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Evaluating the viability of Olfactory Ensheathing Cells transduced to overexpress Glial Cell Line Derived Neurotrophic Factor in a rat model of Dorsal Root Injury

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Preface

From October 2007 until February 2008 I joined the Neural Injury Research Unit at the University of New South Wales in Sydney, Australia. Under the supervision of Prof. Phil Waite. I was mainly working together with Ann Wu, PhD, on a project on Olfactory Ensheathing Cells (OEC) genetically modified to secrete high levels of glial cell line derived neurotrophic factor (GDNF). The OEC cell line used in this study was isolated and transduced at the National Centre for Adult Stem Cell Research, Griffith University, Brisbane, Australia, by Nick Cameron, PhD-candidate, under the supervision of Prof. Alan Mackay-Sim.

The aim of this study was to evaluate the viability of the transduced OEC cell line for therapy of spinal cord injury. For this purpose, GDNF secretion rates and cell characteristics were determined in cell culture. Furthermore the applicability of this cell line was investigated *in vivo* in a rat model of dorsal root injury (DRI). GDNF concentrations and cell survival were evaluated after implantation of transduced OECs into rat spinal cord with DRI in a timecourse study.

I was taught how to perform the following procedures, which I then continued to perform independently:

Basics of cell culture

Enzyme linked immunosorbent assay (ELISA)

Immunocytochemistry

Immunohistochemistry

Furthermore, I assisted Ann Wu and Jenny Lauschke with anaesthesia, animal surgery, perfusion, dissection and postoperative care of the experimental animals.

The ELISA data on *in vivo* concentrations of GDNF after implantation of transduced OECs were obtained in teamwork with Ann Wu and are therefore also part of her PhD thesis, which was submitted to the University of New South Wales in Sydney, Australia.

Munich, March 2011 Maximilian Münchhoff

Meinen Eltern Ricarda und Peter Münchhoff

Summary

After an injury to the central nervous system, regeneration commonly fails due to an inhibitory environment and the lack of supportive factors. However, axonal regeneration in the central nervous system can be enhanced by implantation of a special type of glial cell, the olfactory ensheathing cell (OEC), into the spinal cord. Furthermore, glial cell line derived neurotrophic factor (GDNF) was also shown to be a potent stimulator of axonal outgrowth and regeneration. In order to combine the benefits of both therapeutic approaches, OECs can be genetically modified to secrete high levels of GDNF. In this study, we used a rat model of dorsal root injury (DRI) to evaluate the applicability of an OEC cell line that has previously been transduced to overexpress GDNF.

Initially, transgene expression in cell culture was quantified by ELISA, demonstrating stable overexpression of GDNF *in vitro* over at least one week. OECs were labelled with the fluorescent cell marker carboxyfluoresceindiacetate succinimidyl ester (CFSE), which did not significantly influence GDNF expression. Immunocytochemistry showed good cell survival of genetically modified OECs under culture conditions.

The viability of the transduced OECs was then investigated *in vivo* after injection into the spinal cord of rats in a model of cervical dorsal root injury (DRI). Using ELISA, the amount of GDNF in the spinal cord was measured over one month following OEC implantation. This showed increased levels of GDNF for at least one week. However, at later time points, no significant augmentation of GDNF compared to the baseline level in the rat spinal cord was observed. Immunohistochemical analysis of spinal cord sections showed poor survival of implanted transduced OECs in the spinal cord.

Our findings show that the lentiviral transduction of OECs led to the secretion of constant high levels of GDNF *in vitro*. Yet, *in vivo* survival of genetically modified OECs implanted into the rat spinal cord was poor. Therefore other approaches should be evaluated to combine cellular therapy using OECs with the application of neurotrophic factors in spinal cord injury.

Keywords: Olfactory ensheathing cell – Glial-cell-line-derived neurotrophic factor – dorsal root injury – *ex vivo* gene therapy

Zusammenfassung

Die Regeneration nach einer Verletzung des zentralen Nervensystems versagt aufgrund eines hemmenden Umfeldes und eines Mangels an unterstützenden Faktoren. Die Regeneration von Axonen im zentralen Nervensystem kann jedoch durch die Implantation eines bestimmten Typs von Gliazelle, der Olfactory Ensheathing Cell (OEC), gefördert werden. Weiterhin wurde gezeigt, dass Glial Cell Line Derived Neurotrophic Factor (GDNF) die Regeneration und das axonale Wachstum stark fördern kann. Um die Wirkung dieser beiden Therapiemöglichkeiten zu kombinieren, können OECs genetisch modifiziert werden damit sie große Mengen an GDNF sezernieren. In dieser Studie verwendeten wir ein Tiermodell der Ratte mit Verletzung der hinteren Wurzel um die Anwendbarkeit einer OEC Zelllinie zu untersuchen, die zuvor transduziert wurde um GDNF in hohen Mengen zu exprimieren.

Anfangs wurde die Expression des Transgenes in Zellkultur mittels ELISA quantifiziert, wobei eine stabile Überexpression von GDNF *in vitro* über mindestens eine Woche nachgewiesen werden konnte. Die OECs wurden mit dem fluoreszierenden Zellfarbstoff Carboxyfluoresceindiacetate succinimidyl Ester (CFSE) markiert, wodurch die Expression von GDNF nicht signifikant beeinträchtigt wurde. Mittels Immunozytochemie konnte ein gutes Überleben der genetisch veränderten OECs in Zellkultur dargestellt werden.

Die Anwendbarkeit der transduzierten OECs wurde dann *in vivo* nach Injektion in das Rückenmark von Ratten in einem Modell mit zervikaler Hinterwurzelverletzung untersucht. Mittels ELISA wurde die Menge an GDNF im Rückenmark über einen Zeitraum von einem Monat nach OEC Implantation gemessen. Dabei zeigten sich erhöhte Mengen von GDNF für mindestens eine Woche. Zu späteren Zeitpunkten jedoch konnte keine signifikante Erhöhung von GDNF im Vergleich zum Ausgangswert gezeigt werden. Immunohistochemische Untersuchungen von Rückenmarksquerschnitten zeigten ein schlechtes Überleben von transduzierten OECs nach Implantation in das Rückenmark.

Unsere Ergebnisse zeigen, dass die lentivirale Transduktion von OECs *in vitro* zur konstanten Sekretion von großen Mengen von GDNF führte. *In vivo* zeigte sich jedoch ein schlechtes Überleben von genetisch veränderten OECs nach Implantation ins Rückenmark. Daher sollten andere Strategien untersucht werden, um die zelluläre Therapie mit OECs mit der Anwendung von neurotrophischen Faktoren zu kombinieren.

Schlagwörter: Olfactory Ensheathing Cell - Glial Cell line Derived Neurotrophic Factor - Hinterwurzelverletzungen - *ex vivo* Gentherapie

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I. Abbreviations

AAW	Australian Albino Wistar
AdV	Adenovirus
ANOVA	Analysis of variance
CFDA-SE	Carboxyfluoresceindiacetate succinimidyl ester
CFSE	Carboxyfluoresceindiacetate succinimidyl ester
CNS	central nervous system
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DREZ	dorsal root entry zone
DRI	Dorsal root injury
DRG	Dorsal root ganglion
EDTA	Ethylendiamintetraacetate
ELISA	Enzyme linked Immunosorbent Assay
Et al.	Et alii
FCS	Fetal calf serum
GDNF	Glial-cell-line-derived neurotrophic factor
GFL	Glial-cell-line-derived neurotrophic factor family ligands
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GFR-α1	GDNF receptor-α
HBSS	Hank’s balanced salt solution
I. p.	Intra-peritoneal

Ig	Immunoglobulin
IU	International Units
NGF	Nerve growth factor
NGS	Normal goat serum
NP-40	Phenylmethylsulfonylfluorid
NT-3	Neurotrophin-3
OEC	Olfactory ensheathing cell
ORN	Olfactory receptor neurons
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.2% Triton X-100
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonylfluorid
PNS	Peripheral nervous system
RET	Rearranged during transfection
SEM	Standard error of mean
SCI	Spinal cord injury
TMB	Tetramethylbenzidine
UNSW	University of New South Wales

II. Introduction

II.1 Spinal cord injury

Spinal cord injury (SCI) is an insult to the spinal cord or the spinal nerves that can cause either temporary or permanent decrease in sensory, motor and autonomic functions. Depending on the extent and level of the injury, SCI can be a devastating event for the individual. The acute response of the organism after the damage to the spinal cord is a transient physiologic depression of cord function below the level of injury. This can result in flaccid paralysis, loss of sensorimotor function, autonomic dysregulation, bowel paralysis, priapism and hypotension due to a loss of vasomotor tone. These symptoms are commonly referred to as spinal shock and tend to last several hours to days (Ditunno *et al.* 2004).

In chronic SCI the most apparent consequence is loss of motor function below the level of injury, which can occur in variable degrees from complete to incomplete loss of strength. Other consequences can include loss of sensation, spasticity, autonomic dysreflexia and impaired sexual, bowel and bladder functions. All these complications not only immensely diminish quality of life, but also significantly reduce life expectancy (Strauss *et al.* 2006).

Besides these severe consequences for the individual, SCI is also a major burden for society. The incidence of SCI in the year 2008 was estimated to be around 12,000 new cases a year with an approximate prevalence of 260,000 persons in the United States of America, for example (van den Berg *et al.* 2010). The most common causes of SCI include traffic accidents, falls, violence and sports. SCI causes direct costs for health care and indirect costs, e.g. due to loss of productivity. For example, the lifetime costs for a 25 year old paraplegic are estimated to be one million US dollars, and for a quadriplegic up to three million dollars (DeVivo 1997).

All these facts, but especially the aim to diminish the agony of the affected individual make it a highly desirable goal to find effective therapies for the treatment of SCI. A lot of research has been conducted already, but so far a successful treatment could not be found. But why is it so difficult to find a cure for SCI?

The reason why the outcome of SCI is so severe is the lack of intrinsic regenerative potential in the central nervous system (CNS). In the peripheral nervous system (PNS), however, regeneration has been observed for more than a century. After damage to an

axon in the PNS, the part distal from the cell body undergoes Wallerian degeneration and only the Schwann cells and connective tissue components persist. Secretion of neurotrophic factors promotes sprouting and axonal regrowth from the proximal nerve stump (Terenghi 1999; Boyd *et al.* 2003). Schwann cells provide the axonal growth cones with guidance (Son *et al.* 1995) and a permissive environment allowing successful regeneration under optimal conditions, for example after crush injury where the basal lamina is still intact, and sometimes even under sub-optimal conditions, e.g. after axotomy (Son *et al.* 1995; Fu *et al.* 1997).

In the CNS instead, regeneration fails due to a lack of supportive factors and the presence of an inhibitory environment. Santiago Ramón y Cajal was the first to describe that regeneration can occur, if the peripheral branch of the dorsal root is injured, but if the central branch is injured, the axon will only grow up to the dorsal root entry zone (DREZ) and is unable to cross the CNS barrier to enter the spinal cord (Berciano *et al.* 2001). This observation led him to the conclusion that the CNS environment in the adult mammal is not permissive for axonal regeneration, a concept that is still axiomatic in CNS injury research.

That is why dorsal root injury (DRI) is an interesting model to test therapeutic strategies to overcome the circumstances that prevent regeneration in the CNS. Furthermore, some technical considerations make DRI a convenient experimental model for animal studies compared to other models like complete or incomplete spinal cord transection or spinal cord contusion. Post-injury survival is higher and postoperative care is less extensive in DRI compared to transection and contusion models, because DRI is less harmful. To evaluate regeneration, several methods are available on the electrophysiological, morphological and functional level that are relatively easy to apply, for example conductance studies, retrograde labeling of afferent nerves and mechanical stimulation (Wu *et al.* 2009).

II.2 Dorsal root injury

DRI is not only an interesting experimental model for research on SCI, it also has clinical relevance. In newborns, injury to the dorsal root occurs by traction injuries during difficult childbirth as part of peri-natal brachial plexus palsy (Andersen *et al.* 2006). In the United States the incidence of neonatal brachial plexus palsy was estimated to be approximately 1.5 cases per 1,000 live births (Foad *et al.* 2008). The main risk factors are shoulder dystocia, macrosomia and forceps delivery. Most often the upper brachial plexus with spinal nerves C5 and C6 is affected, resulting in loss of sensation in the corresponding dermatomes and paralysis of the biceps and shoulder muscles which is referred to as Erb or Duchenne palsy. If the lower brachial plexus with spinal nerves C8 and T1 is involved, the forearm flexors and the intrinsic muscles of the hand will be paralysed, which is called Klumpke palsy.

In adults, DRI involving the brachial plexus (Blaauw *et al.* 2008) and only rarely the lumbar plexus (Moschilla *et al.* 2001) most often results from traffic, especially motor cycle accidents. Other causes can be sports injuries, accidents at work, violent assaults or iatrogenic.

The dorsal root contains the axons of primary sensory neurons. The cell bodies of these pseudounipolar neurons are located in the dorsal root ganglion (DRG) from where a peripheral and central branch originates as shown in Figure 1. The peripheral branch conducts somatosensory stimuli including mechanical, nociceptive, thermal, chemical, muscle and tendon afferent information from the peripheral nervous system (PNS). The central branch projects towards the spinal cord to enter the central nervous system (CNS) at the dorsal root entry zone (DREZ), where the transition between PNS and CNS is located (Fraher 1999).

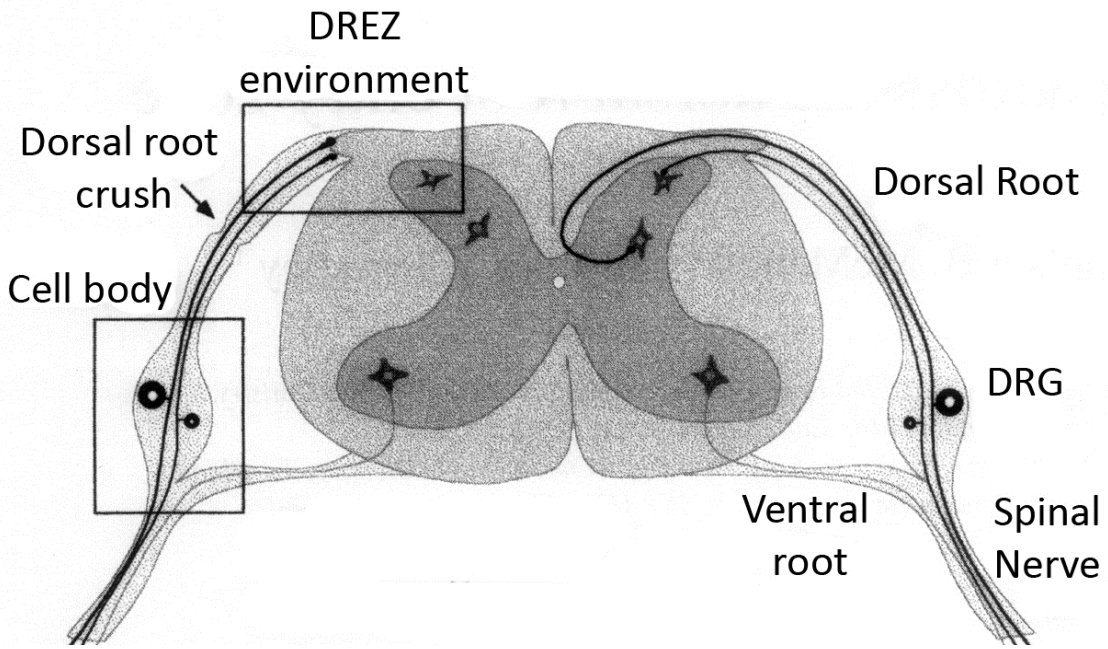

Figure 1: Transversal section of the spinal cord showing the cross-sectional anatomy of the dorsal root and the dorsal root entry zone.

DRG: Dorsal root ganglion, DREZ: Dorsal root entry zone. Adapted from Ramer et al. (2001).

Whereas injury to the ventral spinal roots can result in paralysis, DRI leads to deafferentiation of the spinal cord that causes loss of sensation and proprioception, which also affects fine motor control. Another consequence can be intractable neuropathic pain and allodynia (Bertelli *et al.* 2008).

The management of DRI depends mainly on the location of the injury in relation to the dorsal root ganglion (DRG). For injuries distal to the DRG, successful surgical interventions have been developed (Kline *et al.* 1986; Kim *et al.* 2003; Songcharoen 2008) like neurolysis and nerve grafting. For injuries proximal to the dorsal root ganglion (DRG), a definite therapeutic strategy could not be established yet (Carlstedt 1991; Liu *et al.* 2009), because the regenerating dorsal root axons are not able to cross the PNS-CNS barrier at the DREZ (Perkins 1980; Golding *et al.* 1997).

II.3 Failure of regeneration in the central nervous system and therapeutic approaches

What are the underlying mechanisms that prevent regeneration in the CNS? The non-permissive characteristics in the CNS were first thought to be due to a simple mechanical barrier consisting of astrocytes and connective tissue components organized as the glia

limitans at the DREZ (Windle *et al.* 1950). This suggestion is supported by experiments of three-dimensional astrocyte cultures *in vitro* inhibiting axonal growth (Fawcett *et al.* 1989) and by astrocytes proliferating and migrating into the DREZ after dorsal rhizotomy (Liu *et al.* 2000).

Further studies have shown that activated astrocytes, microglia, fibroblasts, oligodendrocytes and oligodendrocyte precursor cells, which are involved especially in the beginning of glial scar formation, upregulate a plethora of molecules thought to contribute to the failure of regeneration. Generally referred to as axon regeneration inhibitors this group includes semaphorin 3 (Pasterkamp *et al.* 2001), tenascin (Apostolova *et al.* 2006) and different chondroitin sulphate proteoglycans (McKeon *et al.* 1995). These molecules are integrated into the meshwork of the processes and cell bodies of activated astrocytes. Regenerating axons reaching this tight barrier stop growing, develop a synaptoid swelling and are transformed into a resting state not only due to the physical barrier, but also due to activation of their physiological stop pathway (Carlstedt 1985; Liuzzi *et al.* 1987). Another type of inhibitory factors that has gained attention recently are myelin associated proteins like myelin-associated glycoprotein (McKerracher *et al.* 1994) and the Nogo-proteins (Bandtlow *et al.* 2000; Huber *et al.* 2000). They are both ligands to the NgR1-receptor leading to growth cone collapse (Xie *et al.* 2008).

These advances in understanding the underlying causes for the failure of regeneration reveal several points of action for possible therapeutic strategies. Promising results have been obtained by directly inactivating inhibitory molecules, for example neutralizing Nogo-A with the IN-1 antibody (Bregman *et al.* 1995; Bandtlow *et al.* 2000; Chen *et al.* 2000; GrandPre *et al.* 2000; Qiu *et al.* 2000).

Other approaches rather aim at introducing a growth permissive environment by transplanting different tissues or tissue components. Autologous peripheral nerve grafts have been tried to bridge the DREZ with minor success (Saiz-Sapena *et al.* 1997). Implanting purified Schwann cells was shown to be difficult because of their poor survival, migration and ability of remyelination in the CNS-environment (Iwashita *et al.* 2000; Shields *et al.* 2000). Amongst other types of cells like Schwann cells, fibroblasts and stem cells that have been evaluated, the olfactory ensheathing cell (OEC) shows big potential to support regeneration in the CNS.

II.4 Olfactory ensheathing cells

In the olfactory system of mammals, regeneration of primary olfactory receptor neurons (ORN) occurs throughout lifetime (Doucette *et al.* 1983). ORNs are located in the olfactory epithelium and can be replaced by progenitor cells after cell death (Graziadei *et al.* 1978; Mackay-Sim *et al.* 1991). Their axons can regenerate from the olfactory mucosa which is part of the PNS through the lamina cribrosa and enter the olfactory bulb to form targeted synapses in the CNS (Schwob 2002). OECs are thought to play a major role in this successful regeneration from the PNS into the CNS, which is unique in the adult mammalian nervous system.

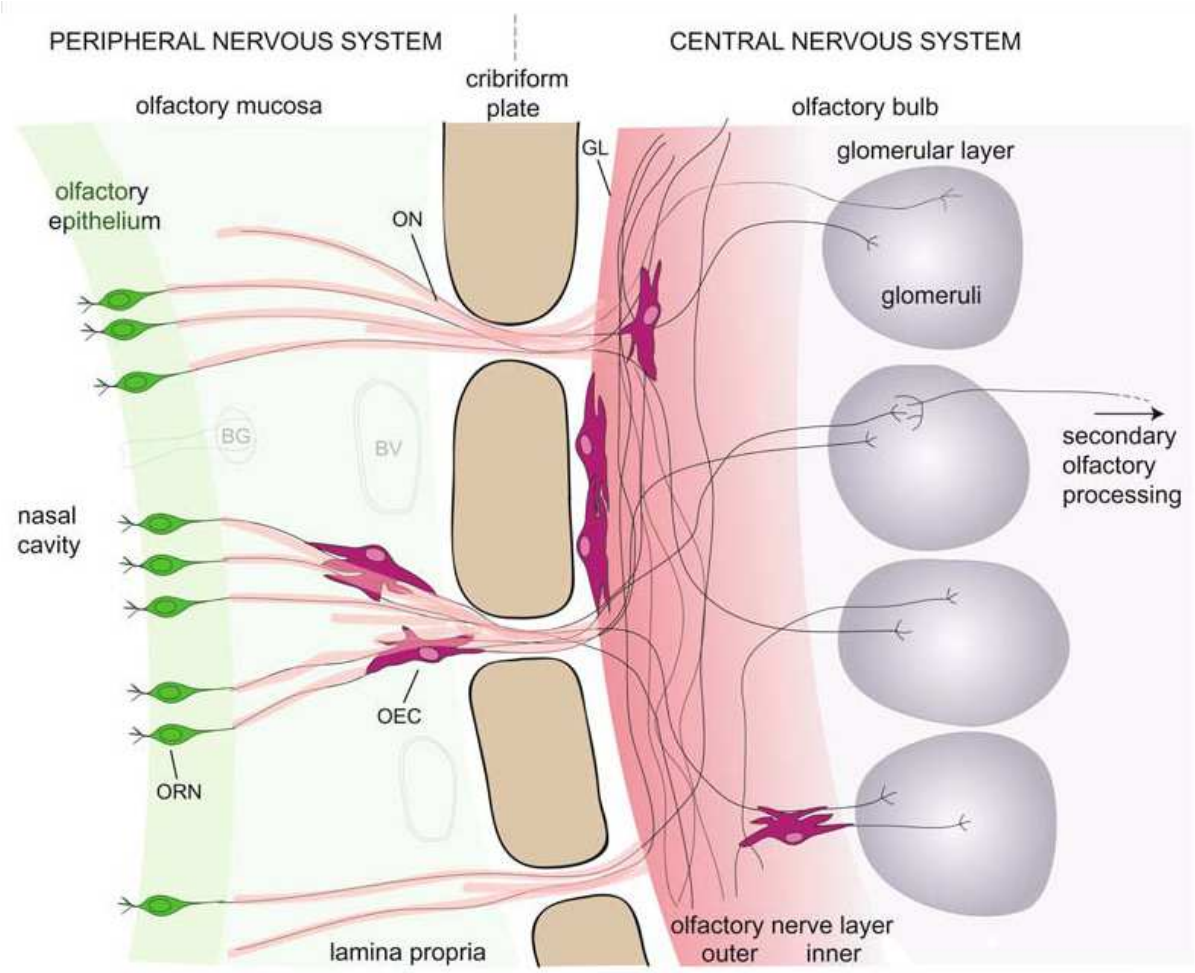

Figure 2: Diagram showing the primary olfactory system in the adult rat.
BG, Bowman's glands; BV, blood vessel; GL, glia limitans; ORN, olfactory receptor neuron. Adapted from Vincent et al. (2005).

OECs are a special type of glia cell that reside along the olfactory nerve and on the surface of the olfactory bulb (Doucette 1990) and share properties of both Schwann cells and astrocytes (Barnett 2004). They ensheath and guide the axons of the ORN along

their way from the olfactory epithelium through the lamina cribrosa into the olfactory bulb where they form synapses with their respective second order neurons in the glomerular layer (see Figure 2). OECs seem to support axonal regeneration in five different ways: Promoting axonal outgrowth and angiogenesis, migration of OECs into the lesion site, remyelination of axons, and axonal and tissue sparing (Franssen *et al.* 2007). OECs can be obtained from the olfactory bulb or the olfactory mucosa which makes them easily accessible and free of ethical concerns compared to stem cells.

These characteristics make OECs a promising candidate for cellular therapy after SCI and many studies have been conducted with varying results. The first *in vivo* experiment was conducted in 1994 by Ramon-Cueto and Nieto-Sampedro in a rat model of DRI. Implantation of purified OECs into the spinal cord at the DREZ led to regeneration of transected dorsal root axons into the spinal cord (Ramon-Cueto *et al.* 1994). After these auspicious results several other experiments followed. Most groups investigated the use of OECs in animal models of complete or partial spinal cord transection reporting regeneration, remyelination and even partial functional recovery (Imaizumi *et al.* 2000; Ramon-Cueto *et al.* 2000; Li *et al.* 2003; Lopez-Vales *et al.* 2006). Other studies concerning DRI include the work of Navarro *et al.,* which showed that recovery of electrophysiological functions after multiple dorsal rhizotomy was promoted by injecting a suspension of OECs into the DREZ (Navarro *et al.* 1999). Li *et al.* (2004) reported axonal outgrowth through the DREZ after providing a matrix containing OECs at the cut dorsal root stump.

After the initial excitement, doubts were raised by several studies questioning the benefits of OEC implantation. Gomez *et al.* (2003) could not observe regenerating axons crossing the DREZ after implantation of OECs into the dorsal column following multiple rhizotomy. Ramer *et al.* (2004) showed increased angiogenesis and decreased astrogliosis after transplanting OECs into the DREZ, the DRG or the dorsal column, but did not observe regenerating axons to cross the DREZ. Riddell *et al.* (2004) injected OECs into the DREZ and the cut dorsal root stump, but did not observe regeneration by electrophysiological or by histological investigations. Several other studies also showed poor survival and migration of OECs after transplantation into the spinal cord following transection (Lu *et al.* 2006) or contusion (Pearse *et al.* 2007).

Despite this lack of consistency between different experiments, implantation of OECs has already been used in clinical trials in humans. A small phase I clinical trial was performed without showing serious adverse effects or complications after implanting autologous OECs into the spinal cord of paraplegics at three years follow up. In the same trial some

improvement in sensitivity was observed (Feron *et al.* 2005; Mackay-Sim *et al.* 2008). Another pilot clinical study showed promising improvement of muscle activity and sensation in seven patients with chronic SCI after receiving OEC autografts, although this study was criticized because it did not include a control group (Lima *et al.* 2006). The largest and probably best known clinical trials have been conducted in China by Huang *et al.* reporting significant improvements in sensation, motor and vegetative functions after transplanting fetal olfactory tissue into the spinal cord of patients with chronic SCI (Huang *et al.* 2006; Huang *et al.* 2006; Huang *et al.* 2009). These publications are subject to heavy criticism, since they seem to not meet international standards (Dobkin *et al.* 2006). Furthermore, as fetal olfactory tissue was used instead of purified OECs, effects could also be attributed to other tissue components like stem cells. However, implantation of OECs should neither be discarded as therapeutic intervention nor employed in humans prematurely, but further research needs to be done in animal models to determine their true value in the treatment of SCI.

II.5 Glial cell line derived neurotrophic factor

Another approach to promote recovery from SCI is to enhance the inherent regenerative capacity of the affected neurons by applying neurotrophic factors. Several neurotrophic factors have been tried as candidates for the treatment of DRI and glial-cell-line-derived neurotrophic factor (GDNF) has proven promising.

Neurotrophic factors include three separate major groups: neurotrophins, neurokines and the GDNF family ligands (GFLs). The GFL consists of four members, artemin, neurturin, persephin and GDNF (Airaksinen *et al.* 2002). Their common main signaling pathway is via the receptor tyrosine kinase RET. GDNF forms a complex with the co-receptor GFR-α1, which then binds to the receptor domain of RET. This leads to the dimerisation and subsequent transphosphorylation of RET mediating intracellular signaling (Sariola *et al.* 2003).

GDNF was initially purified and described as a neurotrophic factor for midbrain dopaminergic neurons in 1993 (Lin *et al.* 1993). Further investigations revealed its trophic effect on motoneurons (Henderson *et al.* 1994) and all types of dorsal root ganglion neurons (Buj-Bello *et al.* 1995; Ramer *et al.* 2000), which makes GDNF a potential candidate for therapy after injuries to the nervous system.

In a rat model of DRI Ramer *et al.* (2000) showed that intrathecal application of GDNF promotes functional regeneration across the DREZ leading to recovery of thermal and

mechanical sensitivity, which was documented by electrophysiological and behavioral studies. In another experiment, bridging transplants enriched with GDNF improved axonal regeneration and remyelination after spinal cord injury (Iannotti *et al.* 2003). GDNF seems to promote regeneration in a dose dependant manner (Mills *et al.* 2007) by primary effects on the sensory neurons (Zhang *et al.* 2009). Moreover, intrathecal injections of GDNF can reduce neuropathic pain after dorsal root injury (Boucher *et al.* 2000).

Administration of GDNF after DRI seems desirable, but is problematic when applied to the patient. Neurotrophic factors are not bio-available, and blood brain barrier and blood-cerebrospinal fluid barrier severely limit their access to the CNS after parenteral application. Local intraparenchymal injection to the target site is invasive and does not provide the desired constant concentrations (Thorne *et al.* 2001). In most animal models, neurotrophic factors were delivered by the use of an osmotic pump (Ramer *et al.* 2000; Mills *et al.* 2007), which is an invasive procedure and does not provide long-term localized concentrations. The implantation of delivery vectors like gel foam enriched with neurotrophic factors is also limited by invasiveness and diffusion restrictions. Intrathecal injections would be necessary repeatedly and the use of an intrathecal catheter is associated with the risk of infections and cannot focus neurotrophic factors to the target site (Blesch 2000).

II.6 The combination of cellular therapy with application of neurotrophic factors

New delivery methods are possible using gene therapy. Two main approaches have been followed so far: direct *in vivo* gene transfer to the nervous tissue and *ex vivo* gene therapy. Direct gene therapy uses a single injection of a non-viral or viral delivery vector to transduce neural tissue *in vivo*. GDNF has been successfully applied after spinal cord contusion in rats using an adenoviral vector resulting in improved functional recovery compared to the control group (Tai *et al.* 2003). *In vivo* gene transfer of GDNF combined with microsurgery has been shown to promote partial recovery of nociception and proprioception in rats after cervical dorsal root section (Liu *et al.* 2009).

In vivo gene therapy is associated with some disadvantages like relatively unspecific distribution of the infectious particles, low concentrations of vectors in tissue and suboptimal conditions during transduction (incubation time, temperature, etc.), which can limit gene transfer efficiency. *In vivo* gene transfer using viral vectors can also result in acute or chronic immune responses of the host organism (Chirmule *et al.* 1999).

Another strategy is administration of neurotrophic factors by *ex vivo* gene therapy. *Ex vivo* gene therapy uses implantation of cells that are expanded and transduced in cell culture to deliver the transgene to the target site.

Several types of cells have been tried as vehicles for neurotrophic factors after spinal cord injury so far. Schwann cells genetically modified to secrete BDNF (Menei *et al.* 1998) and nerve growth factor (NGF) (Tuszynski *et al.* 1998) have been reported to promote axonal regeneration after SCI, but functional recovery could not be observed. Schwann cells do not seem to interact well with a CNS environment and can increase the glial scar by promoting the expression of proteoglycans in astrocytes (Plant *et al.* 2001). Fibroblasts have been modified to secrete GDNF and were implanted in the spinal cord after complete and partial transection leading to regeneration of several spinal systems (Blesch *et al.* 2003). However, fibroblasts do not show regenerative potential themselves and because of their non-CNS origin they might become tumorigenic (Cao *et al.* 2004). Other studies reported promising results using stem cells to deliver neurotrophic factors. For example, marrow stromal cells genetically engineered to overexpress BDNF were observed to facilitate regeneration after implantation to cystic sites of SCI (Lu *et al.* 2005).

Because of their inherent regenerative potential, we decided to employ OECs as a vehicle for GDNF delivery to the spinal cord. Ruitenberg *et al.* (2003) transduced OECs *ex vivo* using an adenoviral vector, and used these cells to deliver BDNF or NT-3 to the spinal cord after unilateral transection of the dorsolateral funiculus. Enhanced axonal sprouting was observed and behavioural studies showed improved functional recovery compared to the control group. In another study Cao *et al.* (2004) used OECs genetically modified to secrete high concentrations of GDNF. OECs were transduced with GDNF using a retroviral-based system and implanted into the spinal cord of adult rats with complete transection. Recovery was analysed on the morphological level and improved regeneration was reported compared to the control group (Cao *et al.* 2004). These results support the idea that combinational therapy is the right approach to overcome the plethora of factors preventing recovery from SCI.

Gene transfer can be achieved either by various transfection methods based on non-viral vectors or by viral transduction. Transfection methods include physical methods like electroporation, ultrasound, liposomal transfection and crystal co-precipitation. These procedures are time and cost effective, but do not result in stable long-term transgene expression, since plasmids are not integrated into the host genome and are therefore subject to rapid degradation. Furthermore, most transfection techniques have negative effects on cell viability and culture expansion (Niidome *et al.* 2002).

These disadvantages motivated the development of several viral transduction methods using adeno-, retro- and lentiviral vector constructs. Adenoviral transduction is widely used and very efficient, but as the adenoviral genome is localized episomally in the cell nucleus, transgene expression is rapidly lost in subsequent cell generations (Marienfeld *et al.* 1999). Retroviral vectors integrate into the host genome and are transmitted to following cell generations. However, most retroviruses can only infect dividing cells (Daly *et al.* 2000).

Lentiviruses belong to the family of retroviruses and integrate into the host genome providing stable long-term transgene expression that is passed on to subsequent cell generations (Cockrell *et al.* 2007). One of the main advantages of lentiviral based methods compared to other retrovirus-derived vectors is the ability to effectively transduce non-dividing cells as well as dividing cells (Naldini *et al.* 1996). Development of novel non-reproductive vectors provides high safety for their use *in vivo* (Kappes *et al.* 2003). Therefore lentiviral vector systems seem to be an ideal candidate for *ex vivo* gene therapy.

II.7 Aim of this study

The overall aim is to develop a successful treatment for spinal cord injury (SCI) by combining the beneficial effects of cellular therapy using OECs with constant local delivery of the neurotrophic factor GDNF. For this purpose the National Centre for Adult Stem Cell Research, Griffith University, Brisbane, generously provided us with an OEC cell line that was genetically modified using a lentiviral vector system to secrete high levels of GDNF. The particular aim of this study is to investigate the applicability of this cell line in a rat model of dorsal root injury before further experiments can be commenced.

First of all, the expression of the transgene GDNF by the genetically modified OEC cell line was verified. GDNF secretion rates were quantified in cell culture using ELISA.

Secondly, we investigated if genetic modification with the lentiviral vector significantly influenced cell survival or cell characteristics of the OECs *in vitro*. Immunocytochemistry was used to provide a general overview of the morphology and cell culture purity of the OECs after transduction.

However, the main focus of the study was to evaluate if transplantation of the modified OECs into the spinal cord of rats indeed resulted in high local concentrations of GDNF over a period of one month. For this purpose, we injected a suspension of OECs into the

rat spinal cord after crushing the cervical dorsal root. GDNF concentrations in spinal cord segments were then quantified over one month using ELISA.

Finally, survival of OECs in spinal cord sections was analyzed using Immunohistochemistry, one and seven days after DRI and OEC injection. In order to visualize the cells in the spinal cord after injection, OECs were labeled with the fluorescent marker CFSE. To establish, if labeling had an influence on GDNF secretion or cell survival, GDNF secretion rates were quantified and Immunocytochemistry was performed again after the cells had been labeled.

III. Material and Methods

III.1 Cell lines and culture

All OECs were generously provided by the National Centre for Adult Stem Cell Research, Griffith University, Brisbane, Australia. They were obtained from the olfactory mucosa of Australian Albino Wistar (AAW) rats as described previously (Bianco *et al.* 2004). After purification of the cell culture with fluorescence activated cell sorting (FACS) based on antibodies against p75, the cells were transduced *in vitro* to constantly secrete high concentrations of GDNF using a lentiviral vector system. All of these procedures were performed at the National Centre for Adult Stem Cell Research, Griffith University, Brisbane, Australia, and the exact protocols are unavailable, since they are patent protected.

After the cells arrived via same-day courier by plane in our facilities at the University of New South Wales, Australia, the transport medium was removed and replaced with pre-warmed DMEM plus 10% fetal calf serum (FCS). The cells were incubated at 37°C / 5% CO_2 until further use and the culture medium was replaced every second day. Cell culturing was carried out in a laminar flow hood (Clyde-Apac BH 2000) under sterile conditions.

III.2 Preparation of conditioned culture medium to determine secretion of GDNF *in vitro*

To quantify the amount of secreted GDNF and to confirm continuous production of GDNF over several days, GDNF secretion rates were assessed after one, three and seven days in our culture conditions. A total number of 14 cell batches were used for these experiments and six of the cell colonies were pre-labeled with CFSE using the Vybrant® CFDA SE Cell Tracer Kit (Molecular Probes) as described in section III.5 to assess the effect of labeling on GDNF secretion and/or cell survival.

Culture supernatant for determination of GDNF concentration using ELISA was collected on day one, three and seven as follows. Cells were incubated for 24 h before culture medium (DMEM plus 10% FCS) was removed from the flask and cells were rinsed twice with 5 ml of pre-warmed Hank's balanced salt solution (HBSS) without calcium, magnesium or phenolred (Lonza). To detach cells from the flask, 1 ml of 0.05% Trypsin /

0.02% Ethylendiamintetraacetate (EDTA) was added and incubated at 37°C for three minutes or until all of the cells were in solution, as judged by visual inspection with an inverted microscope (Olympus CK 40). Then 9.5 ml of culture medium were added and the solution was placed in a centrifuge tube to pellet the cells at 200 × *g* for five minutes. The supernatant was removed and cells were re-suspended in 1 ml culture medium. Cells were counted in a Neubauer-counting chamber under an inverted microscope. The cell suspension was diluted to a concentration of 500 × 10^3 cells in 2.5 ml fresh culture medium and incubated at 37°C for another 24 h. The supernatant was then collected and stored at -20°C until the GDNF concentration was determined using ELISA as described below. This procedure was repeated at day three and seven.

III.3 Enzyme linked Immunosorbent Assay

For GDNF quantification, ELISA was performed using the GDNF Emax™ ImmunoAssay System (Promega Corporations). All necessary buffers and solutions were prepared according to manufacturer's instructions and stored in aliquots at 4°C or -20°C.

Following the manufacturer's protocol, the Anti-GDNF Monoclonal Antibody (m-Ab) was diluted in carbonate coating buffer (0.025 M sodium bicarbonate, 0.025 M sodium carbonate, pH 8.2) and 100 µl were added to each well of a Nunc MaxiSorp™ Immunoassay 96-well plate (Nunc). The plate was covered with a lid, sealed with Parafilm® and incubated overnight at 4°C.

The next morning, all liquid was removed from the plate taking care not to cross contaminate the wells. After adding 200 µl of Block & Sample 1 × buffer to each well, the plate was covered with a lid and incubated at room temperature for one hour without shaking.

In the meantime, the GDNF standard curve was prepared in Eppendorf tubes rather than directly in the plate as suggested by the manufacturer in order to prevent scratching the surface with pipette tips. The supplied GDNF standard was diluted in Block & Sample 1 × buffer to achieve a range of concentrations from 7.8 to 1000 pg/ml.

After blocking the plate, all liquid was removed from the wells and 100 µl of the standard dilutions were applied in triplicates to the first three columns of the plate. Then 100 µl of each sample were added in duplicates to the designated wells and the plate was incubated with a lid for six hours at room temperature shaking at 50 rpm. Phosphate

buffered saline (PBS) was used as negative control in all assays. Samples were added at dilutions of 1:4 and 1:16, since these dilutions fell within the range of the standard curve.

The plate was then washed with 200 µl of Tris-buffered saline Tween-20 (TBST: 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% v/v Tween-20) five times taking care to remove all liquid and avoid cross contamination of wells.

Then 100 µl of the provided Anti-Human GDNF primary antibody diluted in Block & Sample 1 × buffer was added immediately to each well, and the plate was sealed and incubated overnight at 4°C without shaking.

On the third day the provided Anti-Chicken Ig-Y Horseradish peroxidase conjugate diluted in Block & Sample 1 × buffer was prepared. After washing the plate five times, 100 µl of the diluted antibody conjugate were added to each well. The plate was covered with a lid and incubated two hours at room temperature on a plate shaker.

After washing the plate five times with 200 µl of TBST, the color development was initiated by adding 100 µl of Tetramethylbenzidine (TMB) One Solution to each well. Following 15 minutes incubation at room temperature, the enzyme substrate reaction was stopped by addition of 100 µl of 1 N HCl to the wells in the same order in which substrate was added.

Absorbance at 450 nm was recorded within 20 minutes using a SpectraMax Plus plate reader (Molecular Devices) controlled by Softmax Pro version 4.7 software. The GDNF standard curve was generated by linear regression from the standard dilution series in triplicates using Microsoft Excel 2007.

III.4 Immunocytochemistry

Round cover slips (22 mm diameter, 0.13-0.17 mm thickness; SMIEC) were placed in a 12 well tissue culture plate (Linbro 4.5 cm² surface) and coated by adding 200 µl of a 0.1 mg/ml Poly-L-Lysine solution per well. Excess reagent was removed and cover slips were dried for two hours. Cells were passaged and seeded onto the Poly-L-Lysine coated cover slips at 10×10^3 cells for three days of incubation or 20×10^3 cells for one day of incubation per well to achieve approximately 50% confluency. Incubation was at 37°C in DMEM containing 10% fetal calf serum. Half of the medium was replaced every second day to provide sufficient nutrients for the cells.

After one or three days culture medium was removed and wells were washed with 0.1 M PBS before cells were fixed with 4% Paraformaldehyde (PFA) in 0.1 M PBS for five minutes at room temperature. After three wash cycles on a plate shaker at 60 rpm with 0.1 M PBS, the cover slips were blocked for one hour on a plate shaker in 10% normal goat serum (NGS; Chemicon) in 0.1 M PBS. For tissue sections, 0.1 M PBS containing 0.2% Triton X-100 (PBST) was used in all steps instead of 0.1 M PBS.

Then 200 µl of primary antibodies diluted in blocking solution as shown in Table 1 were added and incubated overnight in a humid chamber at 4°C. As negative controls, slides were incubated without the primary antibody in blocking solution only. For glial fibrillary acidic protein (GFAP), immuno-staining samples of a rat ischiadic nerve were used as negative controls and spinal cord sections as positive controls.

Table 1: Primary and secondary antibodies used for Immunohisto- and Immunocytochemistry

Antigen	Primary antibody [manufacturer]	Dilution	Secondary antibody [manufacturer]	Dilution
p75	P75-Ab (Mouse) [Chemicon]	1/200	Goat Anti-Mouse Alexa Fluor 568 [Molecular Probes]	1/500
ED-1	ED-1-Ab (Mouse) [Serotec]	1/1000	Goat Anti-Mouse Alexa Fluor 568 [Molecular Probes]	1/500
GFAP	GFAP-Ab (Rabbit) [Dako]	1/1000	Swine Anti-Rabbit Ig TRITC-labeled [Dako]	1/200

The next day, cover slips were washed three times in 0.1 M PBS for five minutes each. 0.5 ml of secondary antibody diluted in PBST as shown in Table 1 were added and followed by incubation for one hour at room temperature on a plate shaker wrapped in foil.

After another three washes, the nuclei were counterstained with 1 µM Hoechst Blue Nuclear Stain (Hoechst) diluted in 0.1 M PBST for 15 minutes. Following two more washes with 0.1 M PBST, the slides were mounted onto Superfrost® plus glass slides with Dako Cytomation Fluorescent Mounting Medium and sealed with nail polish. Slides were stored in darkness at 4°C until evaluation. Images were captured with an Olympus DP 70 digital camera mounted onto an Olympus BX- 51 microscope and processed with Adobe Photoshop.

III.5 Labeling OECs with CFSE

To be able to detect and visualize OECs after injection into the spinal cord, OECs were labeled with CFSE using the Vybrant® CFDA SE Cell Tracer Kit (Molecular Probes). CFSE is a well-established cell marker, which has intense and long-lasting (over three weeks) fluorescene. It has already been used with OECs and did not have an apparent influence on cell survival (Imaizumi *et al.* 2000; Deng *et al.* 2006).

Cells were incubated with 10 µM CFSE in DMSO for 15 min at 37°C followed by a wash with pre-warmed culture medium (DMEM plus 10% FCS) and incubation in fresh medium for at least 12 hours prior to implantation.

III.6 Animal study

III.6.a Experimental design

A total number of 44 inbreed Australian Albino Wistar (AAW) rats were used for this study. The rats were kept at a 12 h light / 12 h dark cycle in cages with a maximum number of five animals each. At all times procedures were performed according to the University of New South Wales (UNSW) animal ethics committee. Food and water was provided ad libitum.

The animals were divided into four major groups:

1. Tissue samples from seven rats without DRI and without OEC implantation were collected in order to determine the physiological amount of GDNF in the spinal cord using ELISA. This represents the control group.

2. Six rats underwent surgery for DRI without OEC implantation and tissue was collected one or three days after injury in order to determine if DRI itself has an effect on the amount of GDNF in the spinal cord.

3. The main group of 27 rats underwent surgery for DRI and received an implantation of OECs at the same time. Tissue samples were collected after 1, 3, 7, 14 and 30 days to determine the GDNF concentration in tissue over time.

4. Tissue samples from four rats that received DRI followed by OEC implantation were obtained for Immunohistochemistry in order to evaluate cell survival after implantation.

Figure 3: Operation site with stereotactic device

III.6.b Surgical procedure

A hemilaminectomy of the vertebrae C6 and C7 was performed followed by a crush of the dorsal roots and an injection of OECs into the spinal cord segments C7 and C8 as follows:

The rats were weighed and anaesthetised with an intra-peritoneal (i.p.) injection of Ketamine (10 mg / 300 g bodyweight) and xylazine (8 mg / 300 g bodyweight). Respiratory rate, rectal temperature and presence / absence of the hind paw withdrawal reflex were recorded in 15 minute intervals. If anesthetic top-up was required, animals received an i.p. bolus of 5 mg Ketamine.

After shaving the operation site on the back of the rats, animals were put onto the operating table and 0.3 ml of Bupivacaine (5 mg/ml) were applied subcutaneously along the cutting line. Saline was placed on eyes to prevent drying. The incision from C2 to T2 was performed using a no. 22 scalpel blade. The exposed superficial and deep muscles were blunt-dissected away from the midline using rounded-tip scissors to expose the spinous and lateral processes and retractors were inserted. Under the microscope, remaining tissue was removed from the spinal column. Bupivacaine (0.1 ml) was dropped on vertebras C6 and C7 prior to performing a hemilaminectomy by carefully removing the spinous and lateral processes on the right side using rongeurs. Damage to the spinal cord was avoided. The dura mater was cut using iris scissors and the C7 and C8 dorsal roots on the right side were crushed proximal to the dorsal root ganglion with a No. 5 forceps three times for ten seconds each. An additional 0.1 ml of Bupivacaine was dropped onto the cord and root surface before the crush.

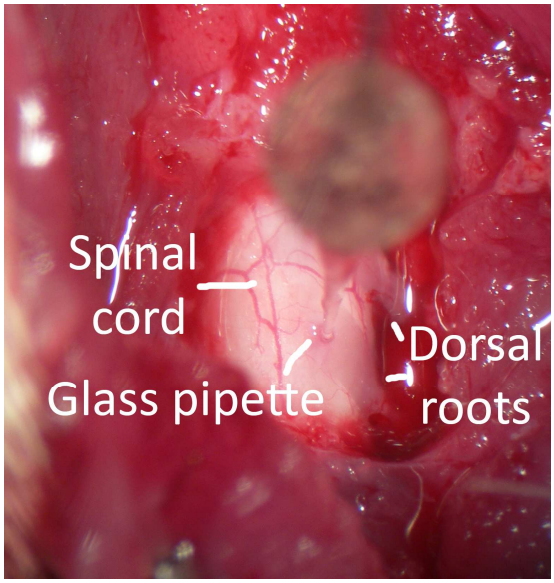

Figure 4: Injection of OECs into rat spinal cord

III.6.c Cell implantation

Cells were washed three times with HBSS, trypsinized, triturated, counted, washed and pelleted as described in Section III.2, and suspended in culture medium (DMEM plus 10% FCS) at a concentration of 120×10^3 cells per µl. The viability of cells treated in such a way was confirmed to be more than 90% by Trypan blue (Sigma) staining. Immediately after the root crush, 0.5 µl of this OEC suspension was injected 1 mm deep and 1 mm to the midline of each segment C7 and C8. For the injection, a glass pipette glued to a 5 µl Hamilton syringe fixed in a stereotactic device was used with an internal diameter at the tip of approximately 80 µm and an external diameter of 100 µm (Figure 3 and Figure 4). The injection was performed slowly over two minutes.

The retractors were removed, deep and superficial muscles were sutured with 4.0 chromic gut absorbable sutures, and the skin was closed with metal clips. The wound was cleaned with a saline swap and the animal was transferred into a cage with the 15 minute interval monitoring continued until the animal awoke.

III.6.d Postoperative care

Immediately after surgery and over the first three postoperative days, animals received a daily bolus of 0.1 ml Keflin (Cephlotin sodium 100 mg/ml) and 0.025 ml Rimadyl (Carprofen 50 mg/ml). The animals were weighed daily for the first week post-op and their behaviour was observed. To prevent autophagia and scratching, bitter spray was applied onto the forepaw and the wound immediately after surgery and daily for the first week. The skin metal clips were removed two weeks after surgery.

III.6.e Perfusion and dissection

Rats were deeply anaesthetized by i.p. injection of pentobarbitone (Lethabarb Virbac 325 mg/ml, 0.3 ml per 100 g of bodyweight). When breathing had stopped, the liver and diaphragm were exposed by a transversal cut through skin and abdominal muscles. The diaphragm was removed from the rib cage and the ribs were cut caudo-rostrally using large blunt-tipped scissors and clamped out of the way.

After clearing the heart of connective tissue and fat, 0.1 ml pure heparin were injected into the heart through the left ventricle. A small hole was cut in the left ventricle and a round-tipped perfusion needle was inserted and advanced to the aorta. The needle was clamped just rostral to the apex. Perfusion was started with 250 ml of 0.9% saline

containing 500 IU heparin/ml. A small hole was cut in the right atrium to allow the perfusate to escape. In rats designated for Immunohistochemistry, perfusion was continued with 400 ml of 4% Paraformaldehyde (PFA) in 0.1 M.

The perfused animals were dissected, and the C7 and C8 segments were removed separately. Samples for ELISA were collected in microcentrifuge tubes, labeled, and immediately snap frozen in liquid nitrogen for storage at -80°C until further use. Tissue samples for Immunohistochemistry were collected, post-fixed for 24 hours with 4% PFA in 0.1 M PBS and cryo-protected with 30% sucrose in 0.1 M PBS at 4°C for at least 48 h or until the tissue sunk. Samples were embedded in Tissue-Tek® optimum cutting temperature (OCT) (Sakura Finetek) and stored at -20°C.

III.7 Tissue sample preparation for ELISA

In order to increase the amount of detectable GDNF, tissue samples were subjected to acid treatment as described previously (Okragly *et al.* 1997).

Lysis buffer containing 20 mM Tris, pH 8.0, 137 mM NaCl, 1% nonylphenoxylpolyethoxylethanol (NP-40), 10% glycerol, 1 mM phenylmethylsulfonylfluorid (PMSF), 0.5 mM sodium Vanadate, 10 µg/ml aprotinin and 1 µg/ml leupeptin was prepared and stored in aliquots at -20°C until use.

Chilled lysis buffer was added to tissue samples thawed on ice at a ratio of 30 µl per 100 mg of tissue. The tissue was then homogenized using a microcentrifuge tube pestle for one minute and centrifuged at room temperature at 13200 rpm for 20 minutes.

The supernatant was diluted 1:5 with Dulbecco's PBS (200 mg/l KCl, 8.0 g/l NaCl, 200 mg/l KH_2PO_4, 1.15 g/l Na_2HPO_4, 133 mg/l $CaCl_2 \cdot 2H_2O$, 100 mg/l $MgCl_2 \cdot 6H_2O$). Then 1 µl of 1 N HCl was added per 50 µl sample. The sample was mixed thoroughly using a mini-vortex and the pH was confirmed to be less than three using litmus paper. After incubation for 15 min at room temperature, 1 µl of 1 N NaOH was added per 50 µl of sample and the pH was confirmed to be approximately 7.6.

Samples were stored on ice for use on the same day. Samples were centrifuged again at room temperature at 13200 rpm for 20 minutes immediately prior to the assay.

III.8 Immunohistochemistry

Tissue blocks were cut to a thickness of 30 µm using a HM 500 OM Microtome at -20°C. Sections were placed on 2% gelatinized glass slides and allowed to dry overnight at room temperature in a fume hood.

Slides were bathed in Histochoice Clearing Agent (Astral Scientific) followed by a series of alcohol washes at 100%, 100%, 95% and 70% ethanol for two minutes each. After washing with 0.1 M PBS for 10 minutes on a shaker, sections were blocked by shaking for one hour with 10% NGS (Chemicon) in 0.1 M PBST. To separate sections on a single slide, Dako™ delineating pen (Dako™) was used. The subsequent steps were performed as described in section III.4 for Immunocytochemistry.

III.9 Statistical analysis

In vitro ELISA data was analyzed for statistical significant ($p<0.05$) differences between cell groups using one-way and uni-variate ANOVA. *In vivo* ELISA results from different animal groups were compared using one-way ANOVA and Tukey post-hoc pair-wise comparison. All statistical analysis was performed with SPSS version 16.0.

IV. Results

IV.1 Immunocytochemistry

IV.1.a Labeling OECs with CFSE

In order to be able to visualize OECs after implantation in the spinal cord and to assess their morphology in cell culture, cells were labeled with the fluorescent marker CFSE. All OECs showed bright fluorescence seven days after labeling (Figure 5). CFSE remained in the cytoplasm and did not leak.

The two typical morphologies of OECs in culture, a spindle-shaped and flattened type (Vincent *et al.* 2005) are represented. Some cells were fixed during mitosis indicating good cell survival and culture expansion (Figure 5).

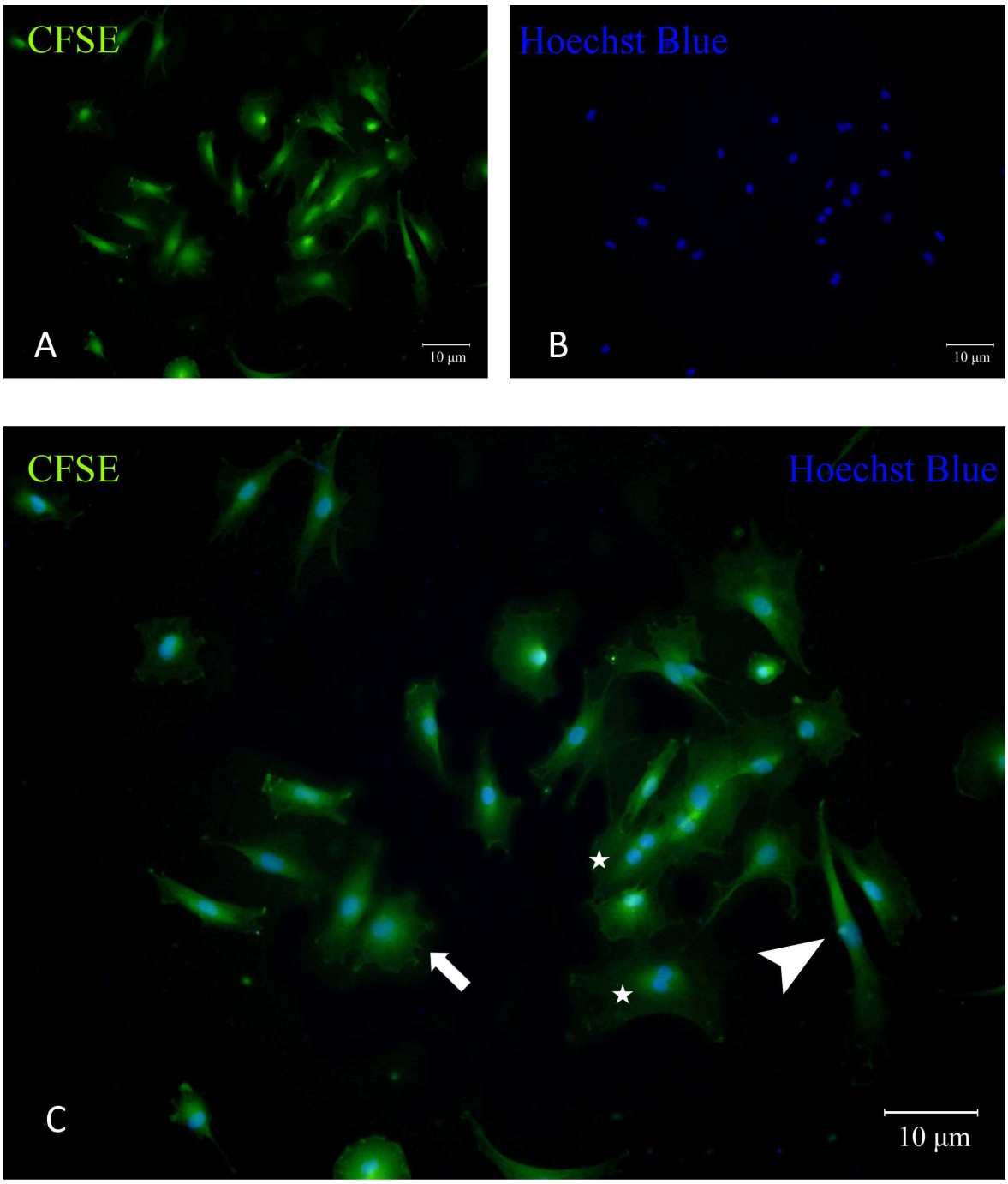

Figure 5: OECs seven days after labeling with CFSE.

A: Green fluorescence of CFSE. B: Hoechst Blue 33342 nuclear counter-stain. C: Merged image. Spindle-shaped (arrowhead) and flattened cells (arrow) as well as cells in mitosis (stars) can be seen.

IV.1.b Cell culture purity

In order to give an overview of the purity of cell culture, ten different batches of cells were stained for p75 as described in section III.4. P75 is a low affinity growth factor receptor and is used as a marker for OECs. In our study, FACS based on antibodies against p75 was used to purify cell cultures prior to viral transduction, which was performed at the Griffith University in Brisbane as mentioned in the preface.

Immuno-staining of cells for p75 was repeated once the genetically modified cells arrived in our facilities in order to confirm the purity of the cell batches that we received. The vast majority of cells was positive for p75 as exemplified in Figure 6. All cells that were negative for p75 had a flattened appearance indicating minor contamination of the cell culture with fibroblasts or other cells.

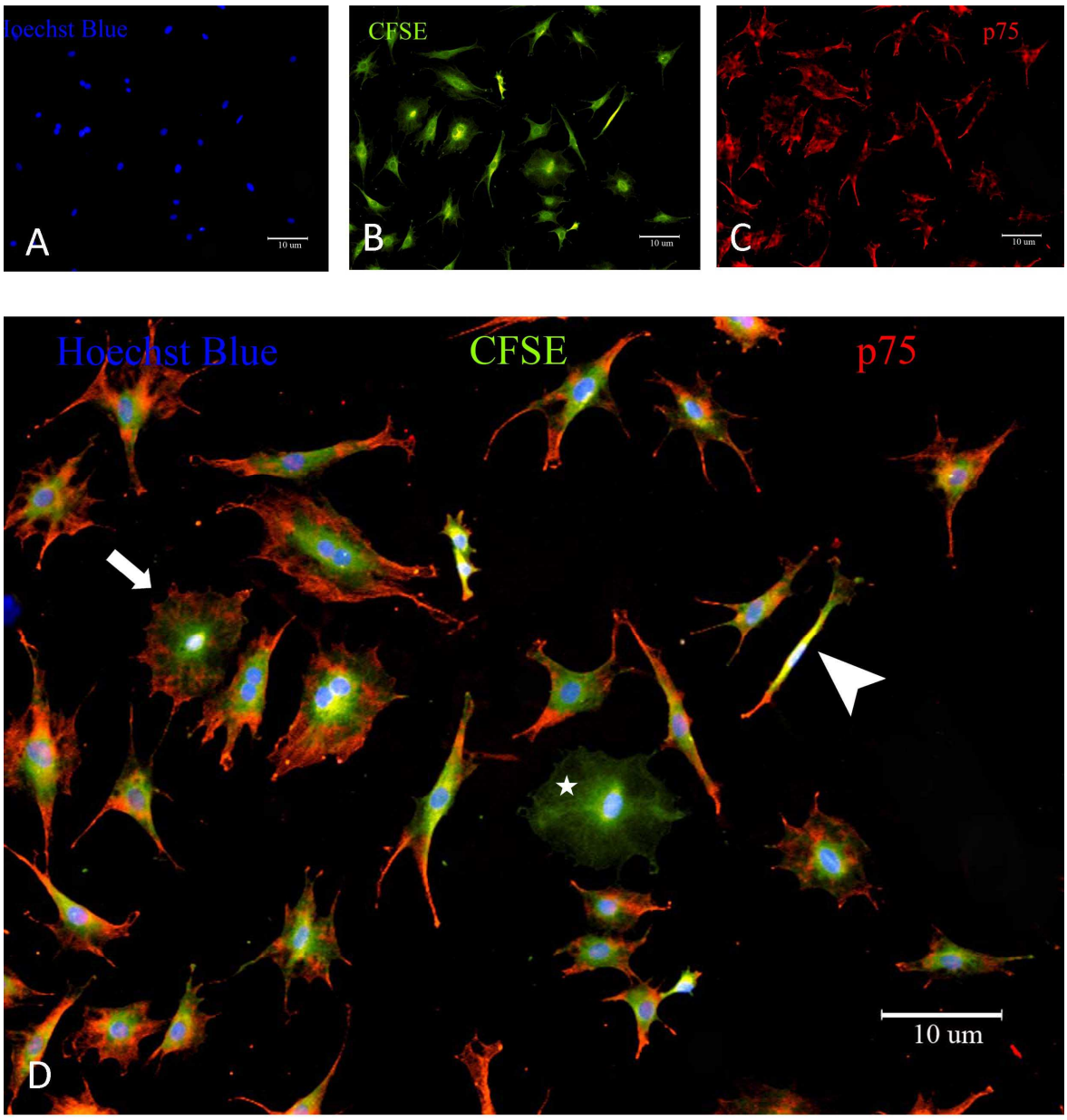

Figure 6: OECs stained for p75.

A: Nuclear counter-stain with Hoechst Blue. B: Fluorescent labeling with CFSE C: Immuno-stain for p75. D: Merged Image. The vast majority of cells are positive for p75 and show flattened (arrow) or fusiform morphology (arrowhead). A single cell (star) in this image is p75 negative and displays fibroblast like features.

IV.2 *In vitro* secretion of GDNF

IV.2.a Secretion of GDNF of unlabeled OECs

In order to quantify the duration and level of GDNF secretion *in vitro* after viral transduction, culture supernatant was collected, and GDNF concentrations were determined using the ELISA protocol as described in section III.3. Figure 7 shows an example of a standard curve that was used for calculation of GDNF concentrations for an ELISA plate.

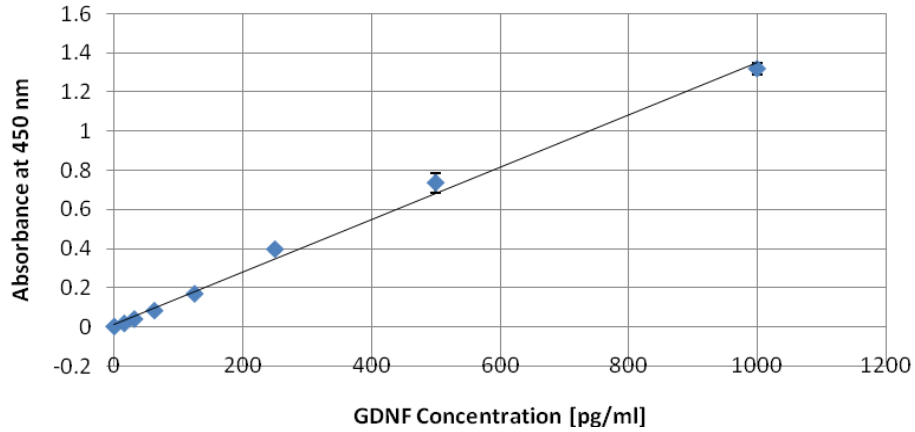

Figure 7: Example of a standard curve, which was used for ELISA.

Error bars represent one-fold standard deviation of the standard dilution triplicates. The graph shows the function derived by linear regression ($y=0.0013406x + 0.0120123$, $R^2 = 0.9951815$).

GDNF secretion rates were determined for six batches of transduced OECs at day one, three and seven in our culture conditions. GDNF secretion rates were calculated to be 13.1 ±0.4, 11.6 ±0.8 and 11.5 ±0.8 pg per 10^3 cells in 24 h (mean ±SEM) at day one, three and seven respectively (Figure 8).

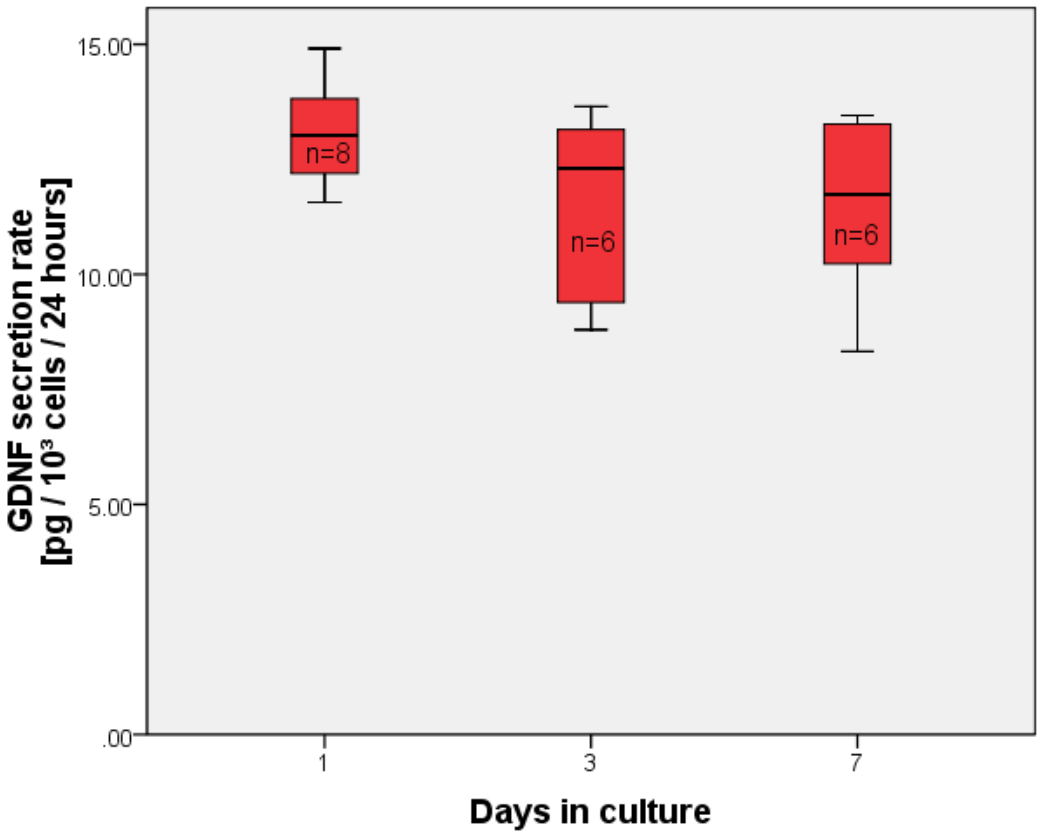

Figure 8: Boxplot showing the GDNF secretion rates of transduced GDNF OECs after one, three and seven days in culture.

N: number of cell batches used for analysis.

Comparison of these GDNF secretion rates at different days in culture in a one-way ANOVA test shows no significant (*p=0.05*) difference of *in vitro* GDNF secretion rates of transfected OECs after one, three and seven days as shown in

Table 2. This shows, that GDNF secretion *in vitro* remains stable for at least one week.

Table 2: One-way ANOVA test on *in vitro* GDNF secretion rates of unlabeled OECs in culture after one, three and seven days.

No significant (p=0.05) difference between varied number of days in our culture conditions can be found.

ANOVA

Dependent Variable: GDNF secretion rate [pg/10³ cells in 24 hours]					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.459	2	5.729	2.02	0.163
Within Groups	48.223	17	2.837		
Total	59.681	19			

IV.2.b Secretion of GDNF of unlabeled OECs

In order to determine if fluorescent labeling affected GDNF secretion, secretion rates were determined for six OEC batches after the cells had been labeled with CFSE. GDNF was secreted at a rate of 14.0 ±0.3, 12.4 ±0.9 and 12.4 ±0.7 pg per 10^3 cells in 24 h (mean ±SEM) at day one, three and seven respectively (Figure 9).

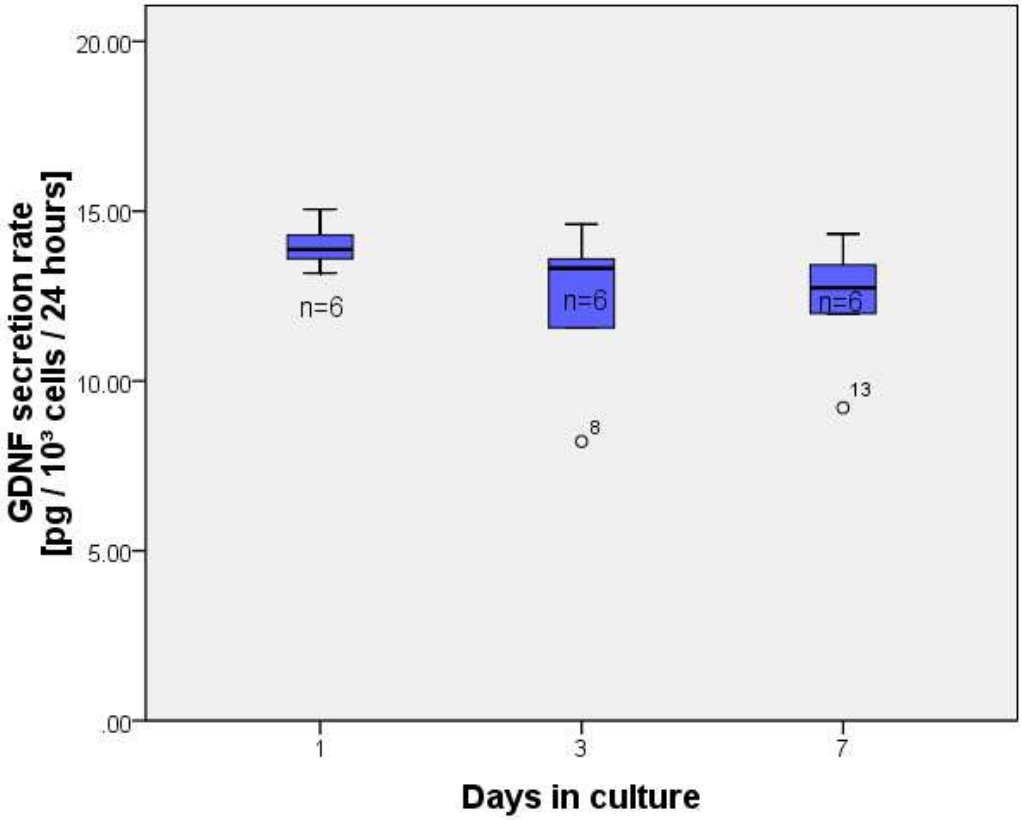

Figure 9: Boxplot showing the GDNF secretion rates of transduced OECs labeled with CFSE using the Vybrant® CFDA SE Cell Tracer Kit (Molecular Probes) after one, three and seven days in culture.

One-way ANOVA did not reveal a significant (*p=0.05*) difference between GDNF secretion rates of OECs labeled with CFSE after one, three and seven days in culture (Table 3).

Table 3: One-way ANOVA test on *in vitro* GDNF secretion rates of prelabelled OECs in culture after one, three and seven days.

No significant difference (p=0.05) between varied number of days in culture can be found.

ANOVA

Dependent Variable: GDNF secretion rate [pg/10^3 cells in 24 hours]					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.678	2	4.839	1.657	0.224
Within Groups	43.811	15	2.921		
Total	53.489	17			

IV.2.c Comparison of GDNF secretion between labeled and unlabeled OECs

GDNF secretion rates of labeled and unlabeled cells were compared to determine if fluorescent labeling with CFSE has an influence on transgene expression (Figure 10).

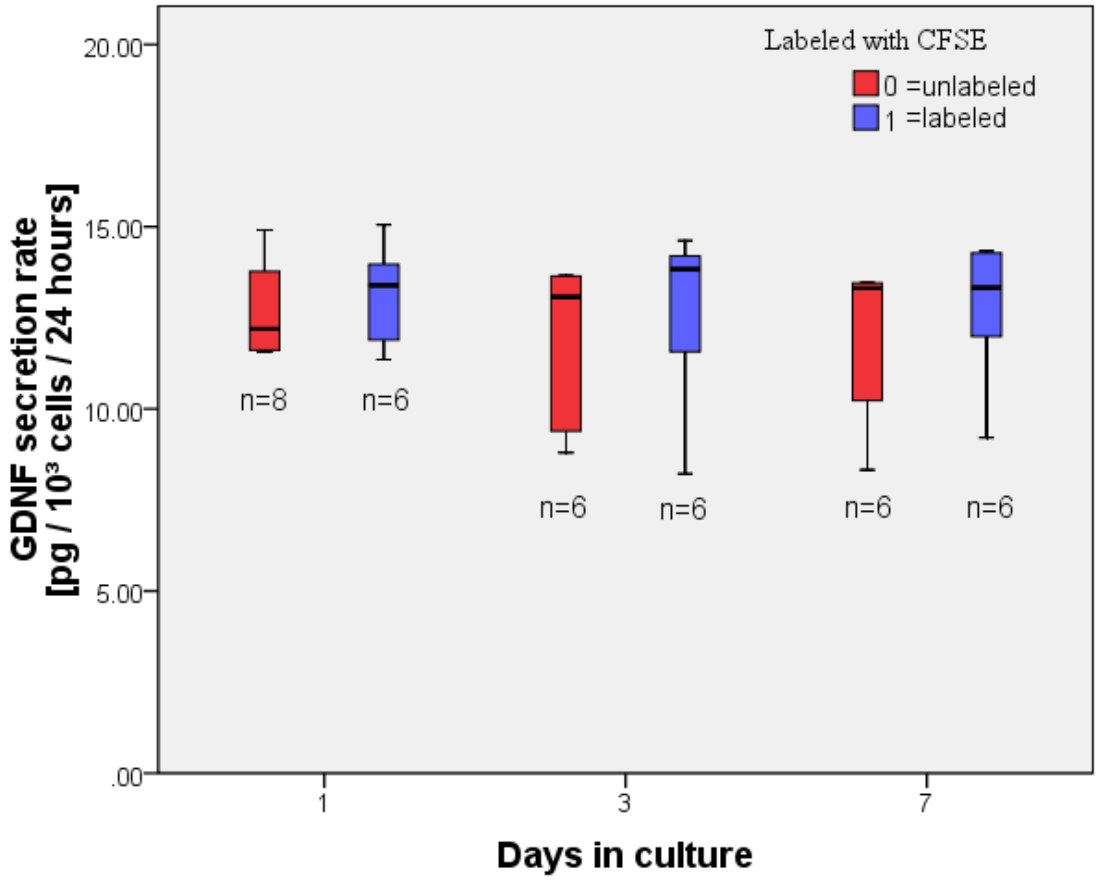

Figure 10: Boxplot showing the *in vitro* GDNF secretion rates after one, three and seven days in culture of labeled (blue) or unlabeled (red) OECs.

Univariate ANOVA did not reveal a significant (*p=0.05*) difference in GDNF secretion rates between labeled and unlabeled OECs (Table 4).

Table 4: Univariate ANOVA with GDNF secretion rate as the dependant variable and days in culture and pre-labeling status as factor variables.

The test shows no significant effect for both factor variables at a level of significance of p=0.05.

Tests of Between-Subjects Effects

Dependent Variable: GDNF secretion rate [pg/10^3 cells in 24 hours]					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	27.165a	5	5.433	1.889	0.124
Intercept	5861.452	1	5861.452	2038	0
Days	20.963	2	10.482	3.645	0.068
CFSE	7.614	1	7.614	2.647	0.114
Days * CFSE	0.017	2	0.008	0.003	0.997
Error	92.033	32	2.876		
Total	6077.368	38			
Corrected Total	119.198	37			

a. R Squared = .228 (Adjusted R Squared = .107)

IV.3 *In vivo* concentrations of GDNF

IV.3.a Control groups without OEC implantation

To determine the physiological amount of GDNF in the spinal cord, tissue samples from seven rats without DRI and OEC implantation were collected and the amount of GDNF was measured using ELISA. Only spinal cord segments between the cervical levels C5 to the thoracic segment T2 were used for analysis to reduce segmental variations. A total number of 26 spinal cord segments showed an average concentration of GDNF of 42.7 ±5.0 pg per g of tissue (mean ±SEM) as shown in Figure 11.

In order to determine if DRI itself without OEC implantation has an effect on the amount of GDNF in spinal cord, six rats underwent surgery for dorsal root injury without subsequent implantation of OECs. After one or seven days, the rats were perfused as described above and the two segments C7 and C8 with unilateral DRI were collected for quantification of GDNF. At day one and day seven after DRI, the amount of GDNF was 24.2 ±7.0 and 31.3 ±7.7 pg per g of tissue (mean ±SEM) respectively (Figure 11).

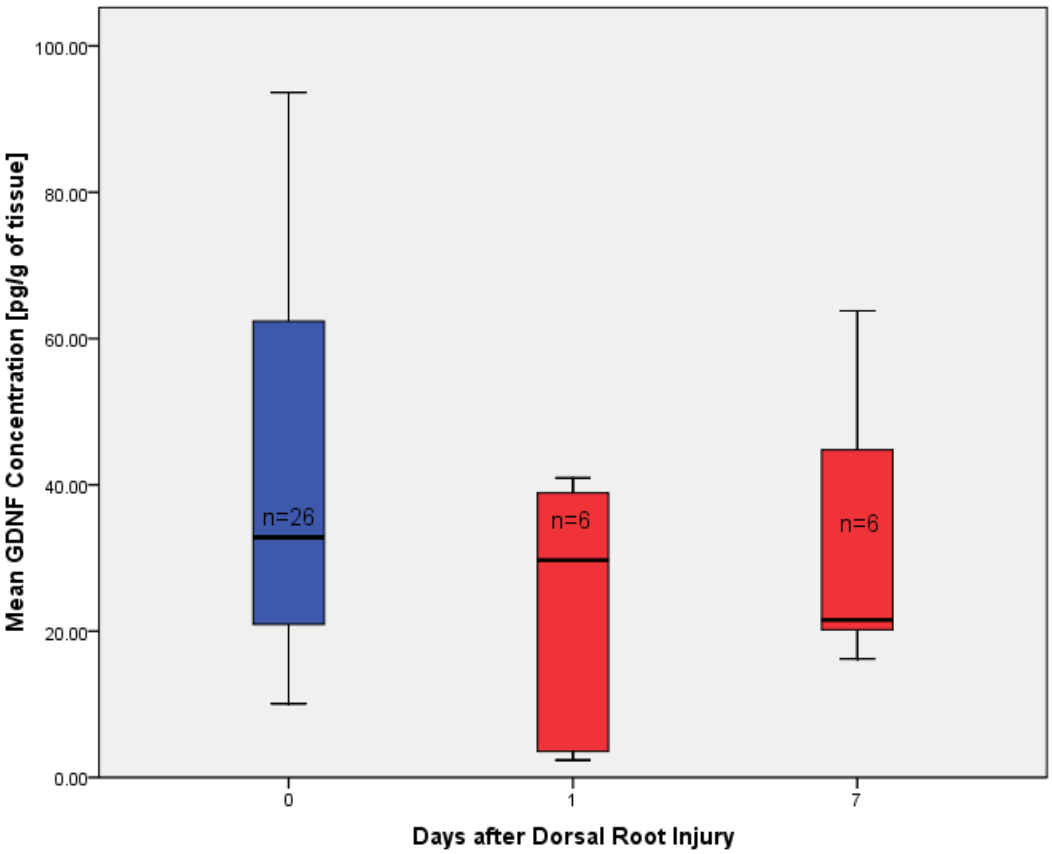

Figure 11: Boxplot showing the amount of GDNF in spinal cord segments of AAW rats without dorsal root injury (blue) and one and seven days after dorsal root injury without OEC implantation (red).

N: Number of spinal cord segments used for analysis.

One-way ANOVA showed no significant (*p=0.05*) difference in the amount of GDNF between the spinal cord segments of rats with and without DRI at any day (Table 5). DRI does not influence the amount of GDNF in the first seven days after the event.

Table 5: One-way ANOVA shows no significant (*p=0.05*) difference in the amount of GDNF between the spinal cord segments of rats without DRI and with DRI but without OEC implantation after one and seven days.

Amount of GDNF in spinal segment [pg/g of tissue]

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1977.03	2	988.515	1.781	0.183
Within Groups	19422.282	35	554.922		
Total	21399.312	37			

IV.3.b Amount of GDNF after dorsal root injury and OEC implantation

To establish, if there is an increased amount of GDNF in the spinal cord after DRI followed by OEC implantation, 27 rats underwent surgery for DRI and received an injection of OECs at the same time. Samples were collected after 1, 3, 7, 14, 21 and 30 days for determination of the amount of GDNF in spinal cord segments over a time-course (Figure 12).

For spinal cord segments, which were examined one day after OEC injection, the amount of GDNF was calculated to be 122.8 ±5.0 pg per g of tissue (mean ±SEM). Samples, which were collected 3, 7, 14, 21 and 30 days after cell implantation contained 87.3 ±13.0, 89.3 ±3.9, 35.0 ±3.2, 34.3 ±10.4 and 53.7 ±8.4 pg of GDNF per g of tissue (mean ±SEM), respectively.

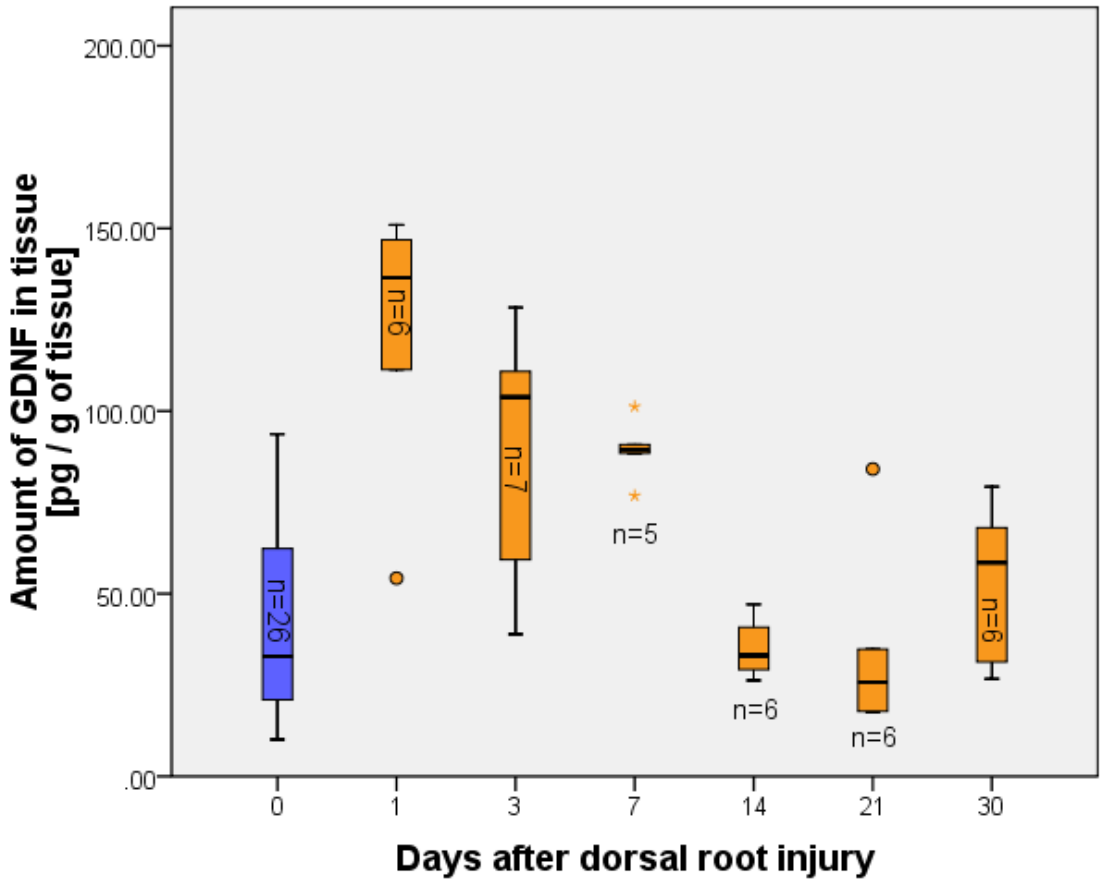

Figure 12: Amount of GDNF in spinal cord tissue of AAW rats without DRI and without OEC implantation as normal controls (blue) compared to AAW rats with DRI and OEC implantation (orange) at day 1, 3, 7, 14, 21 and 30 post-implantation.

N: Number of samples used for analysis; Circles: outliers; stars: far outliers.

Post-hoc Tukey pair-wise comparison (Table 6) shows that there is a highly significant ($p<0.01$) increase in the amount of GDNF in the spinal cord segments C7 and C8 of rats with DRI and OEC implantation at day one, three and seven after implantation compared to the baseline. This shows that implantation of our genetically modified OECs indeed increased the amount of GDNF in the spinal cord significantly for up to one week.

However, beyond 14 days following OEC injection, a significant difference in the amount of GDNF compared to the amount of GDNF in spinal cords without DRI and OEC injection is lacking. Therefore, after one week of augmented GDNF concentrations, the amount of GDNF decreased to baseline level.

Table 6: Tukey post-hoc pair-wise comparison of the amount of GDNF in spinal cord of AAW rats without DRI and without OEC implantation (days 0) compared to AAW rats with DRI and OEC implantation at day 1, 3, 7, 14, 21 and 30 after implantation.

Dependant variable: Mean Amount of GDNF [pg/g of tissue]

(I) days	(J) days	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	1	-80.08038*	11.56661	0	-115.4747	-44.6861
	3	-44.66324*	10.87464	0.002	-77.9401	-11.3864
	7	-46.66238*	12.47104	0.008	-84.8243	-8.5005
	14	7.74128	11.56661	0.994	-27.653	43.1356
	21	8.35462	11.56661	0.991	-27.0397	43.7489
	30	-11.04705	11.56661	0.961	-46.4414	24.3473

IV.4 Immunohistochemistry

To investigate OEC survival after implantation into the spinal cord, spinal cord cross sections were examined histologically one and seven days after OEC implantation. To be able to detect OECs in the spinal cord, OECs were labeled with the fluorescent cell marker CFSE prior to implantation as described above.

In order to visualize the central nervous tissue, immunostaining for Glial fibrillary acidic protein (GFAP) was performed. GFAP is a marker for astrocytes and is widely used in immunohistochemical staining to display central nervous tissue, e.g. in the spinal cord (Deng *et al.* 2006).

To show phagocytosing cells, tissue sections were stained immunohistochemically with antibodies against ED-1. ED-1 is a specific cellular marker for activated microglia, monocytes and macrophages in rats (Deng *et al.* 2006).

IV.4.a Spinal Cord sections one day after surgery

Immunological staining for GFAP was performed on spinal cord cross sections at the level of C8 at one day after surgery for DRI and labeled OEC implantation. Figure 13 shows the injection site, which is characterized by a dense accumulation of CFSE-labeled OECs in the spinal cord at a depth of about 1 mm. The astrocytes in the central nervous tissue show intense immunoreactivity for GFAP. In the dorsal root, the transitional zone can be seen where the CNS environment changes to a PNS environment, which is free of astrocytes.

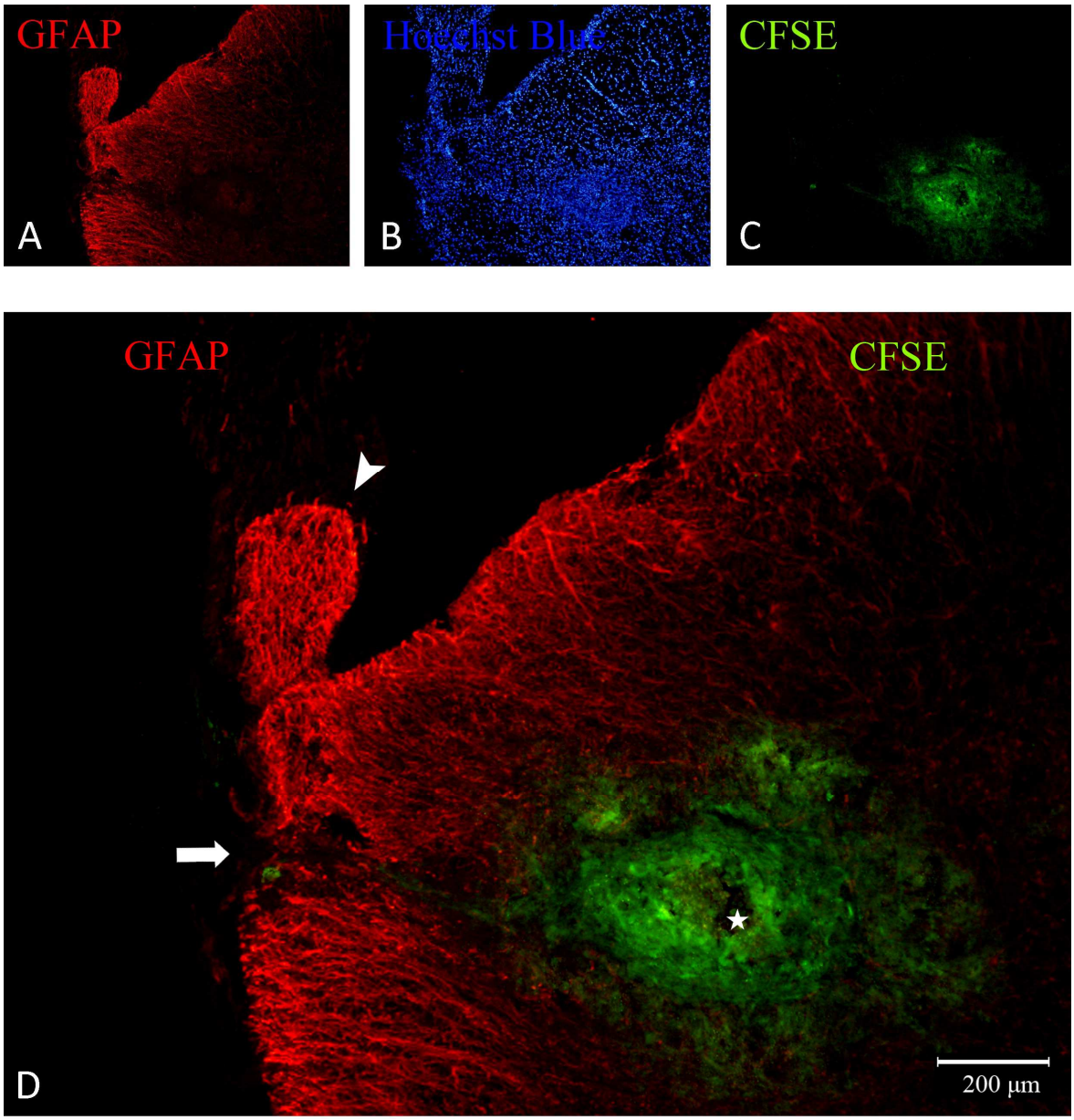

Figure 13: Spinal cord cross section showing implanted OECs one day after DRI.
A: GFAP immunostain with GFAP-Ab. B: Hoechst Blue nuclear counter-stain. C: CFSE-labeled OECs. D: Merged image of A+C. OECs (star) are located in the spinal cord after implantation through the injection site (arrow) at the dorsal surface of the spinal cord. In the dorsal root (arrow head) the transitional zone can be seen, where GFAP immunoreactivity changes from positive to negative.

Figure 14 shows the same section at higher magnification. A disruption of tissue can be seen in the injection pathway. Most CFSE-labeled OECs are localized deep in the spinal cord with only few cells present along the injection trajectory. In some areas, the fluorescent signal of CFSE can be distinguished as the outline of a cell, which also overlaps with a counter-stained nucleus, thus indicating intact OECs after injection.

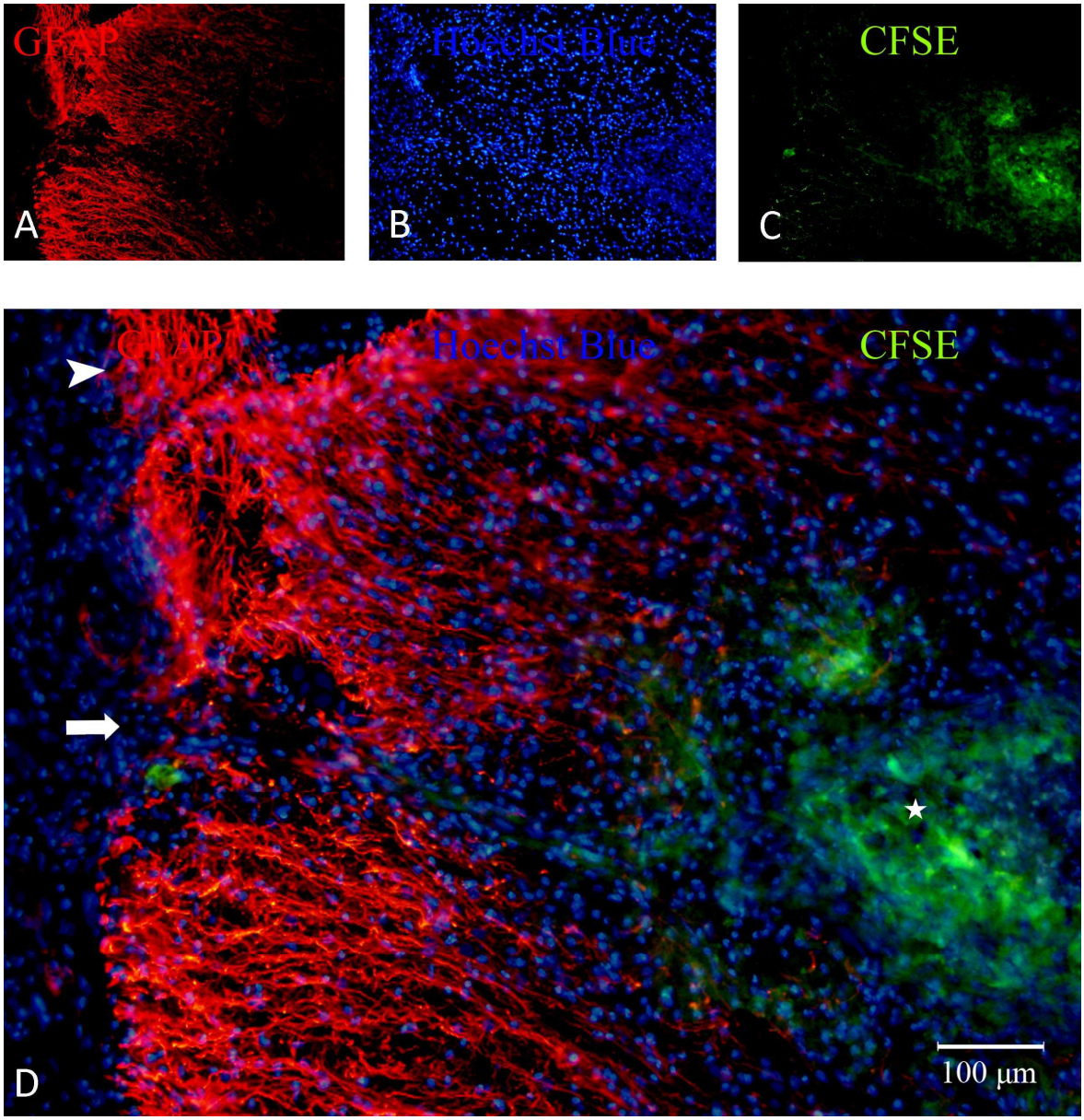

Figure 14: Spinal cord cross section showing OECs one day after implantation at higher magnification.

A: GFAP Immunostain with GFAP-Ab (Rabbit). B: Hoechst Blue nuclear counterstain. C: CFSE-labeled OECs. D: Merged image of A, B, and C. Injection site (arrow), dorsal root (arrow head) and implanted OECs (star).

In order to visualize phagocytosing cells, immuno-staining for ED-1 was performed on spinal cord cross sections one day after surgery and cell implantation (Figure 15). There is an accumulation of ED-1 positive cells in the crushed dorsal root and the corresponding dorsal root entry zone. A high concentration of phagocytosing cells can also be seen at the OEC implantation site whereas the contra-lateral side of the spinal cord remains mainly free of ED-1 positive cells. This indicates phagocytic activity in the injured dorsal root and at the OEC injection site.

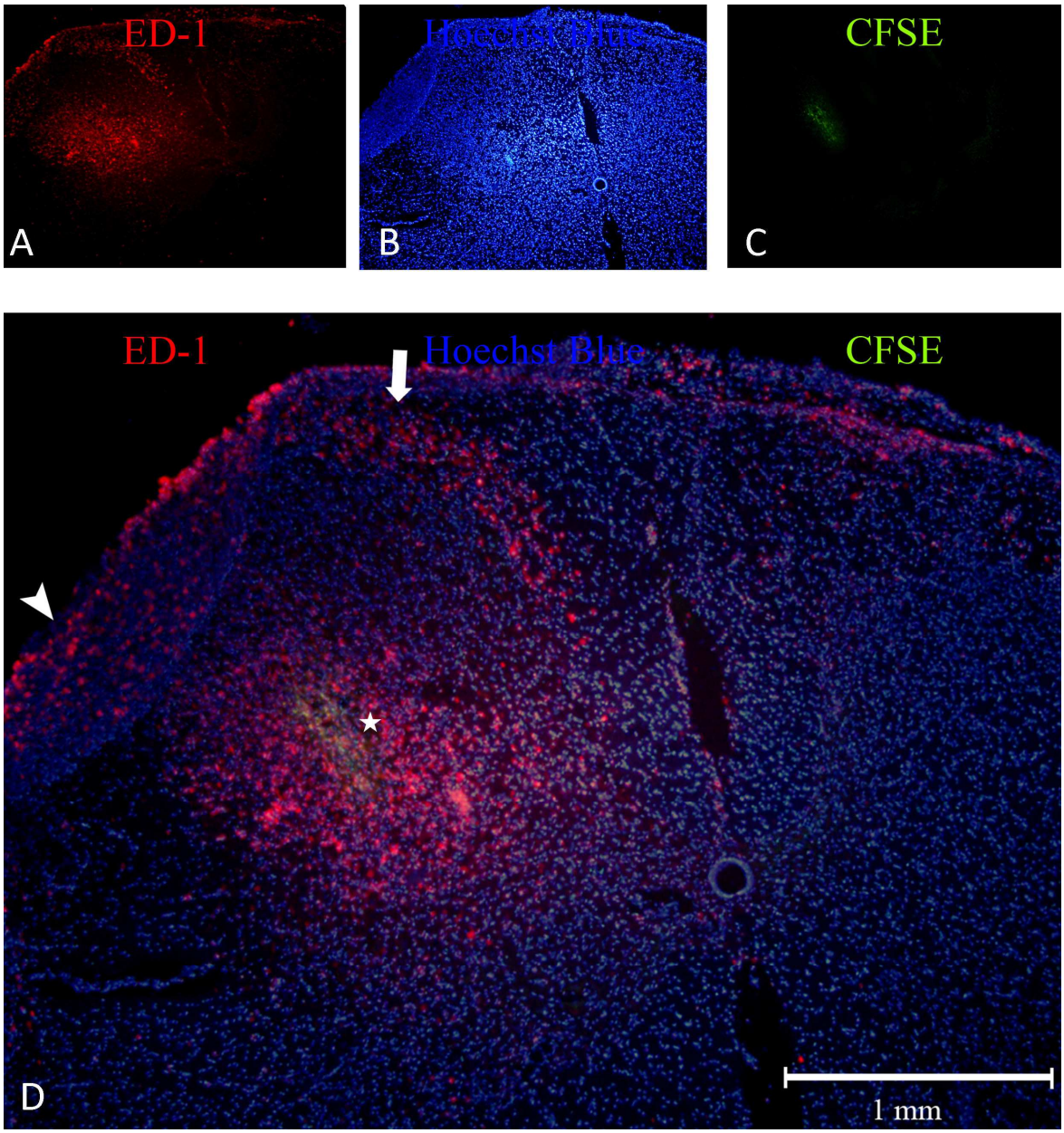

Figure 15: Spinal cord cross section stained for ED-1 one day after surgery.
A: ED-1-Ab. B: Hoechst Blue nuclear counterstain C: CFSE-labeled OECs. D: Merged image of A, B, and C. Accumulation of ED-1 positive cells in the dorsal root entry zone (arrow), the dorsal root (arrow head) and at the OEC implantation site (star).

IV.4.b Spinal cord sections seven days after surgery

In order to assess OEC survival, spinal cord sections were collected seven days after surgery and OEC implantation. Figure 16 shows a cross section of spinal cord immuno-stained for GFAP at high magnification. Green fluorescence of CFSE was only detected at high magnification at the immediate site of implantation. It was impossible to identify cell bodies, instead the fluorescent signal was distributed in the tissue or accumulated as debris indicating disintegration of OECs.

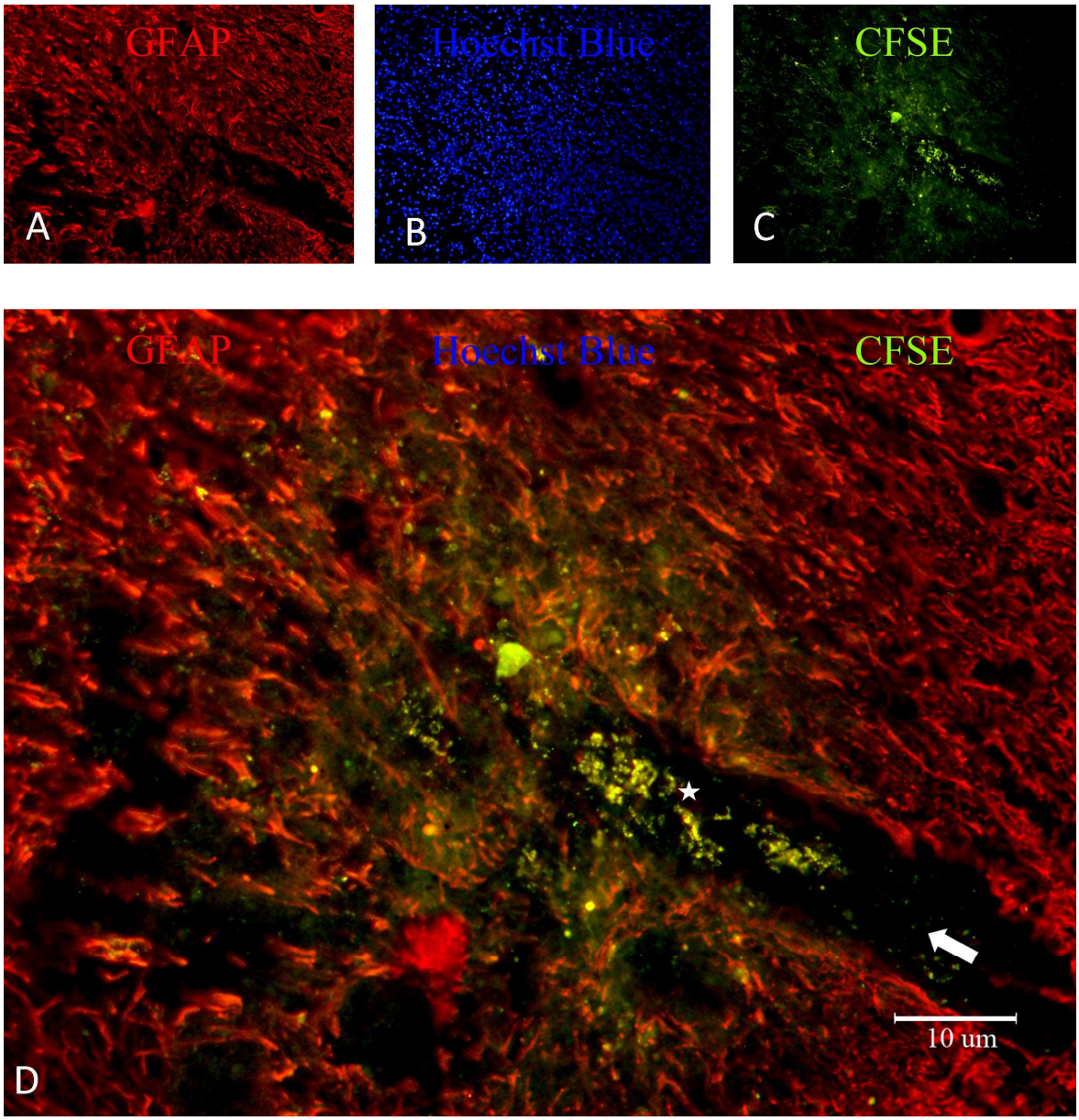

Figure 16: Spinal cord cross section at day seven after surgery and OEC implantation immunostained for GFAP.

A: GFAP-Ab. B: Hoechst Blue nuclear counterstain. C: Fluorescent marker CFSE. D: Merged image of A and C. The arrow indicates the injection channel. The fluorescent signal of CFSE cannot definitely be assigned to cell bodies (star).

Another spinal cord cross section seven days after OEC implantation was stained for ED-1 (Figure 17). A high density of ED-1 positive cells was observed in the dorsal root, the DREZ and the OEC implantation site indicating intense phagocytic activity in these areas. A green fluorescent signal was not detected at this magnification.

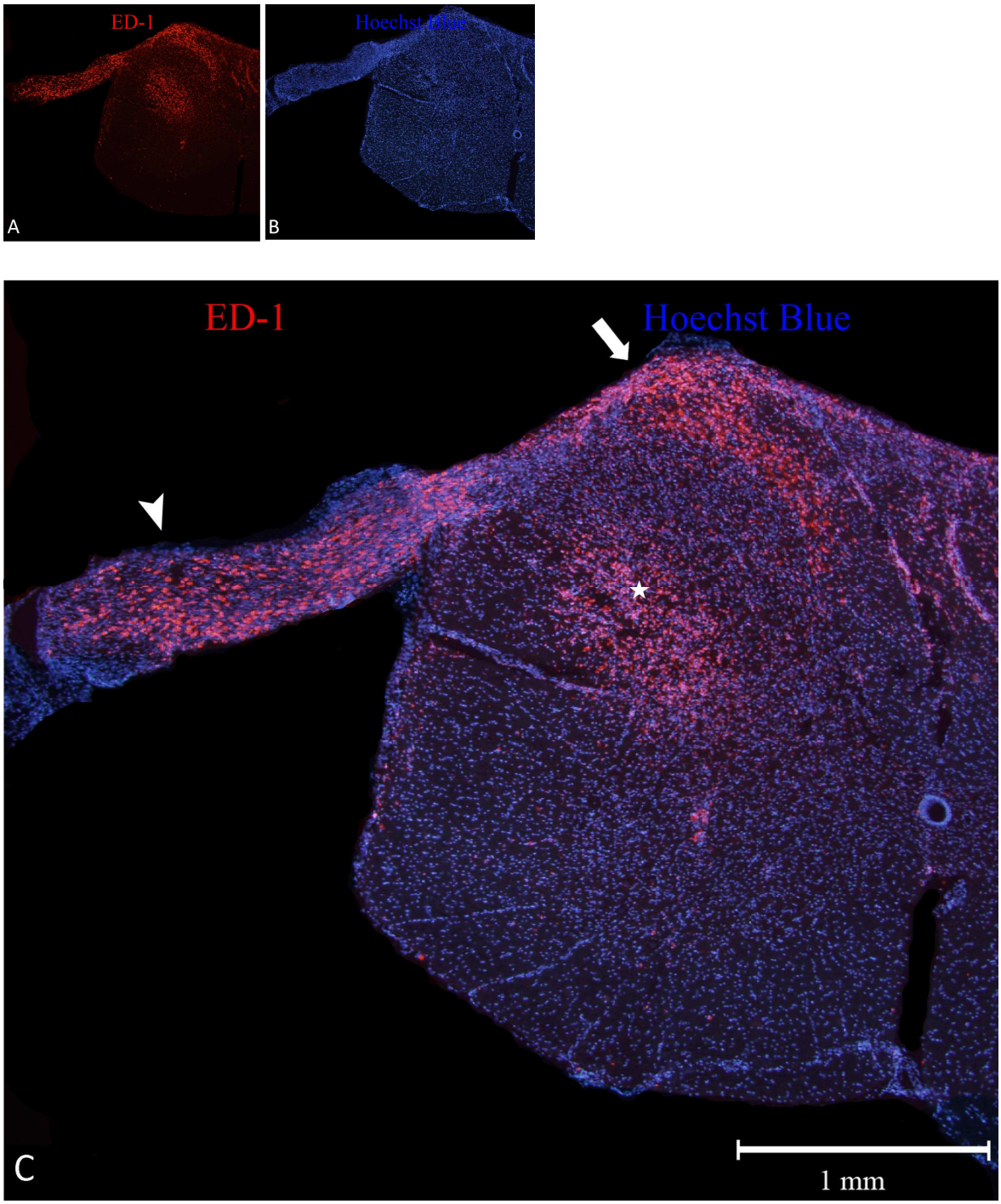

Figure 17: Spinal cord cross section immuno-stained for ED-1 seven days after surgery and OEC implantation.

A: ED-1-Ab. B: Hoechst Blue nuclear counter-stain. C: Merged image A and B. Accumulation of ED-1 positive cells in the dorsal root (arrow head), the DREZ (arrow) and at the OEC implantation site (star).

Figure 18 shows the OEC injection site of the same section at higher magnification. CFSE fluorescence was observed at this magnification, but most of it co-localizes with the signal of ED-1 immuno-stain. This suggests that most CFSE particles have been phagocytosed by activated microglia. Indeed, some phagocytosing cells could be distinguished (Figure 18). Also, CFSE was detected as diffuse background signal outside of cells indicating disintegration of OECs probably due to cell death.

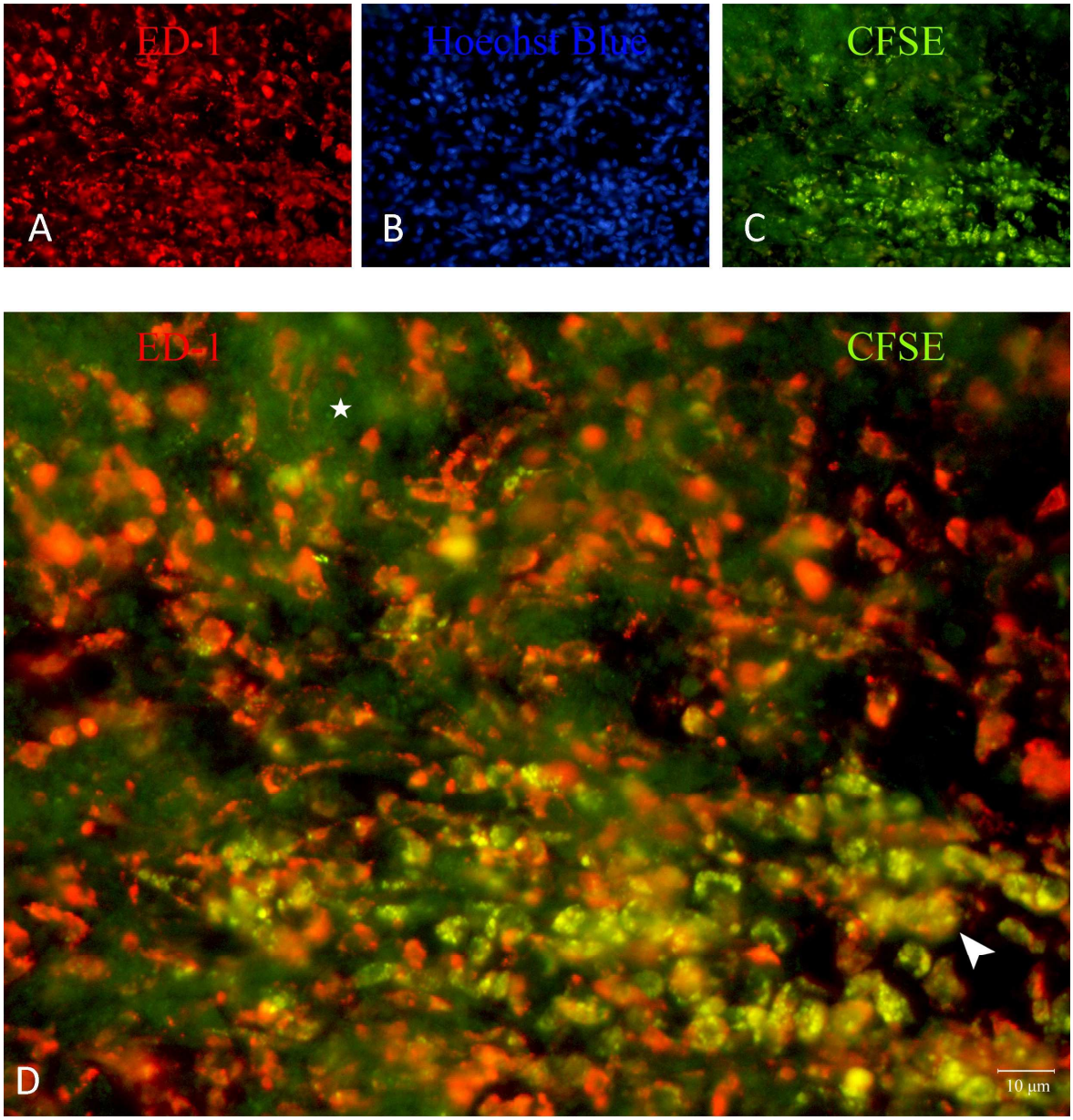

Figure 18: Spinal cord cross section immuno-stained for ED-1 seven days after surgery and OEC implantation at high magnification showing the OEC implantation site.

A: ED-1-Ab. B: Hoechst Blue nuclear counter-stain C: Fluorescent label CFSE. D: Merged image A and C. Orange color results from overlapping red and green signal. Some ED-1 positive cells have taken up CFSE (arrowhead). CFSE is also located outside of cells in tissue (star).

V. Discussion

The combination of cell transplantation and gene therapy is maybe one of the most powerful strategies to promote regeneration in spinal cord injury. In this study, we investigated the effects of implantation of genetically modified OECs into the spinal cord after DRI. We combined two promising approaches to develop treatment for spinal cord injury:

Firstly, implantation of OECs was shown to facilitate regeneration after DRI in several studies (Ramon-Cueto *et al.* 1994; Navarro *et al.* 1999; Li *et al.* 2004). Furthermore OECs showed good survival, migration and integration in spinal cord tissue after implantation (Deng *et al.* 2006) making them an attractive candidate for cellular drug delivery.

Secondly, application of GDNF, a neurotrophic factor, seems to promote functional recovery after DRI (Ramer *et al.* 2000; Iannotti *et al.* 2003). Ideally, GDNF should be present in constant concentrations over a long term at the site of injury. This could be achieved by implantation of OECs that have been genetically modified *ex vivo* to secrete high concentrations of GDNF.

Therefore we decided to investigate the applicability of an OEC cell line that has been transduced with the neurotrophin GDNF using a lentiviral vector system. The cell line was isolated and genetically modified at the National Centre for Adult Stem Cell Research, Griffith University, Brisbane, Australia. The aim of this study was to evaluate the suitability of this cell line before further experiments can be conducted.

First of all, successful transduction and constant secretion of high amounts of GDNF by the transduced OECs had to be verified. For this purpose GDNF secretion rates were determined *in vitro* over a time-course of one week using ELISA.

The second part of the study examined whether transduction influenced cell survival or normal cell function. This was investigated using Immunocytochemistry.

In the third and main part of the project, the amount of GDNF in the spinal cord after implantation of transduced OECs into a rat model of DRI was quantified in a time-course over one month.

Moreover, spinal cord sections were examined one and seven days after OEC implantation using immunohistochemistry to investigate OEC survival in the spinal cord. For this purpose OECs, had to be labeled with CFSE before implantation to visualize

them in tissue. The possibility that CFSE might change the characteristics of the transduced OECs was taken into account and GDNF secretion rates were also quantified for labeled cells.

Our investigations show that stable gene transfer can be achieved with the lentiviral transduction method leading to expression of high levels of GDNF *in vitro*. Labeling the cells with CFSE did not have an apparent influence on GDNF secretion rates. Immunocytochemistry did not reveal alterations in morphology or normal cell functions after transduction and labeling with CFSE.

The *in vivo* ELISA assays carried out over one month show that the amount of GDNF in the spinal cord was significantly elevated over at least one week. Afterwards no significant augmentation compared to the control group could be demonstrated. Immunohistochemical investigations suggest that survival of implanted OECs in the spinal cord was poor.

The results of this study are discussed in detail in the following.

V.1 Characteristics of transduced OECs in culture

OECs can be isolated either peripherally from the olfactory mucosa (Ramer *et al.* 2004) or centrally from the olfactory bulb (Barnett *et al.* 1993). There has been some discussion on whether these different sources might comprise two distinct subpopulations of OECs because of slight variations in their antigenic profile (Pixley 1992; Alexander *et al.* 2002). However, so far no clear distinction has been made between peripherally and centrally derived OECs concerning their functional and antigenic properties (Au *et al.* 2003; Jani *et al.* 2004; Kumar *et al.* 2005).

Most groups have used centrally derived OECs so far, but several studies have been conducted using OECs isolated from the olfactory mucosa as well (Guntinas-Lichius *et al.* 2002; Ramer *et al.* 2004; Feron *et al.* 2005). We chose to use OECs harvested from the olfactory mucosa, since they are more easily accessible. Especially in the context of clinical feasibility, collecting tissue from the olfactory mucosa is far less invasive than accessing the olfactory bulb.

In this study, immunoselection for p75 based on FACS was used to purify cell cultures for OECs prior to viral transduction. OECs are usually identified and isolated based on their antigenic profile. Common markers for OECs are O4, S100 and p75 (Vincent *et al.* 2005). P75 is a low-affinity neurotrophin receptor that is widely used for OEC purification and identification (Barnett *et al.* 2000; Ramon-Cueto *et al.* 2000). It has been shown *in vitro*

that olfactory glia purified for p75, and therefore enriched in OECs, is more effective in supporting axonal regeneration than glia that was not purified (Kumar *et al.* 2005).

Here we showed that OECs can be isolated from the peripheral olfactory mucosa and amplified in cell culture. Furthermore we demonstrated that transduction based on the applied lentiviral vector does not seem to alter normal cell morphology and the expression of p75. Seven days after viral transduction most of the cells displayed immunoreactivity for p75 (Figure 6) indicating good culture purity.

Our cells showed the two morphologies typical for OECs in culture conditions, a flattened and a process-bearing type (Vincent *et al.* 2003). Cells, which were negative for p75, all displayed a flattened morphology and were probably contaminating Schwann cells or fibroblasts. Some cells were fixed during mitosis (Figure 5 and Figure 6) indicating good cell survival and culture expansion. Lentiviral transduction did not change the morphology of OECs in culture and did not have an obvious deleterious effect on cell survival *in vitro*. Other studies also showed that transduction of OECs using retroviral (Cao *et al.* 2004), lentiviral (Ruitenberg *et al.* 2002) or adenoviral systems (Ruitenberg *et al.* 2003) did not alter cell survival or normal cell function *in vitro*.

After labeling the cells with CFSE, bright fluorescence in the cytoplasm could be observed for at least one week as shown in Figure 5 and Figure 6. Cell survival was not affected as also suggested by previous studies using CFSE to label OECs (Imaizumi *et al.* 2000; Deng *et al.* 2006). Therefore, labeling OECs with CFSE is an uncomplicated and reliable way to visualize cells after implantation.

V.2 Transgene expression *in vitro*

Besides the implantation of cells like OECs or stem cells, another strategy in the therapy of spinal cord injury is the application of neurotrophic factors. Several studies have shown beneficial effects for the delivery of neurotrophic factors after spinal cord injury. After DRI, GDNF seems to be the most promising candidate to facilitate regeneration (Ramer *et al.* 2000).

Hence, we decided to use an OEC line that was genetically modified to secrete GDNF. For evaluation of transduction efficiency, two main aspects are important: The level and the stability of transgene expression. Here we report GDNF secretion of transduced OECs between 8 and 15 pg per 10^3 cells in 24 h. The level of GDNF expression of unmodified OECs was undetectable using the same ELISA methods. Cao *et al.* (Cao *et al.* 2004) reported a GDNF production rate of 25 pg per 10^3 cells in 24 h after transduction of purified OECs with a retroviral system, which is in the same range as our results,

suggesting that both, retroviral and lentiviral vectors, are efficient for *in vitro* transduction of OECs. Furthermore, GDNF expression of the transduced OECs stayed stable for at least one week (Figure 8).

Therefore, we were able to demonstrate that transduction of OECs using a lentiviral vector is successful in yielding high levels of GDNF expression, which is stable for at least one week. No significant difference was observed between labelled and unlabelled cells, indicating that CFSE-labelling did not influence GDNF expression (Figure 10).

V.3 *In vivo* GDNF concentrations

The purpose of lentiviral transduction and implantation of OECs was to achieve constant high levels of GDNF in the spinal cord to promote recovery from DRI. In order to test whether this was accomplished, spinal cord segments were collected for determination of GDNF concentration using ELISA at 1, 3, 7, 14, 21 and 30 days after surgery and cell implantation.

V.3.a Baseline GDNF concentration in spinal cord

To determine the baseline level of GDNF in the rat spinal cord, the amount of GDNF in several cervical spinal cord segments of AAW rats was quantified using ELISA (Figure 11). We found that the average GDNF concentration was 42.7 ± 5 pg per g of tissue (mean ±SEM). In another study, the physiologic amount of GDNF in lumbar spinal cord of Sprague-Dawley rats was measured to be 74 ± 22 pg per g of tissue (mean ±SEM) (Tokumine *et al.* 2003). Both values are in the same range. As similar ELISA methods were used, slight variations can be due to the different type of rat or due to the fact, that our samples were collected from the cervical spinal cord instead of the lumbar spinal cord.

In order to examine if DRI itself (i.e. without the implantation of OECs) affects the amount of GDNF in injured spinal cord segments, GDNF concentration was determined at one and seven days after surgery for DRI. The amount of GDNF was determined to be 24.2 ± 7.0 and 32.3 ± 7.7 pg per g of tissue (mean ±SEM), respectively. This value does not differ significantly from the baseline concentration in the uninjured control group (Table 5). Therefore DRI itself does not influence the amount of GDNF in spinal cord. This confirmed that the increase in GDNF concentration after DRI and implantation of transduced OECs was not due to a physiologic increase in GDNF as a response to the injury. Furthermore, it shows, that there is no intrinsic elevation of GDNF levels after injury and that extrinsic application of GDNF is desirable.

V.3.b Concentration of GDNF *in vivo* after dorsal root injury and implantation of transduced OECs

The main aim of this study was to determine if implantation of lentiviral transduced OECs can be used to achieve high levels of GDNF in the spinal cord over prolonged periods. For this purpose, we inflicted DRI onto AAW rats by dorsal root crush and injected genetically modified OECs into the respective spinal cord segment at the DREZ. The amount of GDNF in spinal cord segments was then determined over one month using ELISA.

The results show that the concentration of GDNF in the spinal cord after transduced OEC implantation was initially significantly elevated compared to the baseline GDNF concentration. However, after two weeks the concentration of GDNF in the spinal cord dropped and no longer showed a significant difference compared to the baseline level (Figure 12).

This rapid decline in transgene concentration was unexpected and stands in contrast to the findings of other groups. Ruitenberg *et al.* transduced cultures of purified OECs using adenoviral (AdV) and lentiviral vector constructs with green fluorescent protein (GFP) and examined *in vivo* transgene expression (Ruitenberg *et al.* 2002). They found that transgene expression in OEC implants modified with AdV-based gene transfer gradually decreased over one month after implantation, although numerous implanted OECs were still detected in the tissue. They assumed that this loss of expression might be due to the episomal localization of the transgene after AdV-mediated gene transfer, which is therefore not inherited to subsequent cell generations. In OEC implants subject to lentiviral transduction, however, they observed intense transgene expression for at least four months.

The most likely explanation for the fast decrease in GDNF concentration in the OEC implants in our study is that the implanted OECs did not survive in the spinal cord. In order to validate this hypothesis, we investigated spinal cord sections after OEC implantation histologically at different time points.

V.4 Survival of OECs after implantation into the spinal cord

To be able to investigate the survival of genetically modified OECs after implantation into the spinal cord, tissue sections were examined using immunohistochemistry at one and seven days after surgery for DRI and cell injection.

One day after the surgical procedure, spinal cord cross sections showed that the injected OECs were placed into the spinal cord at a depth of 1 mm. At the injection site and along

the injection trajectory, a disruption of spinal cord tissue was observed, which was caused by the injection. The cell bodies of implanted OECs seemed to be intact, since leakage of the green fluorescent CFSE label was not observed (Figure 13 and Figure 14). Immuno-staining for ED-1 revealed an accumulation of phagocytosing cells in the dorsal root, the DREZ and the OEC injection site (Figure 15).

In contrast, seven days after cell implantation, the CFSE signal was only detected at the injection site under high magnification (Figure 16). CFSE was either taken up by ED-1 positive phagocytosing cells or dispersed throughout the host tissue Figure 17 and Figure 18). Intact OECs were not detected indicating poor cell survival in spinal cord.

These observations contradict the findings of other groups. Long-term survival of OECs transplanted into spinal cord is commonly reported in the literature (Gomez *et al.* 2003; Li *et al.* 2004; Lu *et al.* 2006). For example, Cao *et al.* injected OECs genetically modified to secrete GDNF into the rat spinal cord after SCI (Cao *et al.* 2004). They not only showed good cell survival, but also measured high levels of transgene expression for at least two months after cell implantation. For their experiments they used a cell line that was transduced with a retroviral vector system.

Deng *et al.* (Deng *et al.* 2006) investigated the survival and migration of rat OECs after injection into intact and injured rat spinal cord using OECs also prelabelled with CFSE and a similar injection method. After one day they detected intact rat OECs in the spinal cord together with an accumulation of ED-1 positive cells. However after seven days, the OECs, which were not genetically modified, still survived and even migrated away from the injection site. Cell death after seven days was only observed, when human OECs were injected into the spinal cord of immune competent rats (Deng *et al.* 2006). This was probably due to an immune response. Taking into account that most circumstances in their study resembled our conditions, it seems likely that the poor OEC survival we observed was due to the genetic modification of the OECs.

V.5 Conclusion

In this study we showed that genetic modification of OECs based on lentiviral transduction led to stable overexpression of the transgene GDNF *in vitro*. Normal cell morphology and survival was not influenced in culture. In the rat model of DRI, we showed that injection of transfected OECs into the spinal cord significantly increased the amount of GDNF in the treated spinal cord segments for a couple of days. However, after two weeks GDNF tissue concentrations were not significantly elevated compared to baseline levels. Further histological investigations revealed poor survival of OECs after implantation into the spinal

cord. This explains the rapid drop of GDNF concentration in the spinal cord after a couple of days.

Other groups showed successful survival of implanted OECs that had been genetically modified using other viral vector constructs with concomitant long term *in vivo* transgene expression. In conclusion, the approach to combine the beneficial effects of OECs with the delivery of neurotrophic factors to the injured spinal cord should not be abandoned, but other transduction methods should be applied in further investigations.

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VII. Appendix

VII.1 List of figures

VII.2 List of tables

VII.3 Acknowledgments

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München, März 2011 Maximilian Münchhoff

VII.5 Affirmation

Hiermit erkläre ich, dass die vorliegende Dissertation von mir selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt wurde.

München, März 2011 Maximilian Münchhoff