<td>P2</td>
<td>stimulated with EGF, FGF, ascorbic acid or LPS</td>
<td>ELISA, RT-PCR</td>
<td>Kilroy et al. [112]</td>
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<tr>
<td>IGF-1, HGF, PGE 2</td>
<td>Human liposuction aspirates</td>
<td>P2 &amp; P5</td>
<td>supernatant of 5day cocultures between PBMCs and ASCs</td>
<td>ELISA</td>
<td>Cui et al. [106]</td>
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<tr>
<td>VEGF, HGF, IGF-1</td>
<td>Human</td>
<td>P1</td>
<td>supernatant of 24h incubation with TNF-α</td>
<td>ELISA</td>
<td>Wang et al. [107]</td>
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<tr>
<td>IL-6, VEGF, TNF-α</td>
<td>Rat inguinal fat pad</td>
<td>P2</td>
<td>supernatant of 3d and 6d cultured ASCs</td>
<td>ELISA</td>
<td>Prichard et al. [108]</td>
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<tr>
<td>BDNF, IGF-1</td>
<td>Rat subcutaneous adipose tissue</td>
<td>P0</td>
<td>conditioned medium 24h hours after confluency is reached</td>
<td>ELISA</td>
<td>Wei et al. [113]</td>
</tr>
<tr>
<td>BDNF, NGF, GDNF</td>
<td>Rat fat pad</td>
<td>P2</td>
<td>conditioned medium of ASCs after neuronal differentiation</td>
<td>qRT-PCR</td>
<td>McCoy et al. [114]</td>
</tr>
<tr>
<td>VEGF</td>
<td>Mice inguinal fat pad</td>
<td>P0</td>
<td>tissue samples</td>
<td>immunocytochemistry</td>
<td>Sumi et al. [115]</td>
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<tr>
<td>HGF, VEGF, PGF (placental growth factor), TGF-β</td>
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<td>Not referenced</td>
<td>conditioned medium and cell extracts</td>
<td>qRT-PCR, ELISA</td>
<td>Nakagami et al. [116]</td>
</tr>
<tr>
<td>VEGF, HGF, TGF-β, bFGF, GM-CSF</td>
<td>Human subcutaneous adipose tissue</td>
<td>P0-P2</td>
<td>CM of ASCs cultured under hypoxic conditions for 72 hours</td>
<td>qRT-PCR, ELISA</td>
<td>Rheman et al. [117]</td>
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<tr>
<td>IGF-1, VEGF</td>
<td>Human subcutaneous tissue</td>
<td>Not referenced</td>
<td>conditioned medium</td>
<td>ELISA</td>
<td>Sadat et al. [118]</td>
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(adapted from Salgado et al.: Adipose tissue derived stem cells Secretome: soluble factors and their roles in regenerative medicine) [97]
Besides adipogenic differentiation ASCs display a multilineage differentiation potential in vitro:

Figure 7: The multilineage differentiation potential of ASCs is depicted above.
(Endothelial [119, 120], neuronal-like [98, 121-123], epithelial [124, 125], osteogenic [123, 126, 127], myogenic[128], cardiomyogenic [129, 130], chondrogenic [131, 132])

Neuronal-like differentiation is not required in order to receive a positive effect on nerve regeneration. Neurotrophic factors and surface molecules like different Laminins as well as the immunomodulatory capacities of ASCs are thought to be responsible for this neuroprotective and regeneration-promoting effect in vitro and in vivo. Injection of conditioned media or direct application of undifferentiated ASCs has shown to improve neuronal survival and regeneration in several injury/disease models (Table 3).
<table>
<thead>
<tr>
<th>Study</th>
<th>References</th>
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<tr>
<td>Neurotrophic features of human adipose tissue-derived stromal cells:</td>
<td>Lattanzi et al. [133]</td>
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<tr>
<td><em>in vitro and in vivo</em> studies</td>
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<tr>
<td>Adipose-derived stem cells stimulate regeneration of peripheral</td>
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<td>nerves: BDNF secreted by these cells promotes nerve healing and</td>
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<td>axon growth <em>de novo</em></td>
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<td>Neurotrophic activity of human adipose stem cells isolated from deep</td>
<td>Kalbermatten et al. [135]</td>
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<td>and superficial layers of abdominal fat</td>
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<td>Improvement of neurological deficits by intracerebral transplantation</td>
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<td>of human adipose tissue-derived stromal cells after cerebral ischemia</td>
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<td>in rats</td>
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<td>Autologous transplants of adipose-derived adult stromal (ADAS)</td>
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<td>afford dopaminergic neuroprotection in a model of Parkinson’s disease</td>
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<td>Novel autologous cell therapy in ischemic limb disease through</td>
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<td>growth factor secretion by cultured adipose tissue-derived stromal</td>
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<td>cells</td>
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<td>Delivery of adipose-derived precursor cells for peripheral nerve</td>
<td>Santiago et al. [101]</td>
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<tr>
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<td>Adipose tissue-derived stromal cells-basic and clinical implications</td>
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<td>for novel cell-based therapies</td>
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<td>The conditioned media of adipose stromal cells protect against</td>
<td>Wei et al. [113]</td>
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<td>hypoxia-ischemia-induced brain damage in neonatal rats</td>
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<tr>
<td>Adipose-derived stem cells induce vascular tube formation of</td>
<td>Holnthoner et al. [138]</td>
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<tr>
<td>outgrowth endothelial cells in a fibrin matrix.</td>
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*Table 3: Excerpt of the work on undifferentiated adipose tissue-derived stem cells in regenerative medicine of the last decade.*

Although these studies are very promising, it should be noted that there are some issues regarding the application of adipose tissue-derived stem cells. The *in vitro* doubling time of ASCs is 2–4 days [139], requiring multiple passages in order to obtain sufficient cell numbers for therapy, and with prolonged passage for up to 4 month and longer ASCs may undergo malignant transformation [140]. Karyotype analysis as well as tumorigenesis assays should be performed on a routine basis if expanded ASCs ought to be
used in humans. Additionally, also a serum protein free culture medium needs to be used in order to achieve GMP standards [96].

1.3.4 Neurotrophic factors

The survival of neurons is a prerequisite for regeneration and functional recovery after nerve injury. An array of factors could facilitate these events, amongst them neurotrophins, neurokines and members of the GDNF family. These factors are known as neurotrophic factors and play crucial roles in not only survival and regeneration of neurons but also in the development and maintenance of the nervous system [141, 142].

According to Boyd and Gordon [143], neurotrophic factors can be divided into three classes:

1. Neurotrophins
   - Nerve growth factor (NGF)
   - Neurotrophin-3 (NT-3)
   - Neurotrophin-4/5 (NT-4/5)
   - Brain derived neurotrophic factor (BDNF)

2. Neuropoetic cytokines or Neurokines
   - Ciliary neurotrophic factor (CNTF)
   - Leukemia inhibitory factor (LIF)
   - Interleukin-6 (IL-6)
   - Interleukin-11 (IL-11)
   - Oncostatin-M (OSM)
   - Cardiotrophin-1 (CT-1)
   - Novel neurotrophin-1/B-cell stimulating factor-3 (NNT-1/BSF-3)

3. Glial cell line derived neurotrophic family
   - Glial cell-line derived neurotrophic factor (GDNF)
   - Neurturin (NTN)
   - Persephin (PSP)
   - Artemin (ART)

Brain derived neurotrophic factor (BDNF) and Glial cell-line derived neurotrophic factor (GDNF) are the best studied neurotrophic factors besides NGF. The efficacy of BDNF and GDNF in promoting neuronal survival and regeneration has been proven abundantly. Therefore those factors have been used in this study and the next section will focus on the function and signaling pathways of these factors.
1.3.4.1 Brain derived neurotrophic factor (BDNF)

BDNF is part of the neurotrophin family of neurotrophic factors which mediate strong survival and differentiation stimuli on neurons in both, the CNS as well as in the PNS, respectively. Like other neurotrophins, BDNF is synthesized as a precursor (proBDNF) which is either secreted as a cleaved (mature) or uncleaved (pro) neurotrophin. Secreted proBDNF may undergo extracellular cleavage into mature BDNF, mediated by plasmin and matrix metalloproteinase 7 (MMP7). Lu et al. [144] state that uncleaved secreted proBDNF is already an active neurotrophin opposing the effect of mature BDNF by binding with high affinity to the p75NTR leading to apoptosis of its target cell [145, 146]. Mature BDNF is biologically active as a noncovalent homodimer mediating its function via two receptors: Tyrosine-related kinase B (TrkB) and a member of the tumor necrosis factor-α (TNF-α) receptor family, low affinity nerve growth factor receptor p75NTR. The signaling cascades following binding of neurotrophins (BDNF) to trk (trkB) and/or p75NTR, respectively are depicted in figure 8. Many studies have proven that BDNF is a strong survival factor for injured motoneurons and induces axonal regeneration and sprouting in vitro and in vivo [32, 82, 141, 147-151]. The coexpression of p75NTR and trkB is required for maximal survival responses of motoneurons to BDNF as shown by Wiese et al. [152]. The basal expression of p75NTR, trkB and its ligand BDNF is relatively low in intact motoneurons. Shortly after injury, motoneurons strongly upregulate BDNF, afterwards expression declines until baseline levels are reached at approx. 10 days post injury. The upregulation of p75NTR and trkB is prolonged up to over 30 days after injury [143].

These findings suggest an autocrine mechanism of BDNF at early time points after injury, followed by dependency of motoneurons on BDNF from the distal nerve stump at later stages in order to survive and regenerate their axons.

Exogenous therapeutic administration of BDNF after avulsion injury may therefore lead to enhanced motoneuron survival and increased regeneration.
Figure 8: Intracellular signaling pathways upon binding of BDNF to either trkB or p75 respectively [143, 153]. Following binding of BDNF, trkB receptors dimerize and subsequent auto- and crossphosphorylation provides docking sites for proteins like phospholipase C-γ (PLC), Src homologous and collagen like (shc) adaptor protein, resulting in activation of survival and differentiation pathways. p75 signaling may occur either 1) independently of trkB resulting in induction of apoptosis or 2) in cooperation with trkB to enhance or reduce neurotrophin-mediated trkB activation, respectively [154].
1.3.4.2 Glial cell-line derived neurotrophic factor (GDNF)

GDNF is a secretory protein widely expressed in the CNS and PNS. Alternative splicing produces two different mRNAs: pre-(α)pro-GDNF, which represents the full-length transcript, and pre-(β)pro-GDNF, a truncated form lacking 78 bp in the region encoding the pro-domain [155-158]. These isoforms differ in secretion and processing pathways. The 134 aa mature GDNF resulting after proteolytical cleavage of the pre-pro-GDNF isoforms is identical and is biologically active as homodimers similar to BDNF. Cleavage into mature protein is performed by the furin endoproteinase (paired basic amino acid cleaving enzyme, PACE 4) and proprotein convertases PC5A, PC5B and PC7 [158]. GDNF homodimers mediate their actions via a high affinity ligand-binding subunit (GDNF family receptor α1 [GFR-α1]) and a common signal transduction subunit c-ret (a transmembrane receptor tyrosin kinase similar to trk) as depicted in figure 9.

Mature GDNF is up to 100-fold more potent than BDNF in promoting motoneuronal survival [159], rendering it one of the most potent survival promoting factors known so far [160]. GDNF has been shown to promote survival of sympathetic, parasympathetic and spinal motor neurons in vitro [161], in normal development [157, 160, 162, 163] as well as regeneration of motor neurons after spinal cord injury in vivo [17, 164, 165]. Motoneurons upregulate GDNF receptors GFR-α1 and c-ret early after injury and they remain elevated for either at least 6 weeks (GFR-α1) or 3-5 weeks, respectively (c-ret) [143, 166, 167]. GDNF is most probably not acting in an autocrine manner on motoneurons, because, in contrast to BDNF, it is not expressed in injured or intact motoneurons [168]. Therefore it seems to be essential for regeneration that GDNF is provided by resident cells in the distal nerve stump after avulsion injury, which has direct implications for the validity of ex vivo therapeutic approaches.
Figure 9: Binding of GDNF to GFR-α1 results in c-ret translocation to lipid rafts as well as association of c-ret with pp60src. Dimerization and subsequent phosphorylation of tyrosine residues on the intracellular part of c-ret results in association of e.g. shc and PLC-γ to these docking sites. Similar signaling pathways as in BDNF signaling are activated including the ras/erk pathway, PI3K and Phospholipase C-γ. It should be noted that Saarma and Sariola [159] have shown that GFR-α1 can also signal independently of c-ret by activation of src-family kinases.
1.3.4.3 Direct administration of recombinant BDNF or GDNF

Reimplantation of an avulsed root alone may not be sufficient to produce a long time neuroprotective effect [39, 67]. However, several studies have demonstrated that additional direct administration of either recombinant BDNF or GDNF respectively, leads to increased survival and axonal outgrowth [67, 169]. Due to the injury-related breakdown of the blood spinal cord barrier topical application of these neurotrophic factors at the ventral root exit zone of the spinal cord leads to an immediate supply by uptake through remaining roots and retrograde transport or passive diffusion to injured motoneurons [167]. In addition, BDNF and GDNF have been shown not only to be neurotrophic but also neurotopic factors, meaning, axonal regeneration takes place in a chemotactic, directed manner towards the source of BDNF/GDNF.

In order to achieve a continious delivery of BDNF or GDNF mini osmotic pumps have been used. Kishino et al.[147] have shown that intrathecal administration of recombinant BDNF resulted in increased survival of injured motoneurons as well as in axonal outgrowth. Similar effects of intrathecal administration of GDNF have been shown by Bergerot et al. [67]. Although these results seem promising, intrathecal administration via mini osmotic pumps has several drawbacks. Those pumps are rather expensive, additional surgical procedures are necessary and functional failure as well as infection may occur.

1.3.5 Viral gene therapy with BDNF / GDNF

Long term supply with neurotrophic factors may be achieved by viral vector-mediated forced expression of neurotrophic factors as therapeutic transgenes. Blits et al.[17] performed direct in vivo gene transfer at the defect site. An adeno-associated viral vector (AAV), mediating BDNF or GDNF expression intraspinally was injected in the ventral horn in order to achieve long term gene expression. The results of this study were rather disillusioning; the long term high expression capacity of viral vectors resulted in constantly high levels of neurotrophic factor in the ventral horn. These constant high levels resulted in increased survival and sprouting of motoneurons, but it inhibited directional reinnervation of the avulsed ventral root and therefore led to worse results than in the no treatment group. Concluding it is save to say that expression kinetics obtained by in vivo viral gene delivery is not feasible in a root avulsion model.
1.3.6 Non viral gene therapy with BDNF / GDNF

Although non viral gene therapy is transient and results in lower transfection efficacy than viral gene therapy, the use of plasmids holds important advantages over viral gene therapy in general. First plasmid based gene therapy is considered less immunogenic, secondly the transient expression kinetics may be beneficial in regenerative medicine applications and thirdly plasmids do not tend to integrate into the genome, therefore genotoxicity-associated risks of aberrant cell growth or even malignant transformation of transfected cells is lower [170, 171].

To overcome the problems occurring with the use of osmotic pumps or viral transduction for the delivery of neurotrophic factors, Hell et al.[93] transplanted BDNF expressing mesenchymal stem cells (MSCs) into the lateral funiculus of the lesioned side of the spinal cord. A significant neuroprotective effect of this treatment could be shown. One major drawback in this study was the use of bone marrow derived MSCs which were cultivated in vitro for as long as 10 weeks (approx. 8 passages) in an un-differentiated state. This time consuming procedure makes this approach not only impracticable for therapeutic use but also enhances the chance of malignant transformation of MSCs.

1.4 Summary

Ventral root avulsion is a severe injury to the spinal cord often resulting in a life long impairment of motor and sensory functions of the affected limb accompanied by chronic pain. Therapeutic approaches are limited due to difficulties in diagnosis and the need of early therapy. Recovery is slow and inefficient with mostly unsatisfactory outcome. The massive loss of motoneurons following root avulsion injury is the major issue and is therefore of special interest in the search for new therapeutic approaches.
Aim of the study

Taking the requirements for new treatment strategies and the advantages of non-viral gene therapy into account the aims of this study were to provide a new approach for the treatment of ventral root avulsion injuries.

1. Design of therapeutic plasmids prototypes expressing reporter genes for evaluation

2. Evaluation of an *in vitro* transfection protocol suitable for rat adipose tissue-derived stem cells (rASCs)

3. Proof of temporally restricted expression of biologically active neurotrophic factors (BDNF + GDNF) by transfected rASCs *in vitro*

4. *Ex vivo* gene therapy using transfected rat adipose tissue-derived stem cells as expression systems for neurotrophic factors (BDNF / GDNF) *in vivo*

5. Evaluation of the survival of motoneurons as well as evaluation of elongative growth of ventral root motoneuronal axons after avulsion and reimplantation of the ventral root *in vivo*. 
2. Material and Methods

2.1 Materials

2.1.1 Primers

Primers used in this study were all single stranded DNA oligonucleotides ordered from Microsynth (Balgach, Switzerland) or Invitrogen (Carlsbad, CA, USA). Annealing temperatures, appearance of hairpins, self and cross dimerization as well as GC content were calculated in silico using Beacon Designer (Premier Biosoft International) software. Lyophilized primers were diluted with ddH$_2$O to a final stock concentration of 100pmol/µl. This stock was further diluted to get a working solution with a final concentration of 10pg/µl.

<table>
<thead>
<tr>
<th>Synthesis Primers</th>
<th>Sequence(5’→3’)</th>
<th>Tm(°C)</th>
<th>GC clamp</th>
<th>length(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCBRs</td>
<td>TCATGAGTACCGAGCTTACGCCTGCTAG</td>
<td>73</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>pCBRas +enh</td>
<td>TCATGAGCTGTGGAATGTGTGTCAGTTAGG</td>
<td>73</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>pCBRas -enh</td>
<td>TCATGATAAAAAACCTCCCACACCTCCCCTGAAC</td>
<td>75</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>dsRedBspH1s</td>
<td>TCATGACCTGATTCTGTGGATAACCGTTAT</td>
<td>69</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>dsRedBspH1as</td>
<td>TCATGAGGACAAACCAACACCTAGAATGCGAG</td>
<td>70</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>pTurboRFP s</td>
<td>TCATGAGGATTTTGAGACAACACACTAGAATGCGAG</td>
<td>70</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>pTurboRFPas</td>
<td>TCATGAGAGTTTGAGACAACACACTAGAATGCGAG</td>
<td>69</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>CBRinframe s</td>
<td>GGAATTCAGTAAAGCGTGAGAAAAATGTCATCTATGCCTCCC</td>
<td>74</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>CBRinframe as</td>
<td>CAGGATCCGATTACTAACCACCGCCGCCTTCACCAACAAATTGGTG</td>
<td>79</td>
<td>5</td>
<td>43</td>
</tr>
</tbody>
</table>

*Table 4: Name, sequence, melting Temperature (Tm (°C)), number of GC-clamps and length (in base pairs) of the primers used in this study.*
2.1.2 Plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Size</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDsRed-Express-C1</td>
<td>Clontech (CA, USA)</td>
<td>4.7kb</td>
<td>Discosoma sp. red fluorescent protein</td>
</tr>
<tr>
<td>pTurboFP635-C</td>
<td>Evrogen (Moscow, RUS)</td>
<td>4.7kb</td>
<td>far-red fluorescent protein TurboFP635</td>
</tr>
<tr>
<td>pCBR-Control</td>
<td>Promega (WI, USA)</td>
<td>5.2kb</td>
<td>click beetle red luciferase</td>
</tr>
<tr>
<td>pGFP$\text{max}$</td>
<td>life technologies (GER)</td>
<td></td>
<td>green fluorescent protein GFP</td>
</tr>
<tr>
<td>pVAX1</td>
<td>life technologies (GER)</td>
<td>3kb</td>
<td>eukaryotic expression system</td>
</tr>
<tr>
<td>pVAX1mGDNF</td>
<td>in house</td>
<td>3.7kb</td>
<td>Glial cell-line derived neurotrophic factor</td>
</tr>
<tr>
<td>pVAX1rBDNF</td>
<td>in house</td>
<td>4.1kb</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>pCR-Blunt</td>
<td>life technologies (GER)</td>
<td>3.5kb</td>
<td>blunt end cloning of Phusion polymerase PCR products</td>
</tr>
</tbody>
</table>

Table 5: Name, size and purpose of the plasmids used in this study

Figure 10: pDsRed-Express-C1 vector map. Discosoma sp. red fluorescent protein encoding mammalian expression vector with the C-terminal multiple cloning site (MCS) and subsequent SV40 polyadenylation signal is shown. SV40-Promoter, Kanamycin/Neomycin
resistance as well as a HSV thymidine kinase polyadenylation signal for selection in eukaryotes is depicted.

Figure 11: pCR-Blunt for cloning of blunt end PCR products. E. coli ccdB gene fused to C-terminus of LacZα expression cassette. Kanamycin/Zeocin resistance, bacterial pUC Origin of replication (pUC ori) and the lac promoter (P_{lac}) are shown.
Figure 12: pTurboFP635-C mammalian expression vector with the multiple cloning site (MCS) located between the TurboFP635 coding sequence and the SV40 polyadenylation signal (SV40 polyA). Prokaryotic pUC ori as well as eukaryotic SV40 ori origins of replication are depicted. Kanamycin and Neomycin resistance for selection of stably transfected prokaryotic and eukaryotic cells is shown above.

Figure 13: pCBR-Control Vector containing the synthetic cDNA sequence of the click beetle red luciferase enzyme under control of a SV40 Promoter with a SV40 polyA signal as well as a SV40 enhancer segment. The MCS is located between the synthetic poly(A) signal and the SV40 promoter. Additionally to allow selection of transformed E. coli an ampicillin resistance is present.
Figure 14: pVAX1 vector with high-copy number replication in E. coli due to pUC origin and a Kanamycin resistance for selection of stable transformants. Immediate-early Cytomegalovirus (CMV) promoter results in high-level transient expression of the protein of interest in most mammalian cells. The bovine growth hormone polyadenylation signal (BGH pA) leads to efficient transcription termination and polyadenylation of mRNA.

Figure 15: pVAX1mGDNF mouse glial cell line derived neurotrophic factor eukaryotic expression vector based on the pVAX1 vector. Kanamycin resistance, pUC ori (ColE1ori) and Cytomegalovirus promoter (pCMV) are shown.
Figure 16: pVAXrBDNF rat brain derived neurotrophic factor eukaryotic expression vector based on the pVAX1 vector. Again Kanamycin resistance pUC ori and CMV promoter are depicted.

Figure 17: pmaxGFP is a eukaryotic expression vector which encodes for green fluorescent protein maxGFP an improved variant from copepoda Potellina plumata. It contains an immediate early promoter of cytomegalovirus (PCMV) for protein expression, a simian virus40 (SV40) origin for replication in eukaryotic cells as well as a pUC origin for propagation in E. coli and Kanamycin resistance for selection of stably transfected cells.

2.1.3 Bacterial Strains

Escherichia coli Top 10 Invitrogen/ Life technologies (GER)
Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZ lΔM15 ΔlacX74 recA1 araD139
Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG

**Escherichia coli SURE 2 (Stop Unwanted Rearrangement Events)** Stratagene (CA, USA)
Genotype: e14-(McrA-) Δ(mcrCB-hsdSMR-mrr)171 endA1 gyrA96 thi-1 supE44 relA1 lac
recB recJ sbcC umuC::Tn5 (Kanr) uvrC
[F’ proAB lacIqZΔM15 Tn10 (Tetr)].
2.1.4 DNA size markers

Figure 18: Quantitas Fast marker (Fermentas, GER) (Biozym, GER)

Figure 19: FastRuler middle range ladder

Figure 20: Fast Ruler low range ladder (Fermentas, GER)

2.1.5 DNA polymerases

Hot-Taq Polymerase Peqlab Biotechnology (GER)

Phusion® Hot Start II High-Fidelity DNA-Polymerase Finnzymes (FIN)

2.1.6 Restriction enzymes

All restriction enzymes were Fast Digest Enzymes unless otherwise depicted. The restriction enzymes and their corresponding buffers used in this study were purchased from Fermentas (GER)
2.1.7 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Host</th>
<th>Conjugate</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-p75 AB</td>
<td>p75neurotrophin receptor</td>
<td>rabbit</td>
<td>-</td>
<td>Millipore (USA)</td>
</tr>
<tr>
<td>Anti-S100AB</td>
<td>S100 protein</td>
<td>rabbit</td>
<td>-</td>
<td>Dako (DK)</td>
</tr>
<tr>
<td>Anti-Nestin AB</td>
<td>Nestin intermediate filament</td>
<td>rabbit</td>
<td>-</td>
<td>Abcam (UK)</td>
</tr>
<tr>
<td>Anti-rabbit secondary AB</td>
<td>rabbit IgG</td>
<td>goat</td>
<td>AlexaFluor594</td>
<td>Invitrogen (GER)</td>
</tr>
<tr>
<td>Anti βIII-tubulin AB</td>
<td>βIII-tubulin</td>
<td>rabbit</td>
<td>AlexaFluor488</td>
<td>Millipore</td>
</tr>
</tbody>
</table>

Table 6: Antibodies and their properties and Manufacturers

2.1.8 Recombinant growth factors

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>murine nerve growth factor (NGF)</td>
<td>Promega (WI, USA)</td>
<td>dorsal root ganglia cell culture</td>
</tr>
<tr>
<td>human brain derived neurotrophic factor (BDNF)</td>
<td>Peprotech (NJ,USA)</td>
<td>biological activity assay</td>
</tr>
<tr>
<td>rat glial-cell-line derived neurotrophic factor (GDNF)</td>
<td>Peprotech (NJ,USA)</td>
<td>biological activity assay</td>
</tr>
</tbody>
</table>

Table 7: recombinant growth factors used in this study

2.1.9 Cell culture material

Dulbecco´s modified eagle medium (DMEM) high glucose (4500mg/l)  Sigma Aldrich (UK)
L-glutamine
Penicillin/Streptavidin
Trypsin/EDTA
Accutase  Thermo Electron Corp. (CA, USA)
Fetal Calf Serum (FCS)
10x Phosphate buffered saline w/o Ca++  PAA Laboratories GmbH (AUT)
Cell culture dishes:
   24 well plates  Corning (MA, USA)
   12 well plates  Corning (MA, USA)
   T-175 flasks  Greiner (AUT)
   T-150 flasks  Iwaki (JP)
T-75 flasks Greiner/Iwaki

Coating reagents:
Poly-L-Lysine Sigma Aldrich (UK)
Rat tail Collagen BT-274 Biomedical Technologies, Inc. (USA)
Laminin Sigma (AUT)

2.1.10 Cell lines and primary cells

For all cells used following parameters applied:
Incubation: 37°C, 5%CO₂ humidified environment (ULTIMA incubator, Revco, USA)
Storage: 1.8ml Cryo- aliquots with DMEM (5%FCS) and 10% dimethylsulfoxide (DMSO), liquid N₂

C2C12 mouse skeletal myoblast precursor cell line
Description: mouse C3H muscle myoblast, purchased from the German Collection of Microorganisms and Cell Cultures DSMZ#ACC565 (Braunschweig, GER)
Culture medium:
DMEM high glucose
2mM L-glutamine
5% fetal calf serum (FCS)
Passaging: twice a week

NIH3T3 mouse embryonic fibroblast cell line
Purchased from the German Collection of Microorganisms and Cell Cultures DSMZ#ACC59 (Braunschweig, GER)
Culture medium:
DMEM high glucose
1% L-glutamine
10% FCS
Passaging: once a week

PC12 rat pheochromocytoma cell line
Description: derived from a transplantable rat pheochromocytoma, respond reversibly to NGF by induction of a neuronal phenotype [172].
Purchased from
Culture medium:
DMEM high glucose
15% FCS
1% Penicillin/ Streptomycin
Plating: collagen or laminin-coated plates
Passaging: cells can be split 1:5 without trypsin by thorough washing of lifting with a cell scraper. When splitting approximately 20% of old, conditioned medium was kept.

**Primary rat adipose derived stem cells (rASCs)**
Collected from epididymal fat of Sprague Dawley rats as described in section 2.3.1
Culture medium:
- DMEM high glucose
- 1% Penicillin/Streptomycin
- 1% Glutamine
- 10% FCS
Passaging: once a week with Accutase or trypsin (1:5 diluted in sterile 1x PBS)

**Primary rat dorsal root ganglia (DRGs)**
Collected from Sprague Dawley rats as described in section 2.3.2
Culture medium:
- DMEM high glucose
- 1% Penicillin/Streptomycin
- 10% FCS

### 2.1.11 Microscopes

**Standard phase-contrast and fluorescence microscopy**

<table>
<thead>
<tr>
<th>Microscope</th>
<th>Camera</th>
<th>Software</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observer.A1</td>
<td>AxioCam ICm1</td>
<td>AxioVision Rel.4.8.2</td>
</tr>
<tr>
<td>Zeiss (GER)</td>
<td>Zeiss (GER)</td>
<td>Zeiss (GER)</td>
</tr>
</tbody>
</table>

**Confocal laser scanning microscopy (CLSM)**

<table>
<thead>
<tr>
<th>Microscope</th>
<th>Camera</th>
<th>Software</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axiovert 200M</td>
<td>ProgRes C10plus</td>
<td>LSM 510 META Version 3.2 SP2</td>
</tr>
<tr>
<td>Zeiss (GER)</td>
<td>Jenoptik (GER)</td>
<td>Zeiss (GER)</td>
</tr>
</tbody>
</table>
2.1.12 *in silico* sequence analysis

In *in silico* design of primers, plasmids and restriction simulation was carried out using following freeware bioinformatics tools or websites:

- ApE (A plasmid Editor) [Http://biologylabs.utah.edu/jorgensen/wayne/ape/](http://biologylabs.utah.edu/jorgensen/wayne/ape/)

### 2.2 Molecular Biology Methods

#### 2.2.1 Polymerase chain reaction (PCR)

All PCR-reactions were carried out in a TGradient or T300 thermocycler (Biorad, Hercules CA, USA)

#### 2.2.1.1 Standard *Thermus aquaticus* (Taq)-polymerase PCR

Enzyme activation by denaturing of Hot-Taq blocking antibody and DNA denaturation was achieved by heating to 95°C for 2min. 5-10 ng plasmid DNA/20µl reaction volume was used. Elongation time was chosen assuming an approximate synthesis rate of 1000 base pairs per minute.

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>time</th>
<th>20µl Hot-Taq PCR reaction mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>2 min</td>
<td>10x Buffer with MgCl₂ (25mM) 2µl</td>
</tr>
<tr>
<td>95</td>
<td>30 sec</td>
<td>10mM dNTPs 0,5µl</td>
</tr>
<tr>
<td>Ta</td>
<td>30sec</td>
<td>sense Primer 1µl</td>
</tr>
<tr>
<td>72</td>
<td>60sec/1000bp</td>
<td>antisense Primer 1µl</td>
</tr>
<tr>
<td>72</td>
<td>10min</td>
<td>Hot-Taq Polymerase (1unit) 0,2µl</td>
</tr>
<tr>
<td>13</td>
<td>pause</td>
<td>DNA template (5-10ng) 1µl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ddH₂O 14.3µl</td>
</tr>
</tbody>
</table>

**Table 8: Cycling parameters and reaction master mix of standard Taq-polymerase PCR**

```
2.2.1.2 Hot start high fidelity polymerase PCR

Correct synthesis of long PCR products requires a polymerase with proofreading capability. According to the manufacturer the error rate of Phusion® Hot start II high fidelity polymerase is approximately 50-fold lower than that of Thermus aquaticus DNA polymerase. The high fidelity polymerase is reversibly bound to an inhibitory specific Affibody protein, which inhibits activity of the DNA polymerase at normal temperatures. In addition this Affibody ligand inhibits the 3'\rightarrow5' exonuclease activity of the polymerase, thereby preventing degradation of primers and/or template DNA during the reaction setup. At working temperatures, the Affibody molecule is released leading to a fully active polymerase.

If primers are >20nt and Tm>69°C a 2-step protocol is recommended → annealing = extension = 72°C (according to manufacturer)

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>time</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>30 sec</td>
</tr>
<tr>
<td>98</td>
<td>5-10 sec</td>
</tr>
<tr>
<td>72</td>
<td>15-30 sec/kb</td>
</tr>
<tr>
<td>72</td>
<td>10min</td>
</tr>
<tr>
<td>4</td>
<td>pause</td>
</tr>
</tbody>
</table>

Table 9: Cycling parameters and reaction master mix of Phusion® Hot Start II high fidelity polymerase PCR

<table>
<thead>
<tr>
<th>20µl Phusion polymerase PCR mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Phusion Buffer</td>
</tr>
<tr>
<td>10mM dNTPs</td>
</tr>
<tr>
<td>sense Primer</td>
</tr>
<tr>
<td>antisense Primer</td>
</tr>
<tr>
<td>Phusion® Polymerase</td>
</tr>
<tr>
<td>DNA template(5-10ng)</td>
</tr>
<tr>
<td>ddH2O</td>
</tr>
</tbody>
</table>
2.2.2 Agarose gel electrophoresis

DNA fragments obtained from PCR and/or restriction digests were separated by size on 1% LE Agarose gels (Biozym, GER). Gels were prepared in 1x sodium borate (SB) buffer. The agarose-gel/SB-buffer solution was heated in a microwave and cooled down to approximately 50°C. A DNA intercalating agent (Ethidium bromide [EtBr], GelStar® or GelRed®) for detection was added to a final concentration of 0,3µg/ml. The gels were poured and cooled down for polymerization. DNA samples were mixed with 1/6 volume of gel loading buffer (xylene cyanol FF (XCFF) or bromphenol blue (BPB)) prior to loading. Gels were run with a field strength of 180-250 V in PerfectBlue™ mini L (Peqlab, GER) or PerfectBlue™ mini S horizontal electrophoresis system. Bands were visualized by UV illumination and photographed with a charge-coupled device camera in a gel electrophoresis documentation device (Alpha, Innotech, USA). Buffer and loading dye compositions are depicted in Table 10, 11 and 12.

<table>
<thead>
<tr>
<th>20x SB buffer</th>
<th>pH8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid</td>
<td>45g</td>
</tr>
<tr>
<td>NaOH</td>
<td>8g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>to 1000ml</td>
</tr>
</tbody>
</table>

*Table 10: composition of 20x SB buffer*

<table>
<thead>
<tr>
<th>6x BPB Gel loading buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>bromphenol blue</td>
</tr>
<tr>
<td>glycerol</td>
</tr>
<tr>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

*Table 11: composition of 6x bromphenol blue loading buffer*

<table>
<thead>
<tr>
<th>6x XCFF Gel loading buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylene cyanole FF</td>
</tr>
<tr>
<td>glycerol</td>
</tr>
<tr>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

*Table 12: composition of 6x XCFF loading buffer*
10mg/ml in ddH2O 10000x in DMSO 10000x in DMSO
Sigma-Aldrich, Missouri, USA Cambrex, East Rutherford, NJ, USA Biozym, Oldendorf, Germany

Table 13: DNA intercalating agents in agarose gels used in this study

2.2.3 DNA extraction from agarose gels

The DNA bands of interest were excised from the agarose gel under UV illumination using a scalpel. The gel piece was transferred to a fresh, pre-weighed 1.5ml Eppendorf tube and purified using the Promega Wizard SV Gel and PCR Clean-Up Kit (Madison, USA). According to the manufacturer’s instructions, 10µl of membrane binding solution was used / 10mg of gel piece. DNA extraction was continued following the manufacturer’s instructions. Purified samples were loaded on a 1% agarose gel as control of integrity of DNA.

2.2.4 Restriction digests of DNA

All restriction digests were performed using Fermentas Fast Digest restriction enzymes. A DNA amount of 1µg was used for restriction digests. Generally incubation was performed for approx. 1 hour at 37°C. In the case of linearization of the plasmid with only one restriction enzyme dephosphorylation of the protruding 5´ ends was necessary using calf intestinal alkaline phosphatase (CIAP, Promega, USA). Dephosphorylation is important to prevent re-ligation of the empty vector during transformation, which would be the favored reaction since it is intramolecular ligation compared to intermolecular ligation.

<table>
<thead>
<tr>
<th>DNA restriction</th>
<th>0.5-1µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>restriction enzyme</td>
<td>0.5-1µl</td>
</tr>
<tr>
<td>DNA</td>
<td>1µg</td>
</tr>
<tr>
<td>10x restriction buffer</td>
<td>2µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>up to 20µl</td>
</tr>
</tbody>
</table>

Table 14: general mixture for DNA restriction
2.2.5 DNA ligation

DNA fragments were ligated with a T4 ligase into pre-cut plasmids (pCR-Blunt, pVAX1 and pTurboFP635-C) in 10µl. The molar ratio between insert and target vector was estimated from the band intensity on an agarose gel and was 1:3 in case of ligation of inserts into pVAX1 and pTurboFP635-C backbones and 1:10 in case of pCR-Blunt backbones. All ligations were incubated for 1 hr at 16°C (optimal working temperature for ligase) by a subsequent incubation at 4°C (minimal thermic movement) overnight for improved results.

<table>
<thead>
<tr>
<th>Ligation reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x ligation buffer</td>
</tr>
<tr>
<td>T4 ligase (1 unit)</td>
</tr>
<tr>
<td>insert</td>
</tr>
<tr>
<td>target vector</td>
</tr>
<tr>
<td>ddH2O</td>
</tr>
</tbody>
</table>

Table 16: T4 ligase reaction as performed in this study

2.2.6 Culture of *E.coli*

*E.coli* TOP 10 were cultured at 37°C either in liquid Luria Bertani Broth medium or on Luria Bertani Broth –agar plates O/N (LB, LB-agar, Table 14). Antibiotics were added according to the resistance marker of the transformed plasmid (in general Kanamycin except for pCBR-Control→ Ampicillin). Working concentrations of antibiotics were 30µg/ml in case of Kanamycin and 80µg/ml for Ampicillin. Prior to inoculation or plating of *E.coli* an
appropriate amount of antibiotics stock solution was added to either the LB-medium or the heated LB-agar (hand warm).

<table>
<thead>
<tr>
<th>LB medium liquid</th>
<th>LB-agar for plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>up to 1000ml</td>
</tr>
<tr>
<td></td>
<td>Tryptone</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>Agar-Agar</td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

*Table 17: Bacterial cell culture media*

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Shortcut</th>
<th>working concentration</th>
<th>properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Amp</td>
<td>80µg/ml</td>
<td>killing of dividing cells</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Kan</td>
<td>30µg/ml</td>
<td>bacteriocidal</td>
</tr>
</tbody>
</table>

*Table 18: Antibiotics, their properties and concentrations used in this study*

### 2.2.7 Preparation of chemically competent *E.coli* TOP 10

100ml of Luria Bertani Broth medium were inoculated with 5ml of an O/N culture of *E.coli* TOP 10 and grown at 37°C and permanent shaking to an optical density (OD) of approximately OD₆₀₀= 0.4. Prior to centrifugation at 4000rpm for 10min at 4°C the cells were transferred to a polypropylene (PP) falcon and stored on ice for 10 min. After centrifugation the supernatant was decanted and the pellet was resuspended in 30ml of an ice-cold MgCl₂ (80mM)-CaCl₂ (20mM) solution. A further centrifugation step at 4000rpm, 4°C for 10 min was applied after which the pellet was resuspended in 2ml of an ice-cold CaCl₂ (100mM) glycerol (10%) ddH₂O solution. The chemically competent TOP 10 cells were frozen in liquid N₂ and stored as 50 or 100µl aliquots at -80°C. After thawing on ice, the prepared competent *E.coli* TOP 10 cells were ready for transformation with DNA.

### 2.2.8 Transformation of chemically competent *E.coli*

5µl of ligation reaction or 0.5-1µg of plasmid DNA were added to 20µl of competent *E.coli* (thawed on ice) and incubated on ice for 30 min. A heat-shock at 42°C for 90sec, followed by another incubation of ice for 2min was applied before adding 80µl of SOC (sodium optimal Broth + glucose) and incubation for 1 hr at 37°C. Transformed cells were plated on LB-agar plates containing the appropriate antibiotic.
### SOC-medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>2%</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5%</td>
</tr>
<tr>
<td>NaCl</td>
<td>10mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.5mM</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>10mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>10mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>20mM</td>
</tr>
</tbody>
</table>

*Table 19: Ingredients for SOC-medium*

#### 2.2.10 Mini- and Maxi-preparation of plasmid DNA from *E.coli*

The Promega Wizard® SV miniprep kit (Promega, USA) was used for minipreparation of plasmid DNA following the manufacturer’s instructions. The elution step was performed using 30µl of nuclease free ddH₂O. The average yield was ~500µg/ml at an A₂₆₀/₂₈₀ of 1.7-2.0.

The Endo-free maxiprep kit from Quiagen (Hilden, GER) was used for endotoxin-free maxipreparation of plasmid DNA for further use in cell culture. Maxipreparation was carried out following the manufacturer’s instructions. Endotoxin-free plasmid DNA was dissolved in 500µl ddH₂O and stored at -20°C. The average yield was 1.5µg/µl at an A₂₆₀/₂₈₀ of ~1.9.

#### 2.2.11 Measuring of DNA concentration

A SmartSpec™ 3000 spectrophotometer (Biorad, USA) was used for determination of DNA and RNA concentration. 50µg/ml DNA or 40µg/ml RNA were set as equivalent of an A₂₆₀=1.0. Measurements were performed with 1:50 dilutions of the plasmid in ddH₂O. An A₂₆₀/₂₈₀ of 1.7-2.0 was accepted for nucleic acids indicating minimal protein contamination.

#### 2.2.12 Sequencing of plasmids

Sequencing of the designed plasmids was carried out at Microsynth sequencing service (Microsynth, SUI). 0.8µg of plasmid were diluted in 10µl ddH₂O with a final concentration of sequencing primers of 20pmol.
2.2.13 Design of a CBR-TurboRFP fusion protein expressing eukaryotic reporter system

A plasmid carrying a reporter gene suitable for in vitro as well as in vivo applications seemed to be advantageous for investigation of gene transfer efficacy, kinetics and localization in gene transfer studies. For this study I designed a TurboRFP-CBR fusion protein expressing plasmid (see section 2.2.13). TurboRFP is a fluorescent protein with excitation/emission maxima of 553nm/574nm respectively, in addition TurboRFP is approx. twice as bright as dsRed_Express fluorescent protein (according to the manufacturer). These properties make it an ideal in vitro reporter protein. To visualize gene transfer efficacy and kinetics in vivo by bioluminescence imaging I chose click beetle luciferase red (CBR) as suitable reporter gene.

Phusion polymerase high fidelity PCR was used to amplify the CBR cassette from the pCBR control vector. Amplification primers were designed to introduce an EcoRI restriction site at the 5´end as well as a BamHI restriction site at the 3´end of the amplicon. The blunt end PCR product was loaded on an agarose gel to check integrity, excised and cleaned up for subsequent blunt end cloning into a linearized pCR-Blunt vector. Competent E.coli TOP 10 were transformed with the ligation product and cultured on LB-Kanamycin plates overnight (o/n) at 37°C.

Positive clones were identified by colony PCR or restriction digests of the plasmid following minipreparation. For the final ligation of the CBR inframe cassette into the pTurboRFP plasmid both constructs were cut with EcoRI and BamHI, loaded on an agarose gel and the correct size bands excised and purified as mentioned in section 2.2.3 competent E.coli TOP 10 were transformed with the final construct, incubated o/n at 37°C and screened for positive clones using minipreparation followed by restriction analysis. For long time storage at -80°C glycerine stocks were made of the positive clones. Maxipreparation of pTurboRFP-CBRinframe plasmid was performed for subsequent in vitro testing.
Testing of biological activity in cell culture

Figure 21: Design of a CBR TurboRFP fusion protein expressing reporter-plasmid
2.2.14 Design of reporter & therapeutic gene co-expression plasmids

One of the main goals of this work was to design four reporter systems for the two therapeutic plasmids used in this study. Starting from pCBR, pTurboRFP635 and pdsRedExpressC1 four reporter genes were amplified using Phusion high fidelity PCR:

1. CBR- CBR cassette without enhancer segment
2. CBR+ CBR cassette including enhancer segment
3. TurboRFP cassette
4. dsRed cassette

Primers were designed in a way that at the 5´end and at the 3´end of each cassette a BspHI restriction site was introduced for the final cloning step into the therapeutic vector. The blunt end HF-PCR product was loaded on an agarose gel, excised, purified as mentioned before and ligated into a linearized pCR-Blunt vector for amplification. *E.coli TOP 10* were transformed with the ligation products and plated on LB-Kanamycin plates o/n at 37°C. After minipreparation of several clones positive clones were identified by restriction analysis. pCR-Blunt plasmids with inserts were cut with BspHI and the correct bands were excised from an agarose gel. pVAX1_mGDNF and pVAX1_rBDNF eukaryotic expression vectors were linearized with BspHI and dephosphorylated for subsequent ligation with the linearized reporter cassettes. The resulting eight plasmids

- pVAX1mGDNF_CBR+
- pVAX1mGDNF_CBR-
- pVAX1mGDNF_TurboRFP
- pVAX1mGDNF_dsRed
- pVAX1rBDNF_CBR+
- pVAX1rBDNF_CBR-
- pVAX1rBDNF_TurboRFP
- pVAX1rBDNF_dsRed

were used to transform competent *E.coli TOP 10 (o/n at 37°C)*. Several clones were picked of each LB-Kanamycin plate with subsequent minipreparation of plasmids. Plasmids were then checked for the right insert and correct orientation of the insert by restriction analysis. Positive clones were deep frozen in glycerol at -80°C for long time storage. Plasmids were tested for expression of therapeutic and reporter proteins *in vitro*. 
HF-PCR of reporter cassette inserting BspHI restriction sites, followed by ligation of blunt end PCR products into pCR-Blunt

Linearize with BspHI and dephosphorylate

Excise reporter cassette with BspHI,

Ligate reporter cassette into linearized therapeutic vector

Testing of biological activity in vitro

Figure 22: schematic drawing of the cloning strategy to introduce reporter cassettes into pVAX1mGDNF and pVAX1rBDNF
2.3 Cell biology methods

2.3.1 Isolation of rat adipose derived stem cells

Epididymal fat pads were isolated aseptically from male Sprague-Dawley rats posthum and transferred into 60ml 1xPBS (without Ca\(^{2+}\), Mg\(^{2+}\)) for further procedures. All subsequent steps were carried out under a laminar to avoid infection.

Fat pads were cut into small pieces and blood vessels were removed. Tissue has been washed twice with 1xPBS (w/o Ca\(^{2+}\), Mg\(^{2+}\)) followed by digestion in 10ml collagenase solution (Table 20) for 30min at 37°C and 170U/min shaking. After tissue was transferred into 50ml Falcon-tubes and centrifuged at 400g at room temperature the supernatant was discarded and the pellets resuspended in erythrocyte-lysis-buffer (Table 21). Lysis occurred in fresh Falcon-tubes at 37°C and 170U/min shaking. Following another centrifugation step at 400g at RT pellets were washed by resuspension and centrifugation (400g, RT) in 1xPBS. Cells were resuspended in ~5ml rASC-medium and filtered using a 70µm cell strainer before sewing them on appropriate cell culture dishes.

<table>
<thead>
<tr>
<th>rASC collagenase solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mg/mL BSA (PAA, AUT)</td>
</tr>
<tr>
<td>1,5 mg/mL Collagenase I (Worthington, USA)</td>
</tr>
<tr>
<td>25 mM Hepes (PAA, AUT)</td>
</tr>
<tr>
<td>PBS (+Ca(^{2+})/Mg(^{2+}))</td>
</tr>
<tr>
<td>sterile filtrate the final solution</td>
</tr>
</tbody>
</table>

*Table 20: Collagenase solution for the enzymatic digestion in rASC isolation.*

<table>
<thead>
<tr>
<th>rASC erythrocyte-lysis-buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>154 mM Ammoniumchlorid (Sigma, AUT)</td>
</tr>
<tr>
<td>10 mM Kaliumhydrogencarbonat (Sigma, AUT)</td>
</tr>
<tr>
<td>0,1 mM EDTA (Biochrom, GER)</td>
</tr>
<tr>
<td>Aqua dest.</td>
</tr>
<tr>
<td>sterile filtrate the final solution</td>
</tr>
</tbody>
</table>

*Table 21: Erythrocyte-lysis-buffer for rASC isolation*

2.3.2 Isolation of rat dorsal root ganglia

Male Sprague Dawley rats killed by either decapitation or during anaesthesia by an isofluorane overdose but not ketamin due to its high neurotoxicity.
After incision of the skin alongside the dorsal processes all subsequent steps were carried out aseptically using microsurgical equipment under the microscope. Exposure and laminectomy of column vertebra from C1 to L5/6 (from cranial to lumbal vertebrae), followed by opening of the dura and subsequent preparation of the dorsal root gangliae was examined. Only the dorsal root gangliae without excrescent roots were used.

DRGs were stored in sterile 1xPBS containing 1%P/S at 4°C until further processing. PBS was replaced by 1.8 ml of DRG Medium (see section 2.1.10) and 200 µl collagenase stock solution (Table 22) and incubated for 3h at 37°C in a water bath. After transferring the ganglia in a 50 ml sterile centrifuge tube the collagenase solution was removed and the ganglia were washed 3 times with sterile culture medium using a 5 ml pipette. In order to obtain a single cell suspension the ganglia were triturated with 2ml DRG culture medium using a 5ml pipette. After a centrifugation step (7min at 700rpm) the final volume of the single cell suspension was adjusted and supplemented with NGF (50ng/ml final concentration→ see table 23) and AraC (see table 24). The cells were plated on laminin coated poly-L-lysine coated coverslips in 24 well plates and put in the CO₂ thermostat. Media was changed every 48-72 hours.

**Table 22: Collagenase stock solution for enzymatic digestion in DRG isolation**

<table>
<thead>
<tr>
<th>DRG collagenase stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25% (~ 3000U/ml) Collagenase type IV (Sigma) in DMEM w/o serum (store at -20°C)</td>
</tr>
</tbody>
</table>

**Table 23: Preparation of Nerve Growth Factor aliquots for DRG culture medium**

- Prepare a solution of 100 µg NGF with 50 □L sterile dist. Water
- Dispense it in 20x2.5 µL aliquots, final concentration: 200 ng/µL;
- Working concentration = 50 ng/ml (~ 2 nM) (mix it with 10 mL medium).
- Store in freezer (20 C).
Table 24: Preparation of Ara-C aliquots for DRG culture medium

2.3.3 Cell culturing

Cells were cultured as mentioned in section 2.1.10

2.3.4 Lipofection of cells

Lipofectamine 2000 (Life Technologies, GER) a liposomal transfection agent was used for transient transfection in this study. The negatively charged backbone of DNA (plasmids) is interacting ionically with the positively charged, cationic liposomal particles of Lipofectamine 2000 thereby forming microparticles [173] to cross the cell membrane in a caveolae- or clathrin-dependent manner.

Formation of microparticles 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.

In order to achieve optimal transfection rates of primary rat adipose derived stem cells different Lipofectamine 2000 to DNA ratios has been evaluated. For all other cell lines a transfection protocol similar to that of rat adipose derived stem cells was used. In general 1µg of plasmid DNA was used to transfect cells with a confluence of 80-90% in a 24well plate.

2.3.5 Determining transfection rate using FACS

Fluorescence activated cell sorting (FACS) was used to determine the amount of transfected rat adipose derived stem cells. 0.5 x 10^5 cells / well were sown on a 12-well plate. pGFP_{max} and the corresponding amount of Lipofectamine 2000 were used for transfection. Cells were trypsinized washed with 1xPBS and examined with a FACS CANTO 2 flowcytometer (BD Biosciences, USA). Data were evaluated using FACS DIVA software.
DNA : Lipofectamine 2000

<table>
<thead>
<tr>
<th>DNA : Lipofectamine 2000</th>
<th>0.5 x 10^5 cells/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 0.5</td>
<td></td>
</tr>
<tr>
<td>1 : 1</td>
<td></td>
</tr>
<tr>
<td>1 : 3</td>
<td></td>
</tr>
<tr>
<td>Untransfected negative control</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 23: experimental setup for flowcytometry of transfected rat adipose derived stem cells*

### 2.3.6 MTT assay for determination of survival rate after transfection

The MTT assay is a test for measuring cell vitality by mitochondrial activity. MTT (3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) is a yellow tetrazole which is reduced to purple Formazan by a mitochondrial reductase. The amount of purple Formazan formed is indicative of the vitality of the cells [174].

*Figure 24: MTT is reduced to Formazan by a mitochondrial reductase*[^4]

[^4]: (4)

The experimental setup for the MTT assay performed in this study was the same as for the FACS experiment. Cells were transfected with 2µg/well GFP_{max} using the depicted DNA : Lipofectamine rate. 24h after transfection medium was removed and cells were incubated for 90min at 37°C with preheated (37°C) MTT working solution (0.65mg/ml in rASC medium). Following removal of the MTT working solution 0.5 ml/well DMSO was added and plates
were shaken 20 min in the dark (dissolving the Formazan-crystals). 100 µl Aliquots were transferred to a 96 well plate (if strongly violet, further dilution in DMSO may be necessary) and absorption measured at 550 nm against 690 using a TECAN (SUI) SPECTRA spectrophotometer. Data were evaluated using TECAN Magellan software.

<table>
<thead>
<tr>
<th>DNA : Lipofectamine 2000</th>
<th>rADSCs 0.5 x 10^5 cells/ well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 0.5</td>
<td></td>
</tr>
<tr>
<td>1 : 1</td>
<td></td>
</tr>
<tr>
<td>1 : 3</td>
<td></td>
</tr>
<tr>
<td>Untransfected negative control</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 25: experimental setup of the MTT-assay performed in this study*

### 2.3.7 CBR-assay

*Figure 26: Bioluminescent reaction catalyzed by click beetle luciferase*

To determine the amount of CBR produced by CBR transfected cells a CBR assay was used. Cells were sown at a density of approximately 0.3 x 10^5 cells / well in a 24 well plate, transfected with the plasmid of interest and checked for CBR expression 24 h after transfection.

Medium was removed; cells were washed once with 1x PBS and lysed by adding 100 µl/well 1x cell culture lysis reagent (prewarmed to RT) for ~5 min at RT. 50 µl of lysed cells in lysis reagent were transferred to a white 96 well plate. Immediately before measuring luminescence with a POLARstar Omega Ω luminometer, 50 µl of Luminol (BMG Labtech, GER) were added.
2.3.8 Enzyme linked immuno-sorbent assay ELISA

Expression kinetics of therapeutic vectors was evaluated using ELISA (Boster biological technology, LTD, USA). BDNF and GDNF ELISAs are based on standard sandwich technology; BDNF/GDNF polyclonal antibodies precoated 96-well plates and biotinylated monoclonal detection antibodies. Detection occurred using avidin-biotin-peroxidase complexes by subsequent adding of horseradish peroxidase (HRP) substrate 3,3’,5,5´-Tetramethylbenzidine (TMB). TMB gets catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the amount of target protein. According to the manufacturers 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.

![Figure 27: experimental design for expression kinetics evaluation of transfected rADSCs.](image)

Rat adipose tissue derived stem cells were plated at a density of approximately 0.5x10^5 cells in 24 well plates and transfected at a density of ~ 80% with pVAX1_mGDNF or pVAX1_rBDNF using Lipofectamine 2000. 24 hours previous to sampling, medium was removed / sampled and 500μl of fresh rADSC-medium was added.
2.3.9 Immunocytochemistry

In order to visualize axonal outgrowth of dorsal root ganglia, direct immunocytochemistry was carried out. To perform confocal laser scanning microscopy cells were grown on poly-L-Lysine coated coverslips (VWR international, USA) (Table 25) in a 24 well plate and checked daily for morphological changes under a light microscope. After a washing step with 1xPBS (15min) and subsequent fixation of the cells with 4% paraformaldehyde (10min) 2 additional washing steps (1xPBS 2x5min) were performed. In order to prevent unspecific binding of antibodies cells were blocked using BSA blocking solution (Table 26) for 1hr at RT. Incubation with primary antibody (or AlexaFluor488 conjugated antiß3 tubulin Ab) for another hour at RT took place in the dark and slight shaking (working solutions of the antibodies are depicted in Table 27). Unbound antibodies were washed out by three washing steps with 1xPBS (3x5min). In case of unconjugated antibodies secondary antibody solution was added and shaken for 1hr in the dark to avoid bleaching of the fluorophor. All subsequent steps were carried out in the dark. After 2 washing steps with 1xPBS nuclear staining with a 0,5µg/ml 4´6-diamidine-2-phenylindol (DAPI) solution in dH₂O for 5min was performed. Following 2 further washing steps (2x5min) with 1xPBS coverslips were mounted onto Menzel superfrost microscope slides (Thermo Scientific, USA) using 4-88 Mowiol mounting medium and kept at RT in the dark o/n for examination under a Axiovert 200M confocal laser scanning microscope (Zeiss, Oberkochen Ger) on the next day.

<table>
<thead>
<tr>
<th>Poly –L-Lysine coating of glass coverslips</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6cm coverslips</td>
</tr>
<tr>
<td>- 10min 1M HCl</td>
</tr>
<tr>
<td>- wash 2x dH₂O</td>
</tr>
<tr>
<td>- wash 1x 70% EtOH</td>
</tr>
<tr>
<td>- sterilize by autoclaving</td>
</tr>
<tr>
<td>following steps under the laminar flow bench:</td>
</tr>
<tr>
<td>- prepare poly-L-Lysine (Sigma Aldrich working solution (1:10 with dH₂O)</td>
</tr>
<tr>
<td>- keep coverslips in 50ml working solution for 1hr</td>
</tr>
<tr>
<td>- wash twice with dH₂O to remove free poly-L-Lysine</td>
</tr>
<tr>
<td>- air dry coverslips for 20min</td>
</tr>
<tr>
<td>- store at 4°C for up to 6weeks</td>
</tr>
</tbody>
</table>

Table 25: Protocol for the preparation of poly-L-Lysine coated coverslips
### Table 2: BSA blocking solution with Triton X for permeabilization of the cell membrane

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Host</th>
<th>Conjugate</th>
<th>Stock-dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-p75 AB</td>
<td>p75 neurotrophin receptor</td>
<td>rabbit</td>
<td>-</td>
<td>1:300</td>
</tr>
<tr>
<td>Anti-S100AB</td>
<td>S100 protein</td>
<td>rabbit</td>
<td>-</td>
<td>1:400</td>
</tr>
<tr>
<td>Anti-Nestin AB</td>
<td>Nestin intermediate filament</td>
<td>rabbit</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-rabbit secondary AB</td>
<td>rabbit IgG</td>
<td>goat</td>
<td>AlexaFluor594</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti βIII-tubulin AB</td>
<td>βIII-tubulin</td>
<td>rabbit</td>
<td>AlexaFluor488</td>
<td>1:250</td>
</tr>
</tbody>
</table>

Table 27: Antibodies, their specificity and dilution to working concentrations are depicted above

#### 2.3.10 In vitro functionality testing of constructed plasmids

NIH3T3 cells were seeded in a 24-well plate in a concentration of 28000 cells/cm² and transfected using Lipofectamine 2000 and 1μg/well plasmid DNA.

24h post transfection cells were checked for expression of the reporter cassette using either fluorescence microscopy (dsRed and TurboRFP) or a CBR assay (according to section 2.3.7). Additionally supernatants were sampled and ELISAs for BDNF and GDNF, respectively were performed (according to section 2.3.8) to check for the expression of the therapeutic cassette.
3 Results

3.1 Design and evaluation of therapeutic vectors carrying reporter genes

In order to assess the transfection efficiency in vitro and distribution of transfected cells in vivo therapeutic vectors (pVAX1_mBDNF + pVAX1_rGDNF) were designed carrying various reporter genes (dsRed, TurboRFP, CBR+/−Enhancer) (Table 28)

<table>
<thead>
<tr>
<th>Reporter genes</th>
<th>Therapeutic plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVAX1_mBDNF</td>
<td>dsRed</td>
</tr>
<tr>
<td>pVAX1_rGDNF</td>
<td>dsRed</td>
</tr>
<tr>
<td>TurboRFP</td>
<td>TurboRFP</td>
</tr>
<tr>
<td>CBR w/o enhancer</td>
<td>CBR w/o enhancer</td>
</tr>
<tr>
<td>CBR with enhancer</td>
<td>CBR with enhancer</td>
</tr>
</tbody>
</table>

Table 28: The constructed therapeutic vectors

3.1.1 pVAX1_mBDNF and pVAX1_rGDNF

To prove the integrity of the used pVAX1_BDNF plasmid control digests were carried out.

![Figure 28: Products of restriction digests of pVAX1_BDNF](image)

Lane 1: middle range marker (5µl)
Lane 2: XbaI + HindIII digest (predicted bands: 2919 bp + 1181 bp)
Lane 3: BspHI digest (predicted bands: 4000 bp + 99 bp)
Lane 4: Hind III + NdeI digest (predicted bands: 3673 bp + 427 bp)

In order to ligate pVAX1_BDNF with various reporter cassettes the plasmid was cut with BspHI (see Figure 28) and dephosphorylated. The linearized plasmid was stored on -20°C for further procedures.
3.1.2 Cloning of dsRed cassette into pVAX1_rBDNF + pVAX1_mGDNF

According to chapter 2.2.14 the dsRed cassette of pdsRedExpress-C1 was amplified using high fidelity polymerase. BspHI restriction sites were introduced at the 3´and at the 5´end and the PCR products were loaded on an agarose gel (Figure 29).

![Phusion PCR product of dsRed used for cloning into pCR-Blunt](image)

Figure 29: Phusion PCR product of dsRed used for cloning into pCR-Blunt

Lane 1: middle range marker (5µl)
Lane 2-9: dsRed products, lane 2 was excised and used for the ligation

Primers used: dsRedBspH1s, dsRedBspH1as

![Schematic illustration of the dsRed amplicon with introduced BspHI restriction sites](image)

Figure 30: Schematic illustration of the dsRed amplicon with introduced BspHI restriction sites

Cleaning up and blunt end ligation of the dsRed cassette into the bacterial expression vector pCR-Blunt was followed by transformation of E.coli TOP 10 with the resulting plasmid. 8 colonies were picked for minipreparation followed by a restriction digest to check for the right insert orientation.

![HindIII restriction digest of minipreps of 8 E.coli TOP 10 colonies. Predicted bands of cut pCR-Blunt_dsRed: 4802bp + 327bp or 3729bp + 1400bp.](image)

Figure 31: HindIII restriction digest of minipreps of 8 E.coli TOP 10 colonies. Predicted bands of cut pCR-Blunt_dsRed: 4802bp + 327bp or 3729bp + 1400bp.

Lane 1: middle range marker
Lane 2,4,7&8: pCR-Blunt_dsRed 4802bp + 327bp
Lane 3,5,6&9: pCR-Blunt_dsRed 3729bp + 1400bp
Plasmids 2 and 5 were cut with BspHI to excise the dsRed cassette and for further cloning into pVAX1_rBDNF and pVAX1_mGDNF.

**Figure 32**: BspHI restriction digests of pCR-Blunt_dsRed BspHI. Predicted bands: 1847bp + 1611bp + 1179bp + 492bp

Lane 1: middle range marker
Lane 2,3: restriction digest of miniprep of colonies 2 + 5

Ligation of the excised dsRed cassette into cut and dephosphorylated pVAX1_rBDNF and pVAX1_mGDNF plasmids. After transformation of E.coli TOP 10 with the ligation products 9 colonies per transformation were picked and plasmids were isolated. To examine the insertion of the dsRed cassette, the isolated plasmids were digested with NheI+ NdeI. (Figures 33 + 34)

**Figure 33**: NheI + NdeI restriction digests of pVAX1_rBDNF_dsRed
Lane1: middle range marker
Lane 3,5,6-10: correct insert 2850bp + 1992bp + 412bp + 358bp

**Figure 34**: NheI + NdeI restriction digests of pVAX1_mGDNF_dsRed
Lane 1: middle range marker
Lane 8: correct insert 2401bp + 1992bp + 412bp + 358bp
Lane 3&6: correct insert reverse insertion: 3106bp + 1287bp + 412bp + 358bp
3.1.3 Cloning of a TurboRFP cassette into pVAX1_rBDNF + pVAX1_mGDNF

According to chapter 2.2.14 the TurboRFP cassette of pTurboFP635-C was amplified using high fidelity polymerase. BspHI restriction sites were introduced by primer design at the 3’ and at the 5’end and the PCR product was loaded on an agarose gel (Figure 36).

After ligation of the TurboRFP amplicon into pCR-Blunt and subsequent transformation of competent E.coli TOP 10, eight positive clones picked and plasmids were miniprepped. These plasmids were checked for the TurboRFP insert by restriction digests with PstI and NdeI restriction enzymes (Figure 37).
Correct plasmids were cut with BspHI to excise the TurboRFP cassette for further ligation into linearized and dephosphorylated pVAX1_rBDNF and pVAX1_mGDNF eukaryotic expression systems.

To test the pVAX1_mGDNF_TurboRFP and pVAX1_rGDNF_TurboRFP plasmids for the correct insertion of the TurboRFP cassette, 5 colonies were picked for each plasmid and following minipreparation restriction digests with NheI + NdeI were examined. (Figure 39 + 40)
Figure 39: NheI + NdeI restriction digests of pVAX1_rBDNF_TurboRFP
Lane 1: middle range marker
Lane 2, 4-6: correct plasmids. Predicted band sizes: 2837bp + 2037bp + 412 + 358bp or 3600bp + 1274bp + 412bp + 358bp

Figure 40: NheI + NdeI restriction digests of pVAX1_mGDNF_TurboRFP
Lane 1: middle range marker
Lane 2, 3, 5, 6: correct plasmids. Predicted band sizes: 2388bp + 2037bp + 412 + 358bp or 3151bp + 1274bp + 412bp + 358bp

Figure 41: Schematic illustrations of the constructed pVAX1_rBDNF/mGDNF plasmids carrying the TurboRFP cassette (tandem orientation is referred as right)
3.1.4 Cloning of a Click Beetle Luciferase (CBR) cassette into pVAX1_mGDNF and pVAX1_rBDNF

Two different CBR cassettes were cloned into the therapeutic vectors differing by a stretch of 285 nucleotides representing an SV40 enhancer element of the CBR cassette. The reporter cassettes were amplified from pCBRcontrol and isolated according to section 2.2.14.

After excision of the amplicons and blunt end cloning into the bacterial expression system pCR-Blunt clones were picked followed by plasmid minipreparation and digestion with BspHI to check for the correct insertion of the CBR+/- cassettes. (Figures 43 + 44)

Figure 42: Phusion PCR products amplified from pCBRcontrol are depicted on the left side. On the right side schematic illustrations of the amplicons are shown.
Lane 1: middle range marker  
Lane 2: CBR- amplicon  
Lane 3: CBR+ amplicon

Figure 43: BspHI digests of pCR-Blunt_CBR+ clones predicted band sizes: 2424bp + 1847bp + 1179bp + 492bp  Lane 8: correct insert
Following ligation of excised CBR+/- insert into pVAX1_mGDNF and pVAX1_rBDNF. Plasmids were checked for correct insertion by NheI + NdeI restriction digests. (Fig.45-48)

Figure 44: BspHI digests of pCR-Blunt_CBR- clones predicted band sizes: 2139bp + 1847bp + 1179bp + 492bp. Lanes 2-8: correct insert

Figure 45: NheI + NdeI restriction digests of 8 positive clones of pVAX1_rBDNF_CBR-
Lane 2&4: correct insert predicted bands: 3127bp + 2601bp + 412bp
Lane 1,3,5-8: correct insert reverse orientation predicted bands: 4244bp + 1048bp + 412bp
Lane 9: middle range marker

Figure 46: NheI + NdeI restriction digests of 5 positive clones of pVAX1_mGDNF_CBR-
Lane 1: Quantitas Fast ladder
Lane 2&3: correct insert reverse orientation, predicted bands: 4244bp + 1048bp + 412bp, note the additional band at ~1500bp, indicating incomplete NdeI digestion.
Figure 47: NheI + NdeI restriction digests of 5 positive clones of pVAX1_mGDNF_CBR+
Lane 1: Quantitas Fast Ladder
Lane 2: correct insert reverse orientation, predicted bands: 4526bp + 1038bp + 412bp
Lane 3: correct insert correct orientation, predicted bands: 3412bp + 2152bp + 412bp

Figure 48: NheI + NdeI restriction digests of 5 positive clones of pVAX1_rBDNF_CBR+
Lane 1: Quantitas Fast Ladder
Lane 2,3,4,6: correct insert correct orientation, predicted bands: 3412bp + 2601bp + 412bp
In vitro functionality testing of the constructed plasmids

The constructed plasmids were checked for expression from the reporter cassette as well as expression of the therapeutic gene.

Figure 49: Constructed therapeutic eukaryotic expression systems carrying either CBR+Enhancer or CBR-Enhancer reporter cassette. (Tandem orientation is referred as right and convergent orientation is referred as reverse)
24h after transfection of the cells with the indicated plasmids (see section 2.3.10), photographs, CBR assay and sampling for ELISA was performed.

<table>
<thead>
<tr>
<th></th>
<th>dsRed</th>
<th>TurboRFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive control</td>
<td><img src="image" alt="pDsRed-Express" /></td>
<td><img src="image" alt="pTurboFP-635" /></td>
</tr>
<tr>
<td>pVAX1_BDNF</td>
<td><img src="image" alt="pVAX1_BDNF_dsRed_right" /></td>
<td><img src="image" alt="pVAX1_BDNF_TurboRFP" /></td>
</tr>
<tr>
<td>pVAX1_GDNF</td>
<td><img src="image" alt="pVAX1_GDNF_dsRed_right" /></td>
<td><img src="image" alt="pVAX1_GDNF_TurboRFP" /></td>
</tr>
</tbody>
</table>

Figure 50: Overlays of bright field and fluorescence photographs taken from transfected NIH3T3 cells are depicted above.
Figure 51: CBR assay of NIH3T3 cells transfected with designed plasmids. pCBR-plasmid served as a positive control (n=2)

Figure 52: GDNF ELISA results of 24h conditioned media of transfected NIH3T3 cells.

Figure 53: BDNF ELISA results of 24h conditioned media of transfected NIH3T3 cells.
3.2 Design of a TurboRFP-CBR Fusion Protein expressing plasmid

A reporter plasmid feasible for in vitro (TurboRFP) as well as in vivo (CBR) confirmation of successful transfection was designed.

According to section 2.2.13 a dual reporter expression cassette coding for a fusion protein of TurboRFP red fluorescent protein and CBR a far red luminescent protein was constructed. Phusion polymerase PCR was performed using the pCBR-control vector as template introducing EcoRI and BamHI restriction sites for further cloning into pTurboFP365-C.

![Phusion Polymerase PCR products predicted band: 1647bp](image)

Lane 1: middle range marker
Lane 2-6: CBR\textsubscript{inframe} amplicon

![Schematic illustration of the CBR\textsubscript{inframe} cassette with an EcoRI restriction site at the 5’end and a BamHI restriction site at the 3’end](image)
Blunt end subcloning of the CBR<sub>inframe</sub> cassette into the bacterial expression vector pCR-Blunt.

**Figure 56**: EcoRI + BamHI restriction digests of pCR-Blunt<sub>_CBR</sub><sub>inframe</sub>

*Lane 1: middle range marker*

*Lane 2-9: correct insert predicted band sizes 3469bp + 1638bp + 31bp + 11bp + 10bp*

The target vector pTurboFP365-C was digested with BamHI + EcoRI thereby linearized for ligation with the CBR<sub>inframe</sub> cassette.

**Figure 57**: Agarose gel of the BamHI and EcoRI digested, linearized pTurboFP365-C target vector.

After ligation of the CBR<sub>inframe</sub> cassette into the linearized target vector competent *E.coli* TOP 10 were transformed and 8 positive colonies were picked for further examination. Minipreparation followed by restriction digests of the plasmids with NcoI and Scal was performed.
Figure 58: restriction digest of pTurboFP365-CBR fusion reporter vector with NcoI and ScaI
Lane 1: middle range marker
Lane 2,3,4,6,9: correct insert predicted band sizes: 2745bp + 1903bp + 723bp + 703bp + 251bp

Figure 59: Schematic illustration of the designed pTurboFP365-CBR_Fusion vector
3.2.1 In vitro functionality testing of the pTurboFP365-CBR_Fusion vector

In order to assess the functionality of the plasmid NIH3T3 cells were transfected and examined using fluorescence photography and a CBR assay.

![CBR assay graph](image)

In Figure 60, CBR assay and overlays of brightfield and fluorescence photographs of transfected NIH3T3 cells are depicted above.
3.3 Evaluation of a transfection protocol suitable for primary cells (rat adipose tissue-derived stem cells)

Various lipofection protocols were tested to obtain an optimal survival rate at high transfection efficiency.

6 different protocols were tested (Table 26)

<table>
<thead>
<tr>
<th></th>
<th>Ia</th>
<th>Ib</th>
<th>Ic</th>
<th>IIa</th>
<th>IIb</th>
<th>IIc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incubation time</strong></td>
<td>4h</td>
<td>4h</td>
<td>4h</td>
<td>24h</td>
<td>24h</td>
<td>24h</td>
</tr>
<tr>
<td><strong>DNA : Lipofectamine 2000 ratio</strong></td>
<td>1 : 0,5</td>
<td>1 : 1</td>
<td>1 : 3</td>
<td>1 : 0,5</td>
<td>1 : 1</td>
<td>1 : 3</td>
</tr>
</tbody>
</table>

Table 26: The tested lipofection protocols are depicted above. Two main protocols (I + II) with different incubation times and sub-protocols using different DNA: Lipofectamine ratios were investigated.

Following the incubation time, transfection medium was substituted with rADSC medium and the cells were harvested for FACS analysis or prepared for MTT assay analysis.

The results of the FACS and the MTT assay are depicted in Figure 61 and 62.

![4 hours incubation with lipofection-mix](image)

**Figure 61:** Analysis of transfection protocols with 4 hour incubation are depicted above. The blue bars represent the results of the MTT assay (n = 6) whereas the yellow bars depict the transfection efficiency measured by FACS analysis. (Mean+/− SD)
Figure 62: Analysis of transfection protocols with 24-hour incubation are depicted above. The blue bars represent the results of the MTT assay (n = 6) whereas the yellow bars depict the transfection efficiency measured by FACS analysis. (Mean+/− SD)

In addition to the FACS and MTT analysis, the expression of neurotrophic factors was evaluated using two different lipofection protocols:

1. **Protocol A:** 4 hour incubation with Lipofectamine 2000 with a DNA : Lipofectamine 2000 ratio of 1 : 2

Figure 63: Experimental setup for Protocol A
2. **Protocol B:** 24 hour incubation with Lipofectamine 2000 with a DNA : Lipofectamine 2000 ratio of 1 : 1

**Figure 64: Experimental setup for Protocol B**

Rat adipose-tissue derived stem cells were plated at a density of 0.5x10^5 cells in a 24 well plate. Cells were transfected with pVAX1_mGDNF and/or pVAX1_rBDNF using the indicated lipofection protocol and supernatants were sampled at 24 hours, 70 hours and 5 days (BDNF only) after transfection. Supernatants were then checked for BDNF / GDNF expression using ELISA technique. No medium change was performed 24h prior to sampling. Cotransfection of pVAX1_mGDNF and pVAX1_rBDNF was performed using 0.5 µg of each plasmid resulting in 1µg total plasmid DNA/ well.

**Figure 65: GDNF ELISA of supernatants of transfected rADSCs using different lipofection protocols. Note the reduced expression of GDNF in the cotransfection groups. (Mean+/ SD)**
85

Figure 66: BDNF ELISA of supernatants of transfected rADSCs using different lipofection protocols. Similar protein levels of BDNF in the cotransfection groups compared to the single transfection groups were observed. (Mean+/− SD)

3.4 Proof of temporally restricted expression of neurotrophic factors in vitro using ELISA

One of the main goals of this work was to evaluate the expression kinetics of neurotrophic factors produced by transfected rASCs over a time period of ~3 weeks.

Sampling of conditioned media and ELISAs were performed according to section 2.3.7

3.4.1 Expression kinetics of pVAX1_mGDNF transfected rASCs

Figure 67: GDNF expression kinetics of pVAX1_mGDNF transfected rASCs in comparison to untransfected rASCs. Data are depicted as percentage of the mean value of 24hours post transfection. (Mean+/− SD)
Figure 68: BDNF expression kinetics of pVAX1_rBDNF transfected rASCs in comparison to untransfected rASCs. Data are depicted as percentage of the mean value of 24 hours post transfection. (Mean +/- SD)

3.5 Establishment of an in vitro biological activity assay

To prove the biological activity of the neurotrophic factors produced by transfected rat adipose tissue derived stem cells an in vitro biological activity assay was established.

3.5.1 Establishment of a cell culture protocol for primary dorsal root ganglia

Dorsal root ganglia have been isolated according to section 2.3.2 and cultured as described in section 2.1.10. In order to assess the responsiveness to neurotrophic factors DRG have been cultured either in DRG medium supplemented with nerve growth factor (NGF) or DRG medium without NGF. Cells have been fixated and stained as mentioned in section 2.3.9 using antibodies against p75 / nestin (red fluorescence) and β-III tubulin (green fluorescence) (Figures 69 and 70).
Figure 69: Immunocytochemistry of DRG, cultured in NGF supplemented DRG medium. Anti-nestin antibodies with corresponding Alexa 594 conjugated secondary antibodies (upper left side) as well as Alexa 488 conjugated anti-β-III-tubulin antibodies (upper right side). A merge of the two pictures is depicted above. The scale bar represents 100µm.
Figure 70: Immunocytochemistry of DRG, cultured without NGF. Anti-nestin antibodies with corresponding Alexa 594 conjugated secondary antibodies (red channel) as well as Alexa 488 conjugated anti-β-III-tubulin antibodies (green channel) were used. A merge of the two pictures is depicted above. The scale bar represents 100µm.

Figure 71: Immunocytochemistry of DRG cultured in medium with (left) or without (right) nerve growth factor (NGF) added. Anti-p75 antibodies with corresponding Alexa 594 conjugated secondary antibodies (red channel) as well as AlexaFluor 488 conjugated anti-β-III-tubulin antibodies (green channel) were used. A merge of the two pictures is depicted above. The scale bar represents 100µm.
In order to exclude the possibility of binding of the secondary Alexa-594 conjugated antibody to the primary Alexa-488 conjugated antibody, DRG were incubated with the two antibodies.

**Figure 72:** Immunocytochemistry of DRG using the Alexa-488 conjugated rabbit anti β-III-tubulin primary antibody and an Alexa-594 conjugated goat anti rabbit secondary antibody.

### 3.5.2 Alternative biological activity assay using PC12 cells

PC12 cells are known to respond to a variety of factors in general and to BDNF as well as GDNF particularly [175-177]. To test the biological activity of BDNF and GDNF produced by transfected rASCs an assay was established in which PC12 cells were incubated with conditioned supernatants of transfected and untransfected rASCs respectively. Supernatants were taken 70h after transfection. PC12 cells were checked for neurite outgrowth and length of the neurites using light microscopy. Amount of neurotrophic factors in the added supernatants was evaluated using ELISA technique. Photographs were taken prior to incubation with conditioned medium (0 hour timepoint) and 24 hour after adding of the conditioned medium (24 hour timepoint).

<table>
<thead>
<tr>
<th></th>
<th>Untransfected</th>
<th>GDNF transfected</th>
<th>BDNF transfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg/ml GDNF</td>
<td>129</td>
<td>2000</td>
<td></td>
</tr>
<tr>
<td>pg/ml BDNF</td>
<td>200</td>
<td></td>
<td>776</td>
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</table>

*Table 27: Amount of GDNF/BDNF in the 70h conditioned supernatant used for the biological activity assay*
<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td><strong>Supernatant of untransfected rASCs</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>Supernatant of BDNF transfected rASCs</strong></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>Supernatant of GDNF transfected rASCs</strong></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 73: Representative pictures of the PC12 biological activity assay. Neurite outgrowth can be observed after 24h incubation with conditioned medium of transfected rASCs.**

2 wells of a 24 well plate were used for each condition. Pictures of three random spots per well were taken and cells as well as neurites were counted.
Figure 74: Statistical analysis of the performed PC12 biological activity assay is depicted above. (Mean+/− SD)
3.6 In vivo results

The following chapter will include results of the follow up in vivo study. The study was performed at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in cooperation with the Department of Trauma Surgery Medical University Vienna, Austria and the Department of Ophthalmology, Faculty of General Medicine, University of Szeged, Hungary. Results are presented with kind permission of the cooperation partners.

3.6.1 Outline of the study

The lumbar spinal cords of adult Sprague Dawley rats (n=46) were exposed and L4 ventral root avulsions with lateral reimplantations were performed. 2x10^5 ex vivo transfected rASCs were applied in a final concentration of 2.5mg/ml collagen gel (total volume was 21µl) periradicular i.e: around the reimplanted ventral root. Survival times were either 10 days or 12 weeks.

In vivo groups:
- Avulsion + reimplantation + untransfected rASCs
- Avulsion + reimplantation + BDNF transfected rASCs
- Avulsion + reimplantation + GDNF transfected rASCs
- Avulsion + reimplantation + BDNF transfected rASCs + GDNF transfected rASCs

Figure 75: Graphic illustration of the surgical procedure: the L4 ventral roots of Sprague Dawley rats were avulsed and laterally reimplanted. rASCs in collagen were applied around the reimplanted roots. Adapted from Pintér et al.[73]
3.6.2 *In vivo* fate of implanted cells and evaluation of expression of therapeutic protein

In order to assess the fate and therapeutic gene expression of transplanted rASCs, cells were labelled with CellBrite DiI, a red fluorescent cytoplasmic membrane dye, prior to transplantation. 10 days after surgery rats were sacrificed and ventral roots with the surrounding rASCs were immunolabelled against either BDNF (Figure 76) or GDNF (Figure 77) and counterstained with DAPI.

*Figure 76: (A) Longitudinal view of the ventral root and periradicular transplanted rASCs. (B) DiI labelled transplanted rASCs (C) DAPI counterstaining (D) Immunolabelling against BDNF (E) Merge of the three channels*
3.6.3 Survival of motoneurons and reinnervation of the ventral root

Eleven weeks after grafting of rASCs the spinal nerve was labelled with Fast Blue a blue fluorescent retrograde tracer to check for motoneurons which showed elongative axonal growth and reinnervation. 5 days later animals were sacrificed, perfused and the spinal cord was sliced with a microtome for further immunohistochemistry.

Figure 77: (F) Longitudinal view of the ventral root and periradicular transplanted rASCs.

(G) DiI labelled transplanted rASCs

(H) DAPI counterstaining

(I) Immunolabelling against GDNF

(J) Merge of the three channels
In order to count surviving motoneurons, immunolabelling against cholin acetyltransferase was performed as depicted in figure 78.

![Image of immunolabelling against ChAT and FB retrograde labelling](image)

**Figure 78**: Long term survival (12 weeks) data. Motoneuron pool in the L4 ventral horn was immunolabelled against cholin acetyltransferase (ChAT). Additionally retrograde labelling with Fast Blue (FB) was performed. ChAT positive motoneurons represent surviving whereas FB stained represent motoneurons which actually reinnervated the spinal nerve. Several times greater survival and reinnervation numbers can be observed in either GDNF-rASCs or BDNF-rASCs treated animals in comparison with animals that received untransfected rASCs. n = 6, scale bar is 100 µm
3.6.4 Functional analysis (automated gait analysis)

Functional analysis was performed before surgery with pretrained animals and after surgery at timepoints 4, 6, 8, 10 and 12 weeks using CatWalk automated gait analysis (Figure 79).

Figure 79: Results of the CatWalk automated gait analysis. Animals which received transfected rASCs showed significantly better performances in every parameter tested than animals which received untransfected rASCs. Each group n = 6, p < 0.05
4 Discussion

The main goals of the current study were the production of plasmids for the co-expression of therapeutic genes & reporter genes and to examine the feasibility of an *ex vivo* non-viral gene therapy approach in a root avulsion model in the rat. In order to enhance survival of motoneurons and to induce elongative outgrowth of new motor axons a transient expression system was developed. It was possible to clone functional reporter cassettes into the therapeutic plasmids pVAX1_mBDNF and pVAX1_rGDNF without altering the therapeutic function of the original plasmids. It could be shown that rASCs can be transiently transfected with eukaryotic expression vectors with sufficient efficiency for the production of therapeutically active amounts of neurotrophic factors. In the follow up *in vivo* study it was shown that the transfected rASCs produced detectable amounts of neurotrophic factors still 10 days after transplantation around the reimplanted ventral root.

The *in vivo* results of this work indicate the feasibility of an *ex vivo* gene therapeutic approach using transiently transfected adipose tissue-derived stem cells as expression systems.

4.1 Design and evaluation of therapeutic vectors carrying reporter genes

In order to assess the transfection efficacy and activity of the therapeutic plasmids before implantation of the transfected cells, plasmids for the co-expression of therapeutic genes and fluorescent reporter genes have been designed. Additionally therapeutic plasmids have been designed co-expressing a luciferase to make *in vivo* tracking of the transfected cells possible.

Cloning of reporter genes cassettes into the eukaryotic expression plasmids pVAX1_mBDNF and pVAX1_rGDNF proved to be successful. The cloning strategy using a single restriction site (BspH1) for insertion, resulted in random orientation of the reporter cassettes. Due to the use of complete expression cassettes including promoter, reporter gene and polyadenylation signal, representing independent transcriptional entities orientation of the reporter cassette should not affect expression levels.

NIH3T3 cells transfected with the final constructs showed expression of the reporter genes dsRed, TurboRFP and click beetle luciferase red (CBR) as well as expression of either BDNF or GDNF proving the cloning strategy to be successful.

Interestingly, the expression of CBR was lower in cells transfected with constructs carrying therapeutic and reporter cassettes, compared to cells transfected with the pCBR-control.
plasmid. This effect was especially evident in case of the pVAX1_mBDNF_CBR+Enhancer constructs. Lower transfection rates due to the bigger size of the constructed plasmids (~6000bp to ~5000bp) [178] or transcriptional interference phenomena between the two independent unidirectional expression cassettes [179, 180] may explain the observed differences. Taken into account, that the CBR-Enhancer constructs are only 300bp smaller and performed better in expression of CBR as well as in therapeutic gene expression (BDNF+GDNF) than the CBR+Enhancer constructs, it seems plausible that there is transcriptional interference between the two expression cassettes rather than lower transfection efficiency due to bigger size. Additionally the Enhancer could interfere with the promoter thereby reducing the expression level [181].

4.2 Design of a TurboRFP-CBR Fusion Protein expressing plasmid

*In vitro* as well as *in vivo* evaluations are necessary in order to perform *ex vivo* gene therapy. Most reporter gene systems are convenient for only one of the two. For the *in vitro* evaluation of expression of plasmids over several time points fluorescent protein cDNA carrying plasmids are feasible. Plasmids encoding for bioluminescent proteins allow only end point evaluation *in vitro*, because cells can not be kept in culture for measuring of bioluminescence. *In vivo* however bioluminescence proved to be a feasible reporter system. Therefore a reporter plasmid suitable for *in vitro* as well as *in vivo* use has been designed. The TurboFP635-C plasmid encodes for a far-red fluorescent protein which is, due to its long emission wavelength (635nm), suitable for *in vitro* and especially for *in vivo* imaging [182]. Although far-red emitting fluorescent reporter proteins provide better tissue penetration, there is still a limitation to fluorescence imaging for deep within tissue imaging *in vivo*. The rat root-avulsion model used in this study is not suitable for *in vivo* imaging with fluorescent reporter genes, requiring bioluminescent reporter genes for *in vivo* detection. Post-mortem analysis of gene expression at the therapeutic site by histology, however requires a fluorescent marker rather than a bioluminescent reporter gene. Taking these requirements into account, it was the aim to design a fusion protein capable of fluorescence (TurboRFP) and luminescence (CBR). Fluorescence can easily be monitored under a fluorescent microscope thereby being suitable for *in vitro* evaluations, whereas the luminescence is much brighter and may be capable of penetrating the thick rat skin and allows *in vivo* tracking of the expression levels of transfected cells [183].

It could be shown *in vitro* that the fusion protein expressed by the designed pTurboFP-CBR plasmid was functional with regards to fluorescence as well as luminescence emission.
In order to evaluate the functionality and feasibility \textit{in vivo} (e.g.: in a rat model) further experiments would be required.

\textbf{4.3 Evaluation of a transfection protocol suitable for primary cells (rat adipose tissue-derived stem cells)}

High expression levels of the therapeutic protein are crucial for \textit{ex vivo} gene therapy. A prerequisite for therapeutic levels of protein expression is a well established transfection protocol in order to achieve high transfection rates. Gene therapy using plasmids has one major drawback, which is its low transfection rates [184]. In general, viral transduction results usually in transduction rates of nearly 100 \% whereas lipofection of plasmids can reach efficacies of 20 to 99 \%[185] depending on the cell type.

The highest possible transfection efficiency at a reasonable survival rate of rASCs for further \textit{in vivo} application was achieved using 1µg DNA: 1µl Lipofectamine with an incubation time of 24 hours. Using this protocol a transfection efficacy of approximately 35-40 \% could be achieved. Higher concentrations of Lipofectamine 2000 were avoided due to the observed toxicity of the reagent and subsequent death of the majority of cells, although they may result in higher transfection rates. BDNF and GDNF expression did not differ using different transfection protocols, it could be shown that a Lipofectamine 2000 to plasmid ratio of 1:2 and incubation time of 4h results in similar expression levels of neurotrophic factors as a ratio of 1:1 with incubation for 24h. Interestingly cotransfection with pVAX1_mGDNF and pVAX1_rGDNF resulted in lower amounts of GDNF in the supernatant, when compared to the single transfection protocol. BDNF levels in the cotransfection groups were comparable to the single transfection protocol.
Comparison of GDNF expression using two different lipofection protocols $n=3$

![Chart](chart.png)

**Figure 80**: Comparison of differences in protein levels of BDNF and GDNF using single- or cotransfection. ($n=3$, mean +/- SD)

The reduced amount of GDNF in the cotransfection group most probably reflects the reduced amount of plasmid applied. The similar expression of BDNF protein, independent of the reduced amount of plasmid may indicate a negative feedback loop of BDNF expression or secretion [186]. Further investigation using higher n-numbers would be necessary to confirm this hypothesis.

### 4.4 Proof of temporally restricted expression of neurotrophic factors in vitro using ELISA

The experimental model used in this study demanded transient expression kinetics of the neurotrophic factors BDNF and GDNF in order to achieve not only survival of motoneurons but also elongative directional growth of motor axons. Other groups have observed that a constantly high level of BDNF and/or GDNF induces survival of injured motoneurons but results in sprouting and aberrant growth of axons thereby inhibiting functional reinnervation [17, 187]. Using ELISA technique it could be shown that transfected rASCs display a transient expression profile of the therapeutic proteins BDNF and GDNF of about two weeks. Primary rASCs with low passage numbers show little to none endogenous expression of either BDNF or GDNF [134, 135]. After transfection with pVAX1_rBDNF or pVAX1_mGDNF rASCs showed increased expression of BDNF and GDNF peaking between 48h and 72h post transfection in vitro. This strong initial expression is followed by a decline till baseline levels are reached at day 14 post transfection. This strong decline in expression may be explained by
methylation of the transfected plasmids [188, 189], thereby silencing transcription, loss of plasmids during mitosis, active degradation by DNases [190], or a combination of all three. Changes in posttranslational modifications leading to impaired secretion of BDNF and GDNF can not be excluded, because only the supernatant of cultured rASCs was examined for the presence of these neurotrophic factors.

4.5 Establishment of an in vitro biological activity assay

After the kinetics of neurotrophic factor expression have been evaluated it was of great importance to prove the biological activity of the produced therapeutic proteins. The initial plan to use primary dorsal root ganglia (DRG) cells for this purpose was shattered after the discovery that adult DRG survival or neurite outgrowth does not depend on neurotrophic factors [191]. An alternative biological activity assay was established by culturing PC12 cells in medium either conditioned by transfected (pVAX1_mGDNF or pVAX1_rBDNF) rASCs or untransfected rASCs. The results of this assay indicate a strong biological activity of the BDNF and GDNF protein secreted by rASCs.

4.6 In vivo results

Regeneration as well as development of nerve tissue is based on the tight regulation of expression of growth-permissive and -stimulating factors (figure 81).

<table>
<thead>
<tr>
<th>Factors regulating neurite outgrowth</th>
</tr>
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<tbody>
<tr>
<td><strong>Permissive</strong></td>
</tr>
<tr>
<td>ECM-associated</td>
</tr>
<tr>
<td>HB-GAM</td>
</tr>
<tr>
<td>laminins</td>
</tr>
<tr>
<td>tenascins</td>
</tr>
<tr>
<td>fibronectin</td>
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<tr>
<td>heparan sulfates</td>
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<tr>
<td>hyaluronate</td>
</tr>
<tr>
<td>CAMs</td>
</tr>
<tr>
<td>N-Cadherin</td>
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<tr>
<td>L1-family</td>
</tr>
<tr>
<td>NCAM</td>
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<tr>
<td>Axonin-1</td>
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<tr>
<td>Contactin</td>
</tr>
<tr>
<td>Neurotrophic factors</td>
</tr>
<tr>
<td>NGF</td>
</tr>
<tr>
<td>BDNF</td>
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<td>GDNF</td>
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<tr>
<td>NT-3</td>
</tr>
<tr>
<td>NT-4</td>
</tr>
<tr>
<td><strong>Inhibitory</strong></td>
</tr>
<tr>
<td>CSPGs</td>
</tr>
<tr>
<td>NG2</td>
</tr>
<tr>
<td>MAG</td>
</tr>
<tr>
<td>Nogo</td>
</tr>
<tr>
<td><strong>Guiding</strong></td>
</tr>
<tr>
<td>semaphorins</td>
</tr>
<tr>
<td>cephirs</td>
</tr>
<tr>
<td>netrins</td>
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<tr>
<td>Slits</td>
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</tbody>
</table>

Figure 81: Regulators of neurite outgrowth [192] taken from Kiryushko et al “Regulators of neurite outgrowth” 2004

Direct application of recombinant proteins cannot guarantee long term supply at constant levels/sustained release. There are several approaches to achieve therapeutically active levels of recombinant protein at the site of injury. Continuous administration can be achieved with
osmotic mini pumps [67, 147, 151], application of recombinant neurotrophic factors in a matrix-based release system like fibrin glue [167], slow release polymer implants [164] or repeated injections of recombinant protein. There are several drawbacks to these approaches including the need for additional surgery, the risk of infections and possible foreign body response or failure in case of the miniosmotic pump [167]. Repeated injections require a high compliance of the patient and continuously interfere with regeneration of the injured tissue. Recombinant proteins impose a high economic burden and, additionally, depending on the organism used for production of recombinant protein, protein may not be correctly glycosylated, which may lead to attenuated biological activity [193] and are usually applied in supraphysiological dosing [67, 151, 167].

Sustained release of therapeutic protein in the injured tissue with low economic burden can be achieved by the use of viral vectors. Blits et al. used adeno-associated viral vector-mediated expression of GDNF and BDNF in a ventral root avulsion model [17]. The constant overexpression of these neurotrophic factors resulted in enhanced survival of injured motoneurons but inhibited axonal outgrowth most likely to the high levels of BDNF/GDNF produced for long periods of time for up to 16 weeks. Viral vector-mediated delivery is therefore not feasible in a model of injury, which needs rather transient expression kinetics, than continuous high expression over a long term. This problem could be overcome by the design of inducible system like tetracycline/doxycycline responsive systems [194] or by using a constitutive transient plasmid based approach as envisaged herein. Another issue of therapeutic approaches using viral vectors is the high immunogenicity of viral vectors and the subsequent clearance of virally transduced cells by the immune system [195]. In order to supply injured motoneurons with neurotrophic factors Hell et al. [93] injected BDNF producing mesenchymal stem cells directly into the injured spinal cord. The major issue of this approach was the long in vitro expansion period of 10 weeks before implantation rendering this approach impractical for clinical use. Furthermore, direct injection into the spinal cord may cause additional mechanical damage at the injection site.

In our study we used transiently transfected rASCs as an expression system for local BDNF and GDNF delivery. Using a fluorescent cell tracker, the cells were still found at their initial implantation site 10 days after surgery. No migration of transplanted cells into the ventral root could be observed. Transfected rASCs stained positive for the therapeutic proteins BDNF or GDNF, it should be noted, that some cells which stained positive for BDNF/GDNF were not labelled with DiI. This may indicate either the presence of endogenous neurotrophic factor producing cells or incomplete staining or loss of staining. Besides the transplanted cells, cells
inside the ventral root stained also positive for BDNF/GDNF most likely representing Schwann cells [44, 196].

One of the major issues after ventral root avulsion is the massive loss of motoneurons due to excitotoxicity and deprivation of neurotrophic support. Neurotrophic factors are essential for the regenerative potential of motoneurons after injury [150, 197, 198]. The upregulation of receptors for BDNF and GDNF immediately after injury reflects the dependence of motoneurons on these factors, usually provided by Schwann cells in the distal nerve stump[143].

Compared to the group which received untransfected rASCs, we observed a doubling of motoneuronal survival after transplantation of transfected cells as well as a strong (3-fold) increase in motoneurons which reinnervated through the reimplanted root into the spinal nerve. In other experiments other groups have shown that animals which did not receive rASCs after ventral root avulsion and subsequent re-implantation display a similar regenerative capacity as the group which received untransfected rASCs [39, 72]. The neurotrophic support of the injured motoneurons seems to be partially re-established after transplantation of BDNF/GDNF expressing rASCs. This would explain the enhanced survival of motoneurons as well as the directed elongative outgrowth of axons. It is possible, that neurotrophic factors diffuse through the avulsed and reimplanted ventral root into the ventral horn, thereby generating a gradient, which enables motor axons to show directed growth towards the ventral root and site of transplantation. Due to the fact that constantly high levels of neurotrophic factors at the level of the ventral root would rather inhibit further elongative growth than support it, transient expression, which was observed in vitro, is most probably reflected in vivo. This hypothesis is consistent with observations by Blits et al. [17] and Eggers et al.[187], which have shown that very high levels of GDNF (achieved by lentiviral transduction) in the ventral root may even result in neuroma-like structures instead of regeneration.

CatWalk automated gait analysis was used, in order to evaluate functional regeneration. Several parameters were evaluated. Animals that received either BDNF or GDNF transfected rASCs showed significantly better results at all parameters 10 and 12 weeks after surgery than animals that received untransfected rASCs. The parameters step cycle, stand duration and maximal contact area, were significantly better than the control already 8 weeks after surgery group. We conclude that these improvements in gait are due to the temporally restricted expression of neurotrophic factors by the transplanted transfected rASCs.


5 References


6 Appendix

6.1 Summary

The discovery of an easily accessible and abundant pool of stem cells in adult adipose tissue some years ago, established new opportunities for stem cell therapy. Suddenly it was possible to harvest adult stem cells in great numbers by mere liposuction. These adipose derived stem/stromal cells are regarded as multipotent as they can differentiate into various tissues and have been shown to be immunomodulatory. ASCs are easy to cultivate in vitro and are supposed to be safe if administered into the body afterwards. They enable autologous stem cell therapy in a way that was not possible using bone marrow derived stem cells. The growing knowledge of the in vivo behaviour of ASCs allowed new therapeutic approaches including ex vivo gene therapy using these cells, for a variety of diseases and injuries.

In this study we evaluated the use of ASCs as a delivery system for the local transient expression of the neurotrophic factors BDNF and GDNF. The use of a plasmid-based system enabled a beneficial transient expression profile, simultaneously avoiding negative effects of viral based gene therapy like a strong response of the immune system or the possible integration into the host genome.

It could be shown in vitro that transfected ASCs not only secreted biologically active BDNF and GDNF after plasmid delivery but also that this expression was temporally restricted. Furthermore it was possible to construct plasmids, which contain reporter gene cassettes in addition to therapeutic gene cassettes, in order to evaluate the fate of transfected cells in vitro and in vivo in the future in experimental models.

In the follow up in vivo study, ex vivo gene therapy was carried out in a ventral root avulsion model in the rat. Transfected rat ASCs were applied around the reimplanted ventral root. It could be shown that animals which received BDNF or GDNF producing ASCs, displayed increased survival of motoneurons alongside elongative axonal regeneration and significant functional improvement. This study demonstrated as proof of concept that a plasmid based ex vivo gene therapy using adipose tissue-derived stem/stromal cells as shuttle systems for the in situ production of neurotrophic factors is feasible. Nevertheless, besides a closer look at the long-term behaviour of the transplanted ASCs as well as the effects of delayed transplantation, a further increase in transfection efficiency under good manufacturing practise (GMP) conditions is necessary in order to enable clinical translation of this therapeutic approach in the future.

114
6.2 Zusammenfassung


In der nachfolgenden in vivo Studie, wurde eine ex vivo Gentherapie in einem Wurzelausrissmodell in der Ratte durchgeführt. Ratten Fettstammzellen wurden mit den Plasmid-Konstrukten transfiziert und anschließend um die reimplantierte ventrale Nervenwurzel appliziert. Motoneuronen von Tieren, welche Plasmide, die für BDNF bzw GDNF exprimierten, zeigten sowohl eine erhöhte Überlebensrate als auch elongative axonale Regeneration. Therapierte Tiere schnitten weiters signifikant besser bei funktionalen Tests ab als untherapierte.
Diese Studie zeigt als ein „proof of concept“, dass Plasmid-basierte ex vivo Gentherapie mit Fettstammzellen als System für die in situ Produktion von neurotrophen Faktoren durchführbar ist.

Für die klinische Anwendung dieses Ansatzes ist allerdings eine genaue Überprüfung der Langzeitverhaltens der transplantierten ASCs sowie die Effekte einer etwaigen verzögerten Transplantation von Nöten. Weiters ist eine Verbesserung der Transfektionseffizienz unter GMP Konditionen Voraussetzung um diesen Therapieansatz vom Labor zum Patienten zu bringen.
6.3 Curriculum Vitae

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Forschungsassistent
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Wahlbeispiel Molekulare Medizin

Dez 2010- Aug 2011 Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Department of Thoracic Surgery, Medical University of Vienna, Austria + Ludwig Boltzmann Institut für experimentelle und klinische Traumatologie
Studie “Anti-alpha-Gal antibody titres remain unaffected by the consumption of fermented milk containing Lactobacillus casei in healthy adults.”

Seminare & Symposien

ESGCT European Congress, Brighton Oktober 2011
Poster presentation: “BDNF gene therapy promotes motoneuron survival and elongative axonal growth and regeneration in a rat root avulsion model”

TERMIS Weltkongress Wien, September 2012
Mitarbeit bei der Organisation der SYIS Veranstaltungen
- Career Session
- SYIS Young Investigator Night
- Scientific Co-Chair

**Weitere Berufserfahrungen:**

Sommer 2000  Malermeister Paul Hörbranz, Ferialpraktikum
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