NEUROTROPHINS AND THE NEURONAL RESPONSE TO AXOTOMY

by

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Abstract

Unlike facial motoneurons (FMNs), which successfully regenerate after axotomy, rubrospinal neurons (RSNs) undergo a marked atrophy in the second week following cervical axotomy. This delayed atrophy is accompanied by a decline in the expression of regeneration associated genes (RAGs), such as GAP-43 and Tα1-tubulin, which are initially elevated after injury. This observation correlates with the growth of only a small percentage of rubrospinal axons into the permissive environment of a peripheral nerve (PN) implanted into the cord at the level of transection. After low thoracic axotomy, there is no increase in RAG expression which also correlates with complete failure of rubrospinal to regenerate into a PN transplant. The overall hypothesis is that these contrasting outcomes are attributed to differences in availability of trophic support between axotomized FMNs and RSNs. Due to their central roles in the nervous system, this thesis focuses on neurotrophins and their receptors. In situ hybridization and reverse transcription-polymerase chain reaction reveal that intact FMNs as well as RSNs express receptor tyrosine kinase (trk) B, a receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-4/5, and low level of trkC, a preferred receptor for NT-3, but little trkA. After facial nerve transection, FMNs markedly increase the expression of BDNF and trkB. Whereas this increase is not seen in RSNs after cervical axotomy, supporting the concept that axotomized RSNs receive limited trophic support. These observations lead to the further hypothesis that axotomized RSNs will be responsive to exogenous application of BDNF, NT4/5 or NT-3 based on the trk receptor expression profiles. Application of BDNF or NT-4/5 fully prevents atrophy of RSNs and a decline in the expression of GAP-43 and Tα1-tubulin after cervical injury. Moreover, there is a 3 fold increase in numbers of regenerating RSNs into the PN transplants. Even after
thoracic axotomy, BDNF infusion stimulates the expression of GAP-43 and Tα-1 tubulin and overcomes the complete failure of RSNs to regenerate into the PN transplants. This thesis supports the concept that application of an appropriate trophic factor promotes regenerative propensity of injured CNS neurons and provides insights for a development of therapeutic strategies after spinal cord injury.
## Table of Contents

Abstract ........................................ ii
List of Tables .................................. viii
List of Figures ................................ ix
List of Abbreviation ......................... xi
Statement of Original Contributions ....... xiii
Acknowledgments ............................... xv
Dedication ..................................... xvi

**CHAPTER 1**

**INTRODUCTION** .................................. 1

1.1. Overall Hypothesis .......................... 1

1.2. General Background ......................... 1

1.2.1 Problem 1: Atrophy and Death of CNS Neurons after Axotomy ..................................... 1

1.2.2. Problem 2: Failure of Regeneration Associated Gene (RAG) Expression in Axotomized CNS Neurons ............. 3

1.2.3. Trophic Factors Prevent Death and Atrophy of Axotomized CNS Neurons ......................... 5

1.2.4. The Neurotrophin Family ................ 6

1.2.5. The Neurotrophin Receptors ............ 9

1.3. Model Systems of PNS and CNS Neurons .... 11

1.4. Specific Background and Hypothesis ....... 13

1.4.1. Potential Sources of Trophic Support for Axotomized FMNs and RSNs ........................... 13

1.4.2. Regeneration Associated Gene Expression (GAP-43 and Tα1-tubulin) in Axotomized FMNs and RSNs ........ 14

1.5. Objectives ................................. 15

**CHAPTER 2**

**MATERIALS AND METHODS** .................. 17

2.1. Animals and Surgical Procedures .......... 17
CHAPTER 3

EXPRESSION OF BDNF AND TRKB IN AXOTOMIZED FMNs
AND RSNs

3.1. Overview 36

3.2. Introduction 36

3.3. Results 36

3.3.1. Axotomized FMNs Increases The Expression of BDNF
and TrkB after Facial Nerve Transection 40

3.3.2. Axotomized RSNs Fail to Increase The Expression of
BDNF and TrkB after Cervical Injury 44

3.4. Discussion 45

3.4.1. Chapter Summary 45

3.4.2. BDNF and trkB mRNA Expression in Axotomized
Peripheral Neurons 46

3.4.3. Regulation and Exon Usage of BDNF after Axotomy 47
3.4.4. Functional Importance of BDNF and TrkB in Axotomized Neurons ........................................... 48

CHAPTER 4

RESPONSE OF AXOTOMIZED RSNs TO NEUROTROPHIN APPLICATION

4.1. Overview ..................................................... 69
4.2. Introduction ................................................ 69
4.3. Rationales for Experimental Paradigms .................... 71
4.4. Results ...................................................... 72
   4.4.1. Cervical Axotomy Paradigm .......................... 72
   4.4.2. Thoracic Axotomy Paradigm ......................... 78
4.5. Discussion .................................................. 81
   4.5.1. Chapter Summary ..................................... 81
   4.5.2. Receptor Expression and Effects of Neurotrophins on Neuronal Atrophy ......................... 82
   4.5.3. Effect of the Neurotrophin Application on Regeneration Associated Gene Expression ........... 84
   4.5.4. Neurotrophins and CNS Regeneration ................. 85

CHAPTER 5

CHRONICALLY INJURED RSNs REMAIN RESPONSIVE TO BDNF

5.1. Overview ..................................................... 118
5.2. Introduction ................................................ 118
5.3. Rational for Experimental Paradigm ......................... 119
5.4. Results ...................................................... 120
   5.4.1. BDNF Partially Reverses Atrophy of Axotomized RSNs after Chronic Cervical Lesion .......... 120
   5.4.2. BDNF stimulates Trα1-tubulin and GAP-43 expression in Cervically Axotomized RSNs ........ 121
5.5. Discussion .................................................. 122
   5.5.1. Assessment of Neuronal Atrophy: Limitations and Considerations ................................. 122
   5.5.2. Axonal Regeneration Requires Cell Body Response of Injured Neurons ............................ 124
CHAPTER 6

OVERALL SUMMARY GENERAL DISCUSSION 143

6.1. Overall Summary 143

6.2. General Discussion 144
   6.2.1. Axotomized PNS and CNS Neurons Differ in their Expression of Trophic Factors 144
   6.2.2. Gene Expression and Growth Potential of Neurons after Axonal Injury 146

REFERENCES 150
List of Tables

Table 1. The mean cell profile sizes of RSNs 14 days after cervical axotomy
List of Figures

Figure 1. ISH for BDNF and trkB in FMNs 51
Figure 2. Quantification of BDNF ISH signals - Scatterplots 53
Figure 3. Quantification of trkB ISH signals - Scatterplots 55
Figure 4. Serial dilution of RT-PCR for BDNF and trkB - autoradiography 57
Figure 5. Densitometric quantification of BDNF and trkB PCR products 59
Figure 6. Western blotting for BDNF - time course study 61
Figure 7. RT-PCR for BDNF - differential expression of BDNF exons 63
Figure 8. ISH for BDNF and trkB in RSNs 65
Figure 9. Serial dilution of RT-PCR for BDNF and trkB - autoradiography 67
Figure 10. Expression of the neurotrophin receptors in RSNs 88
Figure 11. Schematic diagram showing red nucleus and osmotic minipump 90
Figure 12. Immunohistochemical assessment of infused neurotrophins 92
Figure 13. Histology of RSNs after cervical axotomy - effect of neurotrophin treatment 94
Figure 14. ISH for GAP-43 in BDNF- or NT-4/5- treated RSNs after cervical axotomy 98
Figure 15. ISH for Tα1-tubulin in BDNF- or NT4/5 treated RSNs after cervical axotomy 100
Figure 16. Quantification of GAP-43 and Tα1-tubulin ISH signals 102
Figure 17. Regeneration of RSNs into PN transplanted into the cervical cord 104
Figure 18. ISH for GAP-43 in BDNF-treated RSNs after thoracic axotomy 106
Figure 19. ISH for Tα1-tubulin in BDNF-treated RSNs after thoracic axotomy 108
Figure 20. Quantification of GAP-43 and Tα-tubulin ISH signals 110
Figure 21. ISH for GAP-43 and Tα1-tubulin in intact RSNs treated with BDNF 112
Figure 22. Immunocytochemistry for GAP-43 in rubrospinal tracts 114
Figure 23. Regeneration of RSNs into PN transplanted into the thoracic cord . 116
Figure 24. Histology of RSNs 8 weeks after cervical axotomy . . . . 127
Figure 25. Histology of RSNs 24 weeks after cervical axotomy . . . . 129
Figure 26. Histology of RSNs 52 weeks after cervical axotomy . . . . 131
Figure 27. ISH for T\(\alpha\)1-tubulin in RSNs 8 weeks after cervical axotomy . . 133
Figure 28. ISH for T\(\alpha\)1-tubulin in RSNs 24 weeks after cervical axotomy . . 135
Figure 29. ISH for T\(\alpha\)1-tubulin in RSNs 52 weeks after cervical axotomy . . 137
Figure 30. ISH for GAP-43 in RSNs 8 weeks after cervical axotomy . . . . 139
Figure 31. ISH for GAP-43 in RSNs 24 weeks after cervical axotomy . . . . 141
List of Abbreviations

-/- - homozygous null mutaion
BDNF - brain-derived neurotrophic factor
bp - base pairs
cDNA - complementary deoxyribonucleic acid
CNS - central nervous system
cts - counts
d - day or days
DNA - deoxyribonucleic acid
DRG - dorsal root ganglion
EDTA - ethylenediaminetetraacetate
FG - FluoroGold
FMNs - facial motoneurons
GAP - growth associated protein
h - hour or hours
ICC - immunocytochemistry
ISH - in situ hybridization
kDa - kilo daltons
LNTR - low affinity neurotrophin receptor
mRNA - messenger ribonucleic acid
NGF - nerve growth factor
NT - neurotrophin
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate buffered saline
PN - peripheral nerve
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>rh</td>
<td>recombinant human</td>
</tr>
<tr>
<td>RAG</td>
<td>regeneration associated gene</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSNs</td>
<td>rubrospinal neurons</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodiumdodecylsulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
</tr>
<tr>
<td>Trk</td>
<td>receptor tyrosine kinase</td>
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Statement of Original Contributions

This thesis includes materials which have been published in refereed journals. Details are given below.


Ms. Bedard was a technician for Dr. W Tetzlaff, who prepared tissue sections for ISH in the preliminary stage of this study. Dr. Hincke (Dept. of Anatomy, Univ. of Ottawa) performed western blotting for BDNF, which I subsequently learned to perform on my own. Overall, the majority of work included in this study was carried out by the thesis author.


Dr. Fan was a postdoctoral fellow in our laboratory, who performed part of a surgical procedure involving peripheral nerve transplantation into the spinal cord. Dr. Giehl was a postdoctoral fellow in our laboratory, who assisted in quantification of GAP-43 and Tα1-tubulin ISH in the initial stage of this study. Ms. Bedard prepared tissue sections for some series of ISH. Dr. Wiegand (Regeneron Pharmaceuticals Inc.) performed immunocytochemical assessment of infused neurotrophins. Overall, the majority of work was completed by the thesis author.
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CHAPTER 1. INTRODUCTION

1.1. Overall Hypothesis

Neurons projecting into the peripheral nervous system (PNS) regenerate their axons successfully after axonal injury, while neurons confined within the central nervous system (CNS) do not regenerate. Failure of CNS regeneration may be attributed to a combination of, at least, three problems: 1) axotomized CNS neurons undergo severe atrophy and/or death. 2) axotomized CNS neurons fail to express or maintain the appropriate regeneration associated genes (RAGs), which are involved in axonal regrowth. 3) the extra-neuronal environment of the CNS is not favorable to axonal outgrowth. The overall hypothesis of my thesis is that differences in the availability and/or response to trophic factors by PNS versus CNS neurons are fundamental to problems 1 and 2. In the following General Background section, a review of problems 1 and 2, neurotrophic factors and their receptors is given.

1.2. General Background

1.2.1. Problem 1: Atrophy and Death of CNS Neurons after Axotomy

As mentioned above, one of the major problems after a traumatic CNS injury is neuronal atrophy and/or cell death. Cell death is more commonly observed when the axon is severed close to the cell soma (a few millimeters in the rat). For example, it is well established that neurons of the septum degenerate when their axonal projections to the hippocampus are severed by a fimbria fornix lesion (Daitz and Powell, 1954; Gage et al., 1986; Heftr, 1986; Sofroniew et al., 1990; Williams et al., 1986). Similarly, nigrostriatal neurons die after axotomy of the nigrostriatal projection (Reis et al., 1978;
Weiser et al., 1993). Furthermore, the majority of retinal ganglion cells undergo cell death within 2 weeks of the transection of the optic nerve (Doster et al., 1991; Villegas-Perez et al., 1993). Massive cell death of corticospinal neurons occurs during the first week after subcortical injury with only 50% neuronal survival by day 7 (Giehl and Tetzlaff, 1996). The surviving corticospinal neurons typically exhibit massive cellular atrophy (i.e. reduction of cell size).

Little cell death is observed when an axon is severed at "intermediate" distances from the cell body (e.g. 15-20 mm). For example, corticospinal neurons can survive axotomy, after section of the corticospinal tract at the pyramidal decussation, but still exhibit massive neuronal shrinkage (Giehl and Tetzlaff 1991). Interestingly, no cell death or minimal atrophy is observed when corticospinal axons are severed at greater distance from their cell bodies (e.g. >40mm), as in a thoracic spinal cord injury (Barron et al 1989).

The CNS neuronal model system used in this thesis, rubrospinal neurons (RSNs) undergo severe atrophy, but little cell death, two weeks after cervical axotomy (i.e. intermediate injury - about 20 mm) of the spinal cord (Egan et al., 1977; Tetzlaff et al., 1991). Axotomy of RSN at low thoracic spinal cord levels (i.e. distal injury - about 60 mm) results in little if any atrophy and no cell death (Egan et al., 1977, McBride et al., 1989). As a general rule, the greater the distance between the site of the axonal injury and the parent cell soma, the less severe the reaction by the neuron to that injury. The precise reason for this distance dependency is not known, however, it can be hypothesized that differences in the availability of trophic factors may play a central role. The latter may come from sustaining collaterals or glial cells along the proximal stump of the nerve (Ramon y Cajal 1928/1991). This thesis will address the expression of trophic factors in and around axotomized neurons and the role of trophic factors in the atrophy
of axotomized RSNs as well as the expression of regeneration associated genes (see below).

1.2.2. Problem 2: Failure of Regeneration Associated Gene (RAG) Expression in Axotomized CNS Neurons

A small group of genes show consistent changes in expression within axotomized PNS neurons, which successfully regenerate. These include the transcription factor, c-jun (Leah et al., 1991; Herdegen et al., 1992); the cytoskeletal genes, actin, α- and β-tubulins (Hoffman and Cleaveland, 1988; Hoffman 1989; Miller et al., 1989; Tetzlaff et al., 1988, 1991; Jiang et al., 1991; Moskowitz et al., 1993; Moskowitz and Oblinger, 1995) and the growth associated protein, (GAP-43) (Skene and Willard 1981; Skene et al., 1986; Bisby 1988; Schreyer and Skene; 1991; Tetzlaff et al., 1989, 1991). Although the precise functional roles of these genes in axonal regeneration are not completely understood, the expression of these genes by axotomized PNS neurons correlates with the "regenerative potential" of these neurons.

GAP-43 is the most prominent RAG and has been shown to be involved with growth cone motility and the growth cone's response to environmental signals (for review see Skene, 1989; Strittmatter, et al., 1992; Benowitz and Routtenberg; 1997). Over-expression of GAP-43, in a transgenic mouse, leads to spontaneous sprouting at the neuromuscular junction (Aigner et al., 1995). High levels of GAP-43 in growth cones of cultured DRG neurons enhance their resistance to growth cone collapse as a result of the presence of an inhibitory myelin substrate (Aigner and Caroni, 1995). Only 10% of mice carrying a null mutation for GAP-43 survive for longer than 3 weeks, and those which survive show distinctive axonal path-finding errors in the CNS (e.g. optic chiasm; Strittmatter et al., 1995). The data suggest that GAP-43 gene expression may not be
essential for axonal growth in the permissive environment of the embryonic brain, but it appears to be involved in growth cone guidance. Consequently, the absence of GAP-43 may be compensated by other genes to allow axonal elongation during development. It is likely that a large number of, as yet uncharacterized, growth associated genes and their related proteins such as CAP-23 (Widmer and Caroni, 1990) and microtubule associated proteins (Fawcett et al., 1994; Nothias et al., 1995), also have a role during axonal regeneration. Nevertheless, GAP-43 is the best characterized RAG representative, and thus used as a marker for regenerative potential of injured neurons.

The importance of GAP-43 and possibly other RAGs for axonal regeneration is supported by the observation that central dorsal root ganglion (DRG) projections only regenerate into the permissive environment of a peripheral nerve (PN) graft when their peripheral axons have also been injured (Richardson and Issa, 1984; Richardson and Verge, 1986). This outcome is consistent with the increased GAP-43 expression in DRG only after a peripheral injury, but not after a central axotomy (Schreyer and Skene, 1993; Chong et al., 1993). A tight correlation has also been found between the successful regeneration of CNS axons into a PN transplant and their expression of GAP-43 (Campbell, 1991; Tetzlaff et al., 1991, 1994; McKerracher et al., 1993a; Schaden et al. 1993; Vaudano et al. 1995). Interestingly, CNS neurons only spontaneously regenerate into a grafted PN when the axotomy occurs “close” to the parent cell bodies and fail when the PN is implanted at greater distances from the neuronal cell bodies. This finding may be explained by the observation that only those axotomies proximal to the parent cell bodies stimulate increased RAG expression, whereas distal axotomies fail to do so (Doster et al. 1991; Tetzlaff et al., 1991; McKerracher et al., 1993b; Giehl et al., 1995). For example, RSNs regenerate into a PN transplant when these are grafted into cervical spinal cord injury sites but not when these PN grafts are inserted into the thoracic spinal cord (Richardson et al. 1984).
correlates with an increased expression of GAP-43 and other RAGs in RSN after cervical but not after thoracic axotomy (Tetzlaff et al. 1994). However, the neuronal upregulation of RAGs after cervical axotomy is only transient and this type of injury will often result in neuronal atrophy (Tetzlaff et al., 1991). Therefore, to obtain axonal regeneration in the CNS, it is necessary to prevent neuronal atrophy and to induce appropriate RAG expression. These are the central issues addressed in Chapter 2 and 3 of this thesis focusing on the role of neurotrophic factors. The rationale for this emphasis on trophic factors is given in the next section.

1.2.3. Trophic factors prevent death and atrophy of axotomized CNS neurons

A large number of studies support the concept that axotomized CNS neurons fail to receive sufficient trophic support, as evidenced by the rescue from cell death of axotomized CNS neurons by the infusion of trophic factors. Several families of trophic factors have been shown to be effective, including: neurotrophins (NGF, BDNF, NT-3, NT-4/5, NT-6; see section 1.1.3. below), fibroblast growth factors (e.g. FGF-1, 2, 5), cytokines (e.g. CNTF, LIF/CD1, IL-6), and TGF-β superfamily (e.g. TGF-β, GDNF).

For example, axotomy-induced cell death of septal cholinergic neurons after fimbria-fornix transection can be prevented with intraparenchymal injection of either nerve growth factor (NGF) (Hefti, 1986; Williams et al., 1986; Gage et al., 1988), brain derived neurotrophic factor (BDNF) (Knusel et al., 1992; Morse et al., 1993; Widmer et al., 1993), neurotrophin-4/5 (NT-4/5) (Alderson et al., 1996), basic fibroblast growth factor (bFGF or FGF-2) (Unsicker, 1989; Gomez-Pinilla et al., 1992; Araujo et al., 1993; Emmet et al., 1995), ciliary neurotrophic factor (CNTF) (Hagg et al., 1992), or glia cell line-derived neurotrophic factor (GDNF) (Williams et al., 1996). Likewise, axotomized nigrostriatal neurons have been rescued with CNTF (Hagg and Varon, 1993), NT-4
(Alexi and Hefti, 1996), or GDNF injections (Beck et al., 1995). Furthermore, survival of axotomized retinal ganglion cells can be temporarily facilitated by the intra-ocular injection of BDNF (Mey and Thanos, 1993; Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996), NT-4/5 (Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996), CNTF (Mey and Thanos, 1993), or FGF2 (Sievers, 1989). Finally, neuronal cell death of axotomized corticospinal neurons is prevented by BDNF, neurotrophin-3 (NT-3) (Giehl and Tetzlaff, 1996), or CNTF (Dale et al., 1995). Taken together, in the recent years, it has become evident that different types of CNS neurons are responsive to different families of trophic factors. Among these the neurotrophin family stands out due to its prominent and consistent survival effects. In addition to these rescue effects, neurotrophins have also been shown to prevent the axotomy-induced atrophy. For example, BDNF fully prevents the atrophy of axotomized corticospinal neurons (Giehl and Tetzlaff, 1996). This thesis will examine the atrophy of axotomized RSNs and their responsiveness to the neurotrophins, which was hitherto unknown. Thus, in the following section, I provide a brief introduction to the neurotrophin family.

1.2.4. The Neurotrophin Family

The first member of the neurotrophin family, NGF was discovered almost 50 years ago by Levi-Montalcini and her colleagues and it was reported to exert a robust growth and survival promoting activity for sensory and sympathetic neurons (for review see Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1987). In the late 1980's, BDNF, purified from porcine brain (Barde et al., 1982), was identified as the second member of the neurotrophin family having approximately 50% homology to NGF (Leibrock et al., 1989). The recent molecular cloning of NGF and BDNF, as well as the additional members, NT-3, NT-4/5 and NT-6 have provided us with a diverse number of
molecular tools to investigate the trophic dependencies of various PNS and CNS neurons (NGF: Scott et al., 1983; Ullrich et al., 1983; BDNF: Leibrock et al., 1989; Hohn et al., 1990; NT-3: Ernfors et al., 1990a; Maisonpierre et al., 1990; Rosenthal et al., 1990; NT-4/5: Berkemeier et al., 1991; Hallbook et al., 1991; NT-6: Gotz et al., 1994).

In both the developing and adult nervous system, neurotrophins have been shown to influence neuronal survival, neurite outgrowth as well as neuronal phenotype, transmitter/peptide expression and modulation of synaptic efficacy. (for review see Davies, 1994; Lindholm et al., 1994; Lindsay et al., 1994; Lewin and Barde, 1996; Henderson 1996). The specific requirement for a neurotrophin by a particular neuronal phenotype was demonstrated almost 40 years ago by depletion of endogenous NGF using anti-NGF-antibody injections. This procedure resulted in the loss of more than 90% of the NGF-dependent superior cervical ganglion cells (Cohen 1960; Levi-Montalcini and Booker 1960). More recent NGF gene disruption (i.e. knockout) experiments confirmed and extended these findings by showing that sympathetic as well as the nociceptive subpopulation of DRG cells critically depend on NGF (Crawley et al., 1994). Surprisingly, no apparent deficits in neuron survival were observed within the CNS in these NGF−/− mice, including basal forebrain cholinergic neurons, which are responsive to exogenous NGF application (Crawley et al., 1994).

Subsequent gene disruptions of the other members of the neurotrophin family revealed additional specific neuronal deficits in the PNS, whereas changes in the CNS appear to be moderate and highly localized (Crawley et al., 1994; Jones et al., 1994; Patterson et al., 1996; for review Snider 1994) To provide one illustrative example, the disruption of the BDNF gene (i.e. knockout) resulted in somatosensory deficits due to loss of cutaneous DRG afferents (mediating touch and vibration), but not proprioceptive muscle spindle afferents (Ernfors et al., 1994a; Jones et al., 1994), which are primarily affected by NT-3 gene disruption. Moreover, a 50% reduction of nodose-petrosal
ganglion neurons (Conover et al., 1995; Liu et al., 1995; Erickson et al., 1996) and the almost complete deletion of primary vestibular afferents were observed (Ernfors et al., 1995). Interestingly however, the number of cranial and spinal motoneurons in BDNF deficient mice remained unaltered (Jones et al., 1994) despite its reported rescue effect on axotomized motoneurons in neonatal rodents (Sendtner et al., 1992; Henderson et al., 1993; Koliatsos et al., 1993) indicating redundancies in the trophic support for motoneurons (for review see Oppenheim, 1996). This seems to contrast sensory and autonomic ganglion cells which require specific subsets of neurotrophins as outlined above.

In the developing CNS, only minor neuronal cell losses after neurotrophin gene disruption have been reported indicating functional compensation during development, redundancy between neurotrophin family members, or influences by other trophic factor families (Korte et al., 1995, 1996a, 1996b; McAllister et al., 1995, 1996; Marty et al., 1996 Patterson et al., 1996; Snider and Lichtman, 1996; Elmer et al., 1997). Only recently, Schwartz et al. (1997) have reported more pronounced deficits in cerebellar development in BDNF -/- mice such as the death of cerebellar granule cells, abnormal dendritic development in Purkinje cells, and defects in foliation pattern of cerebellar cortex. In the mature nervous system, neurotrophins play a role in neuronal plasticity (i.e. neuronal connection) and synaptic efficacy of the mature CNS neurons. For example, BDNF -/- mice exhibit functional deficits such as reduced long term potentiation, (Korte et al., 1995, 1996a, 1996b; Patterson et al., 1996). In addition neurotrophins are involved in axonal sprouting during development and after kindling, denervation or direct injury of the adult brain (Kawaja and Gage, 1991; Diamond et al., 1992; Van der Zee et al., 1992; Heisenberg et al., 1994). Interestingly the role of neurotrophins in neural regeneration of the CNS is far less understood and while my thesis study was underway, several studies demonstrated that local application of neurotrophins to the injury site can
enhance regenerative sprouting of various CNS axons (Schnell et al., 1994; Tuszynski et al., 1994; Oudega and Hagg; 1996; Ye and Houle; 1997).

1.2.5. The Neurotrophin Receptors

Using \(^{125}\text{I}\)-labeled NGF, early kinetic studies by Shooter and his colleagues indicated the presence of two characteristic NGF binding sites displaying high (Kd = \(10^{-11}\)) or low (Kd=\(10^{-9}\)) affinities within immortalized cells of pheochromocytoma lineage (PC12) and primary sensory ganglia cultures (Suter et al., 1979; Landreth and Shooter; 1980; Layer and Shooter; 1983; Vale and Shooter, 1983). Molecular cloning has allowed the discovery of two distinct gene products encoding NGF receptors, namely p75, a low-affinity neurotrophin receptor (LNTR) (Chao et al., 1986; Radeke et al., 1987) and secondly, the Trk (receptor tyrosine kinase) receptor, that exhibit properties of low- and high affinity receptors, respectively (Martin-Zanca et al., 1989; Klein et al., 1991a; Meakin et al., 1992a, 1992b). The other members of the Trk receptor family, TrkB and TrkC were subsequently shown to be high affinity receptors for BDNF and NT-4/5, and NT-3, respectively (Kline et al., 1991b; Lamballe et al., 1991; Ip et al., 1993). An affinity labeling of the neurotrophin receptor largely confirmed the colocalization of high-affinity binding sites of a neurotrophin and the presence of corresponding trk receptor expression in the PNS and CNS (Verge et al., 1992; Altar et al., 1993, 1994; Lapchak et al., 1993; Yan et al., 1994).

The binding of a particular neurotrophin to a corresponding high-affinity trk receptor and subsequent activation of intracellular signaling cascades are suggested to be sufficient to mediate the appropriate biological response (Jing et al., 1991; Kaplan et al., 1991a, 1991b; Loeb et al., 1991; Clary et al., 1994). Although all neurotrophins bind
to p75LNTR with low affinity (Rodriguez-Tevar et al., 1990, 1992; Ryden et al., 1996) its functional role in vivo remains unclear.

The ligand-receptor chemical cross-linking and in vitro transfection experiments suggest an association between p75LNTR and TrkA and a possible role of p75LNTR to modulate TrkA function in response to NGF binding (Hempstead et al., 1990, 1991; Barker and Shooter, 1994; Hanzopolous et al., 1994; Kahle et al., 1994; Mahadeo et al., 1994; Verdi et al., 1994; Huber and Chao, 1995, Ross et al., 1996). When p75LNTR is expressed in the absence of the Trk receptors, sphingomyelin hydrolysis and ceramide production are observed in response to neurotrophins, indicating activation of a signal transduction cascade distinct from trk receptors (Dobrawsky et al., 1994). Furthermore, an involvement of the p75LNTR in neuronal death is suggested due to its sequence homology to tumor necrosis factor receptor and has been demonstrated in vitro and in vivo (Rabizadeh et al., 1993; Barrett and Bartlett, 1994; Cassaccia-Bonnefil et al., 1996; Frade et al., 1996; Van der Zee et al 1996; for review see Bredesen and Rabizadeh, 1997; Kaplan and Miller, 1997).

In recent gene disruption studies of the trk receptors, phenotypic deficits in the PNS are similar to the corresponding neurotrophin gene knockout experiments, confirming a primary involvement of the trk receptors in a neurotrophin action (see above) (for review see Snider, 1994; Barbacid, 1994, 1995; Bothwell, 1995; Lewin and Barde, 1996) (Klein et al., 1993; 1994; Smeyne et al., 1994; Erickson et al., 1996).

The discoveries of TrkB and TrkC splice variants lacking a cytoplasmic tyrosine kinase domain (truncated isoforms) and TrkC isoforms with 14, 25, or 39 amino acid insertions in the kinase domain (insertion isoforms) have added an additional level of complexity in the cellular responses to neurotrophins (Klein et al., 1990; Middlemas et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993). A functional role of truncated TrkB and TrkC as well as TrkC insertion isoforms is not well understood. Recent in vitro
studies indicate that these isoforms may influence intracellular signaling events initiated by the full-length TrkB or TrkC receptors, thereby modulating the cellular responsiveness to the neurotrophins (Tsoufas et al., 1993, Valenzuela et al., 1993, 1996; Guiton et al., 1995, Eide et al., 1996; Baxter et al., 1997).

The in vivo expression profiles of the trk receptors has been crucial to the prediction of neurotrophin dependencies for a variety of neuronal phenotypes, including CNS neurons. As described above, the expression of trkB by retinal ganglion cells is in agreement with their responsiveness to BDNF and NT-4 after axotomy (Jelsma et al., 1992; Mansour-Robaey et al., 1994). Furthermore, corticospinal neurons express both trkB and trkC receptors which may explain why both BDNF and NT-3 can ameliorate corticospinal cell death after axotomy (Giehl and Tetzlaff., 1996). Moreover, the activation of TrkA by infusion of specific TrkA polyclonal antibodies, which act as a functional TrkA agonist, is sufficient to rescue axotomized basal forebrain cholinergic neurons (Lucidi-Phillipi et al., 1996). The expression profile of trk and p75 receptors within RSNs and facial motoneurons was unknown and has been a focus of my initial investigations.

1.3. Model Systems of PNS and CNS Neurons

Two model systems of neuronal injury were used in my thesis: 1) axotomized rat facial motoneurons (FMNs) as a PNS model exhibiting robust and spontaneous neuronal survival and successful axonal regeneration, and 2) axotomized rat RSNs as a CNS model where neuronal survival and axonal regeneration is a rare spontaneous occurrence.

FMNs originate ventrolaterally within the facial motor nucleus of the brainstem and, via the seventh cranial nerve, they innervate muscles of the face, eye, ear and
neck. In brief, axons of the FMNs project dorsally within the brainstem bend around the nucleus abducens to form the inner facial genu and exit at the ventrolateral surface of the brainstem. The facial nerve exits the skull at the stylomastoid foramen where it is readily accessible for surgery. In the rat, the estimated numbers of FMNs range from 3400 to 5800 (Martin et al., 1977; Contreras et al., 1982; Friauf and Herbert, 1985; Semba and Egger, 1986).

The RSNs originate within the red nucleus of the midbrain and project to the spinal cord. RSNs are involved in motor control, especially flexor motor responses. Over 97% of their fibers decussate at the midbrain and project within the dorsolateral funiculus to give rise to the rubrospinal tract (Brown et al., 1974; Masson et al., 1991). RSN projections are typically divided into parvocellular and magnocellular subpopulations, with some parvocellular RSNs projecting to the cord (Huisman et al., 1983; Shieh et al., 1983; Kennedy et al., 1987, 1990; Wang et al., 1992) and the magnocellular RSNs projecting throughout the entire length of the cord (Flumerfelt and Gwyn, 1974; Muray and Gurule 1979). The majority of the magnocellular RSNs, approximately 3000 in number, are localized in the most caudal pole (400 μm) of the rat red nucleus (Cabana et al., 1986; Strominger et al., 1987; Wang et al., 1992).

Methodologically, the advantages of these two neuronal injury models are: 1) both neuronal populations are confined to a circumscribed nucleus (region of the CNS) which can be easily identified histologically (e.g. with cresylviolet staining); 2) both neuronal populations can be readily isolated by microdissection, with minimal contamination by other neuronal populations; 3) both neuronal populations have comparable numbers of neurons with similar cell diameters (15-50 μm); 4) the axonal projections of both populations can be experimentally injured, with only minor damage to other tracts; and 5) the functional impairment of the animal after unilateral axotomy of
the FMNs or RSNs is minimal. In addition, my initial observations indicated that RSNs, similar to FMNs, expressed the mRNAs for trkB and trkC, but not trkA or p75. These observations allowed me to also hypothesize similar neurotrophin responses for these two neuronal populations. All these similarities support the rationale for comparing these 2 neuronal injury models.

1.4. Specific Background and Hypotheses

1.4.1. Potential Sources of Trophic Support for Axotomized FMNs and RSNs

After axotomy, FMNs of the adult rat not only survive, but transiently increase their soma size (i.e. hypertrophy). Since axotomy physically disconnects FMNs from their muscle targets, they are presumably isolated from their source of trophic factors. However, It has been shown that Schwann cells within the distal stump of the crushed PN also produce a variety of trophic factors, including: NGF, BDNF, and NT-4 (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993). These Schwann cell factors may provide some trophic support after a nerve crush lesion where there is continuity between the basal membranes of the proximal and distal nerve. In contrast, if the PN injury involves the removal of a segment of the PN (i.e. no basal membrane continuity), motoneurons still survive and transiently hypertrophy. Even though it has been proposed that the proximal stump may become a substitute source of trophic support (Heumann et al., 1987), the actual amount of NGF provided and transported from the proximal nerve stump has been shown to be very low (Raivich et al., 1991). Reactive perineuronal glia cells (microglia or astrocytes) surrounding the FMN cell bodies within the brainstem could also be a paracrine source of trophic support. Finally, an autocrine source of trophic factor support may result from the increased expression
within the injured FMN cell bodies themselves. Therefore, *I hypothesize that paracrine and/or autocrine trophic support for axotomized FMNs may originate within the CNS and test this in Chapter 3 of my thesis.*

In contrast to FMN axotomy, RSNs undergo severe atrophy after axotomy at the cervical cord level, but not after a thoracic level injury (Tetzlaff et al., 1991). Since RSNs have axon collaterals emanating from the primary axon at all levels of the cord, after a thoracic injury, sustaining trophic support may be arising via collateral targets at rostral levels (i.e. within the cervical spinal cord). BDNF and NT-3 expression has been observed within the adult rat spinal cord (Ernfors et al., 1993). After a cervical cord axotomy, the number of sustaining axon collaterals would be insufficient to prevent atrophy of RSNs. In addition, trophic support could originate from glia cells associated with the proximal primary axon, little is known about the trophic factor production by central glia cells after spinal cord injury. Since RSNs undergo atrophy after a cervical axotomy, it is unlikely that axotomized RSNs obtain a local paracrine or autocrine source of trophic support within the red nucleus. Therefore, *I hypothesize that local trophic support of axotomized RSNs is attenuated and this is examined in Chapter 3.*

1.4.2. Regeneration Associated Gene Expression (GAP-43 and Tα1-tubulin) in Axotomized FMNs and RSNs

Axotomized FMNs markedly increase GAP-43 and Tα1-tubulin expression after facial nerve transection (Miller et al., 1989; Tetzlaff et al., 1991). The expression of these genes remains elevated for several months after injury (Miller et al., 1989, Tetzlaff et al., 1989, Mathew et al., 1993, Wu et al., 1993). In contrast, RSNs only transiently upregulate GAP-43 and Tα1-tubulin expressions after cervical axotomy. These expressions start to decline during the 2nd week after axotomy, in parallel with the onset
of neuronal atrophy (Tetzlaff et al., 1991). After a more distal axotomy of RSNs, within the low thoracic level (T10) of the spinal cord, no increase in GAP-43 and Tα1-tubulin mRNA expression was observed (Tetzlaff et al. 1991; 1994). Thus, after thoracic axotomy, the possible trophic support from RSN axon collaterals within the cervical cord may be sufficient to prevent RSN atrophy, but insufficient to stimulate the gene expression.

I hypothesize that a lack of local trophic support within the red nucleus hampers ability of axotomized RSNs to express RAGs. Furthermore, I hypothesize that the provision of exogenous trophic support through the infusion of the appropriate trophic factors will counteract the neuronal atrophy after cervical axotomy and will stimulate RAG expression in RSNs after cervical as well as thoracic axotomy.

If successful, this should stimulate the regenerative propensity of RSNs into peripheral nerve transplants.

1.5. Objectives

At the beginning of my thesis project, I examined the expression of the neurotrophin receptors in FMNs and RSNs and found that both FMNs and RSNs express mRNA for trkB and trkC, but little trkA and p75LNTR, suggesting a similar responsiveness to neurotrophins in these neuronal systems. The following specific hypotheses were tested in this thesis based on the trk receptor expression profile and the previous studies, which were introduced in the relevant chapters.

1) Test the hypothesis that axotomized FMNs receive trophic support in paracrine and/or autocrine manner while axotomized RSNs gain only limited trophic support.
Specifically, examine the expression of neurotrophins and their receptors in the FMNs and RSNs after axotomy.

2) Test the hypothesis that providing an appropriate trophic support for axotomized RSNs by administration of an exogenous neurotrophin 1) prevents atrophy and decline of RAG expression after acute cervical injury, 2) stimulates RAG expression in acute thoracic injury, and 3) reverses atrophy and RAG decline after chronic injury.

3) Test the hypothesis that application of an appropriate neurotrophin enhances the propensity of axotomized RSNs to regenerate into the permissive environment of PN transplants.
CHAPTER 2: Materials and Methods

The materials and methods section is organized according to the experimental techniques used in this thesis.

2.1. Animals and Surgical Procedures

Young adult male Sprague Dawley rats (200-250g, 8-9 weeks old) were used throughout this thesis. They were supplied by Charles River (Quebec, Canada) or local animal breeding colony (University of British Columbia), housed in groups of two to four with a 12:12 h dark-light cycle and fed a standard rodent diet ad libitum. All experimental protocols were reviewed by the local Animal Care Committee according to the guidelines of the Canadian Committee on Animal Care.

2.1.1. Facial Nerve Transection

The rats (n=90) were anaesthetized with 32 mg/kg sodium pentobarbital plus 150 mg/kg chloral hydrate (at the University of Ottawa) or ketamine (60mg/kg) plus xylazine (7.5mg/kg) (at the University of British Columbia). Under sterile conditions, the left facial nerve was exposed at the stylomastoid foramen and a 5 to 7 mm long nerve segment was excised. Subsequently the skin was closed with wound clips. The rats were killed by an overdose of chloral hydrate (at 3, 8, 16, 24 hours and 2, 3, 4, 7, 14, 21 days post-injury (at each time point, n=3 for in situ hybridization, n=4 for reverse transcription-polymerase chain reaction and n=2 for western blotting). The brainstems containing the facial nuclei were frozen on dry ice.
2.1.2. Spinal Cord Hemisection

The neck musculature was split in the midline and left hemi-laminectomy was performed at C3/C4 or T10/T11 of the spinal cord. After opening the dura, the dorso-lateral funiculus of the spinal cord was cut with a pair of iris scissors and the incision was verified with a fine dumont #5 forceps. In some animals, a small piece of gelfoam (Upjohn) soaked with 0.5 to 1 μl of 2 % FluoroGold (FG) (Fluorochrome Inc., Eaglewood, CA) was applied to the injury site. The muscles were sutured with prolene (6-0) (Ethicon, Somerville, NJ) and the skin was closed with wound clips. The rats used for the in situ hybridization (ISH) and reverse transcription polymerase chain reaction (RT-PCR) study for BDNF and neurotrophin receptor expression (n=14) were sacrificed by an overdose of chloral hydrate 7 days after cervical axotomy. The fresh midbrains were immediately frozen on dry ice. In all other experiments, rats were injected with an overdose of chloral hydrate and perfused transcardially with phosphate buffered saline followed by freshly hydrolyzed paraformaldehyde (4%) in 100 mM phosphate buffer.

2.1.3. Neurotrophin Application

For the acute cervical axotomy paradigm, on day 7 post-axotomy, the rats (n=90) were anesthetized and cannulae (28G, 8 mm, Plastic One Inc., Roanoke, VA) were inserted stereotaxically into the vicinity of the red nucleus at the following coordinates: 6.3 mm posterior to Bregma, 1.7 mm from midline to the right side, and 6.5 mm deep from the cortical surface. The cannulae were anchored in position with two watchmaker screws and dental cement. Osmotic minipumps (ALZET #2001, 1μl/hr) were filled with either vehicle alone (20 mM sterile phosphate buffered saline (PBS) supplemented with 100 U Penicillin/Streptomycin and 0.5% rat serum albumin, (Sigma, # A-6272)) recombinant human (rh) NGF, rhBDNF, rhNT-3 or rhNT-4/5 at a
concentration of 500 ng/μl. This concentration of neurotrophins has previously been documented to be effective for in vivo application (Verge et al., 1992). Osmotic minipumps were connected to the infusion cannulae with 6-8 cm of Silastic tubing (#508-003, VWR) and the entire assembly was pre-incubated for 4-12 hours in sterile 20 mM PBS at 37 °C prior to implantation. Thus, each neurotrophin- treated animal received 12 μg of rhNGF, rhBDNF, rhNT-3 or rhNT-4/5 per day in a total volume of 168 μl over a period of 7 days (between day 7 and 14 post-axotomy). On day 14, the animals were overdosed with chlortal hydrate and transcardially perfused with freshly hydrolized 4% paraformaldehyde. The midbrains were kept in 4% paraformaldehyde overnight and subsequently cryoprotected with 16% and 22% sucrose in 10mM PBS. The brains were quickly frozen in dry ice cooled isopentane for histology and for GAP-43 and Tα1-tubulin ISH.

For acute thoracic axotomy paradigm, an osmotic minipump containing either BDNF (500ng/μg/hr for 7 days) or vehicle was implanted as described above on the same day that lateral funiculotomy at T10/11 was performed (n=20). These animals were transcardially perfused 7 days later and midbrains were processed as above for GAP-43 and Tα1-tubulin ISH.

For chronic cervical axotomy paradigm, the rats (n=60) were left untreated after cervical axotomy for 9, 16, 25 or 51 weeks. Subsequently, BDNF (500ng/μg/hr) or vehicle only was infused via osmotic minipump for 7 days as described above during weeks 10, 17, 26 or 52. They were transcardially perfused and processed as above for histology and GAP-43 and Tα1-tubulin ISH.

2.1.4. Peripheral Nerve Implants

To evaluate the effect of BDNF treatment on the regenerative capacity of
axotomized RSN, a segment of the peripheral nerve graft was implanted into the site of lesion to offer a permissive environment for regrowth. This part of the surgical procedure was performed by Dr. D-P Fan, a postdoctoral fellow in our laboratory. The right sciatic nerve was transected at the level of the obturator tendon and left in situ to allow Wallerian degeneration of the distal stump. Ten days later, a 30-35 mm segment of the pre-degenerated nerve was resected and its proximal end inserted into a C4 or T11 left spinal cord lesion site. It was held in place with two Prolene 10-0 sutures (Ethicon, Somerville, NJ) and the distal free end was marked with a Prolene 6-0 suture (Ethicon, Somerville NJ) and left in the subcutaneous tissue. The spinal column from C3 to C5 was immobilized by placing 3 watchmaker screws into the right vertebral pedicles of C3, C4 and C5 and stabilized with bone cement (Ethicon, Peterborough, ON, Canada). During the same surgical session, an osmotic minipump was implanted as described above in order to administer BDNF (500ng/µl/hr) into the vicinity of the axotomized RSN. The rationale to apply BDNF for 7 days at the time of cervical spinal cord injury and transplantation was based on the animal care regulation which restricts the number of surgical interventions (3 instead of 4 operations). We documented a prolonged effect of BDNF beyond the period of application which further justifies our design. Ten weeks after spinal cord injury, pump implantation and transplantation, the rats were anaesthetized again and the free end of the nerve transplant was identified and mobilized. The distal end (1-3 mm) of the nerve graft was resected and the freshly exposed end of the nerve placed into a small polyethylene tubing filled with 5% FG for 1 hr. Fourteen days later, the rats were overdosed with chloral hydrate, and the midbrains were processed as above. FG-labeled neuronal profiles were counted throughout the caudal 1mm of the red nucleus in series of 14 µm coronal sections. Care was taken to avoid double counting in adjacent sections. A Students’ t-test was used to compare the
number of retrogradely filled, i.e. regenerating, neurons in the BDNF treated versus untreated rats.

2.2. Histology and Cell Size Measurements after Cervical Axotomy of RSNs

Most neurotrophin-treated and vehicle-treated rats were sacrificed on day 14 except for six rats which were left beyond the cessation of neurotrophin application i.e. until day 21 or 28. They were overdosed with chloral hydrate, followed by transcardial perfusion as described above. Frozen sections were cut coronally at 12 μm through the midbrain and mounted onto Superfrost Plus slides (Fisher Scientific). Each slide contained a section from 2 control animals that received no pump or a vehicle pump, and from 1 or 2 animals treated with a neurotrophin. The order in which they were cut and positioned onto the slide was randomized. The midbrains were cut from caudal to cranial and collection of sections was begun when 6-10 RSN appeared at the caudal pole of the red nucleus and the following 35 sections were gathered. Sections #15, #25 and #35 were stained with 0.2% cresylviolet for cell profile measurement and photography. The remaining sections were stored at -80 °C for ISH analysis.

The cell profile sizes were measured using a computerized image analysis system. Two techniques involving computerized digitization of the cell image and profile measurement were used. In the first method, a 16x oil immersion objective (Zeiss) was used to crop the images and the contour of each neuron containing a visible nucleus was traced. This contour was filled in and the number of pixels was measured in order to obtain an arbitrary value for the cross-sectional cell area. In the second technique, a field comprising about half of the red nucleus was digitally captured using a 10x objective. Subsequently the density threshold was determined to discriminate the stained neurons from the background. Only those cell profiles which were twice the size
of an average glia cell were included in our analysis. Confluent neurons were separated
with a digitized paintbrush. In both procedures, the operator was unaware of the
treatment condition. Since both procedures yielded very similar results the latter
technique was used routinely. The size of neurons within the red nucleus is
heterogeneous, with smaller neurons located predominantly towards the cranial end of
the nucleus. Thus, the average cell size of axotomized RSN was normalized to that of its
intact, contralateral counterpart in sections #15, #25 or #35 (typically total of 120-150
neurons/red nucleus), and values were expressed as a percentage of contralateral
control for each level of the nucleus. These percentages from individual animals were
subjected to statistical analysis (see statistical analysis) and the median percentage
from the individual treatment groups plus the 25th/75th percentiles are presented.

Cell counts of the RSNs 52 weeks after cervical axotomy were performed using
the cresylviolet stained sections used for the cell profile size analysis (i.e. 15th, 25th and
35th sections). The criteria were based on the presence of visible nucleus and a cut-off
size of twice the size of average glia cells.

2.3. In Situ Hybridization (ISH)

2.3.1. BDNF and Neurotrophin Receptor ISH

Fresh frozen brainstems or midbrains were cryostat sectioned in the coronal plane
at 12 μm, thus each tissue section contained the axotomized and contralateral control
facial or red nuclei. Alternating sections were collected at 2 sections per slide and were
kept in -85° C until use.

For all oligonucleotide probes, no similarities were found for other molecular
sequences at a 75% cut off level in a BLASTN database search at the National Center
for Biotechnology Information (Altschul et al., 1990). A 50-mer oligonucleotide 5'-AGTT
CCAGTGCTTTTTGCTATGCCCCTGCAGCTTCCTTCGTGTAACCC-3', Complementary to bases 645-694 of the rat BDNF sequence (Maisonpierre et al., 1991, Genbank accession # M61175) was used, which was previously described by Ernfors et al., 1990. The trkA probe was complementary to bases 1198-1245 and was previously used by Verge et al. (1992). For the full length trkB, a 45-mer oligonucleotide, 5'-GAGAGGGCTGGCAGAGTCATCGTCGTTGCTGATGACGGAAGCTGG-3', complementary to the base pairs 1363-1407 of the rat trkB sequence was used (Middlemas et al., 1991). This oligonucleotide is derived from the sequence between the truncation site and the tyrosine kinase domain. The trkC probe was complementary to bases 2109-2272 (excluding 2134-2250), bridging the insertion site in cytoplasmic tyrosine kinase domain (Valenzuela et al., 1993) and has been used by Giehl and Tetzlaff (1996). A 50-mer p75LNTR probe was 5'-ACAAGGCCCACGACCACAGCCAAGATGGAGCAATAGACAGGAATGAG-3', which was previously used by Verge et al., (1992).

These oligonucleotide probes were end-labeled with $^{35}$S-ATP (NEN-Dupon) by deoxynucleotide terminal transferase (Gibco BRL) and labeled oligonucleotides were separated by the purification column (NEN-Dupon, cat # NLP-022) achieving a specific activity of 2.5x10$^9$ cpm/ug (Ausubel et al., 1987).

Fresh cryostat sections (4-6 slides per probe for each animal; n=3 at each time point) were removed from the freezer (-85 °C). For hybridization of an oligonucleotide probe, a modified procedure of the protocol given by Verge et al. (1992) was used. Sections were air dried for 20 minutes and directly hybridized to $10^6$ cpm of probe in 100 μl of the hybridization cocktail containing; 50% formamide, 4x saline-sodium citrate (SSC), dextran sulfate (100gm/litre), 1% sarcosyl, salmon sperm DNA (250mg/liter), yeast tRNA (500mg/liter), 50mM dithiothreitol(DTT) and 20mM phosphate buffer (pH 7.0). For autoradiography, the sections were dipped in Kodak NTB-2 emulsion, diluted 1:1 in H$_2$O
and exposed for 8-10 weeks for BDNF, 7-9 weeks for trkA and trkC and 3-4 weeks for trkB. They were subsequently stained with 0.2% cresylviolet acetate (Sigma), dehydrated in the series of alcohol and embedded in Permount or DePeX (BDH).

Controls were performed with a sense probe from the identical part of the BDNF or trkB sequence, showing that a probe of similar GC content does not give any signal (data not shown). Displacement with 100-fold excess of unlabeled probe virtually abolished the hybridization signal, indicative of the specificity of our method (data not shown). In parallel to the ISH experiments, RT-PCR for BDNF, trkB and trkC was performed using total RNA isolated from microdissected facial nuclei or red nuclei (see RT-PCR section for more detail).

2.3.2. GAP-43 and Tα1-tubulin ISH

The GAP-43 probe was complementary to bases 220-270 described in Basi et al., 1987 and the Tα1-tubulin probe was a 50 mer oligonucleotide complementary to the 3'-untranslated sequence of Tα1-tubulin 5'AAACCCATCAGTGAAGTGGACGGCTCGG GTCTCTGACAAATCATTCA-3'. These oligonucleotide probes were end-labeled as described above (Ausubel et al., 1987).

Perfusion-fixed sections (14 μm) were air dried for 20 minutes and pretreated as follows: 4% paraformaldehyde in 10mM phosphate buffer for 20 minutes, rinsed 3 times in 10mM PBS (5 minutes each), 20μg/ml protainase K in 50mM Tris-HCl (pH 7.6) plus 5mM EDTA for 7.5 minutes, rinsed twice in 10mM PBS, 4% paraformaldehyde for 5 minutes, rinsed twice in 10mM PBS and once in DEPC-treated water, dehydrated in 70%, 90% and 100% ethanol (30 seconds each) and air dried. Subsequently sections were pre-hybridized for 2 hours at 43 °C with the hybridization cocktail containing 50% formamide, 5x PIPES, 5x Denhardt’s solution (consists of 1mg/ml Ficoll, 1mg/ml
polyvinylpyrrolidone, 5mg/ml bovine serum albumin (BSA)), 0.2% sodium dodecyl sulfate (SDS), 100mM DTT, 250μg/ml salmon sperm DNA and 250μg/ml yeast RNA. Finally, they were hybridized to $10^6$ c.p.m. of an appropriate probe in 100μl of the same hybridization cocktail for 16-18 hours at 43 °C. Sections were then washed as described above.

These slides were dipped in Kodak NTB-2 emulsion as above and were exposed for 2 days for Tα1-tubulin, 7 days for GAP-43. The control experiments were done previously that the GAP-43 and Tα1-tubulin probes gave a single band of expected size in northern blots of RNA from facial nuclei under identical or less stringent hybridization conditions (Tetzlaff et al., 1991).

2.3.3. Quantification of BDNF and trkB ISH Signals

For each probe, 2-3 slides from each experimental animal (n=3 at a time point) were analyzed. The slide labels were coded so that the user was blinded, i.e. unaware of time point and an oligonucleotide probe applied to the slide. Under oil immersion brightfield illumination (40x oil immersion lens, Zeiss), only those FMNs with the visible nucleolus were analyzed, resulted typically in 35-50 cells from each facial motonucleus. The same criteria was used for the ISH signal quantification in RSNs, resulted typically 20-30 cells per red nucleus. Computer assisted image analysis system allowed digitization of the captured image. Silver grains (ISH signals) were recognized by user-interactive density level thresholding. The background grain counts were assessed by taking grain counts from tissue area without visible cells. A correction factor for overlapping grains was included and the area fraction occupied by grains never exceeded 0.15.

As mentioned in the introduction massive changes in neuronal cell size can occur after axonal injury. Shrinkage of a neuron without changes in grain density — as seen in
the cross section—would reflect a decreased amount of mRNA per neuron. Minor changes in cross sectional area lead to larger changes in cell volume. Therefore, I wished to determine the changes of mRNA levels per neuron using its volume. Due to the complex nature of the neuronal shape its is very difficult to determine its volume without serial reconstruction. Hence, I decide to calculate the estimated neuronal volume as a sphere based on the measured cross sectional area. This approximation yields a conservative measure since small shrunken cells are likely not spherical and their volume might be somewhat overestimated. To obtain the “grain counts/cell” the fraction of the cross sectional area occupied by silver grains minus grain-background was multiplied by the estimated neuronal volume. Subsequently, scatterplots representing the grain counts/cell versus cell volume were generated.

To generate a plot of the time course of the trkB ISH signal, the ratio of the mean grain count (± SEM) of the axotomized versus contralateral control motoneurons was plotted against different days after axotomy.

2.3.4. Quantification of GAP43 and Tα1-tubulin ISH Signals

The Northern Exposure Image Analysis system (EMPIX, Mississauga, Ont., Canada) equipped with a frame grabber for the integration of multiple frames was used to capture pictures of the fluorescent FG filled RSN. This integration allowed further visualization of the contours of the unstained contralateral neurons. 50-100 RSNs from each animal (2-4 slides per animal) were analyzed for each probe. In order to obtain this number, we collected and analyzed 3-5 digitized images of the FG-filled RSNs from each section through the red nucleus. The fluorescent pictures of RSNs were loaded into one frame buffer and corresponding darkfield images of the silver grains representing the ISH signal were captured into another frame buffer. The contours of the fluorescent labeled cells were traced with a digitized mouse and this contour was
used as a mask in the other frame buffer which contained the darkfield illuminated silver grains. The area fraction occupied by the silver grains within this mask was measured and corrected for a background of the tissue. This corrected fraction was subsequently multiplied by the estimated volume of the neuronal cell body in order to generate the “ISH signal/cell” (see section 2.3.3. for rationale for using an estimated cell volume). The ISH signal/cell values of the axotomized RSN were expressed as multiples of the mean ISH signal/cell of the contralateral RSN. Only perfusion-fixed brain tissues were included in ISH quantification since fixation was necessary to retain FG-labeling after ISH procedures. Therefore, the numbers of animals for ISH quantification were smaller than those included in the cell size profile study (see histology and cell sizes in this section).

2.4. Reverse transcription - Polymerase Chain Reaction (RT-PCR)

2.4.1. RNA Extraction

During microdissection of the facial nucleus, contamination was avoided as much as possible by carefully excluding any neighboring tissue. Thus, the yields of total RNA extracted from facial nuclei pooled from two animals was only 1 to 1.5 µg total RNA, which was insufficient for northern blotting. For microdissection of the red nucleus, FG-filled axotomized RSN were visualized with a fluorescent microscope and microdissected from 70-80 µm thick serial sections through the caudal pole (400 µm) of the red nucleus, containing the magnocellular population. The contralateral red nuclei were identified and dissected under darkfield illumination. Typically 5 red nuclei were pooled to obtain 1 to 1.5µg of total RNA. Earlier experiments in our laboratory determined that retrograde-
labeling of RSN with FG had little effect on RNA expression (W. Tetzlaff, B. Tsui, A.M. Bedard and S. Cassar, unpublished observation).

The total RNA was extracted using the acidic phenol procedure of Chomzynski and Sacchi (1987) or using Trizol (Gibco BRL) according to manufacture’s protocol. To ensure the RNA samples were free of genomic DNA contamination, they were treated with DNAsel (10 ng/ml, Gibco BRL) for 20 minutes at room temperature. DNAsel was inactivated by adding 1 μl of 50 mM EDTA and incubating the reaction mixtures for 10 minutes at 65°C. The total RNA was reverse transcribed into first-strand cDNA using deoxythymidinol oligomer (oligo-dT) priming. In brief, 0.5 μg of RNA was transcribed for 1 hr at 42°C in 10 μl of a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 60 mM KCl, 2 mM MgCl₂, 7.5 mM DTT, 2.5 μM oligo-dT (16-18 mer, Pharmacia), 2 mM of each deoxynucleotide triphosphate, 0.1 mg/ml nuclease-free bovine serum albumin, 5 U of RNasin (Promega), and 200 U of mouse muloney leukemia virus reverse transcriptase (Superscript, Gibco BRL). Typically, each reaction yielded enough cDNA for 10 amplifications by PCR, representing the equivalent of 50 ng total RNA in each. For the serial dilution series, 50, 25, 12.5, 6.25, and 3.12 ng were amplified. 50 μl of the PCR reaction mixture contained 60 mM Tris-HCl (pH 8.3), 15 mM NH₄SO₄, 2.5 mM MgCl₂, 0.1 mg/ml nuclease-free bovine serum albumin, 0.6 mM of each deoxynucleotide triphosphate, 1U of Taq polymerase (Gibco BRL), and 60 ng each of the 3’ and 5’ primers.

2.4.2. RT-PCR for BDNF

The primers used for BDNF were 5’-CGGATCCGCTGCAAACATGTCCATG-3’ for the left primer and 5’-GCCACTATCTTTCCCTTTTAATGG-3’ for the right primer, according to nucleotides 437-455 and 825-803 of the rat BDNF sequence respectively (with two mismatches each plus a restriction site) (Maisonpierre et al., 1991, Genbank
access # M61175). The rat BDNF sequence was kindly provided to us by Dr. Freda Miller (Montreal) prior to publication. PCR amplification for BDNF was performed initially for 17, 21, and 25 cycles (1 min at 94 °C, 2 min at 42 °C and 3 min at 72 °C). Since 21 cycles (facial) or 25 cycles (rubral) yielded satisfying linear BDNF amplifications based on densitometric analysis of autoradiographic signals or phosphoimaging (Molecular Dynamics), these cycles was used routinely. The BDNF PCR products were run on 1% agarose gels, Southern transferred onto a nylon membrane (Zeta-probe, Bio-Rad) using a standard capillary transfer protocol in 0.4M NaOH for overnigh (Sambrook et al., 1989). The membranes were prehybridized with the hybridization solution containing 50% formamide, 5x Denhardts’ solution, 5x SSPE, 0.5% SDS, 250μg/ml salmon sperm DNA and 250μg/ml yeast tRNA at 43°C for 2 hours and then probed with 10^6 cpm/ml of the 35S-labeled BDNF oligonucleotide probe used for the BDNF ISH for 14-18 hours at 43°C. This ISH probe was not overlapping with the PCR primers and had few homologies with the other neurotrophin sequences (see ISH section).

Subsequently, the membranes were washed in the dilution series of SSC; starting with 2x SSC and ended with 0.1x SSC. All the wash solution contained 0.2% SDS and 2ml/liter β-mercaptoethanol except for the last 2 washes. Each wash was at 47°C for 20 minutes. The membranes were air dried and exposed to Kodak XAR-5 film for 4-5 days or to a phosphoimager (Molecular Dynamics) for 4-5 hours. The film autoradiographs of the PCR products were quantified by laser-densitometry (LKB-Ultrascan XL). The values obtained by the densitometric measurement were plotted in terms of input cDNA concentration to determine linearity and a slope of the regression line. Relative comparisons of the slope of the contralateral control side versus axotomized side revealed the relative change in mRNA level after injury.
Since the sequence of BDNF primers used in the time course analysis were located on the same exon (V) several controls were performed in order to preclude the false positive from genomic DNA contamination. First, amplifications of RNA without the reverse transcription step was blank (data not shown); second, treatment of the extracted RNA with RNase-free DNase did not abolish the signal (data not shown); third, primers from different exons were used (Timmusk et al., 1993; Nakayama et al., 1994) revealing different promoter usage after axonal injury (see blow for detail). Taken together, these controls confirm specificity of BDNF PCR results without genomic DNA contamination. Additional controls were carried out by restriction enzyme digestion of the PCR product; treatment of the BDNF product with Nci I, and trkB product (see below) with Sca I, generated the expected sizes of the fragmented DNA products (data not shown).

For the analysis of exon specific BDNF mRNA expression, the left primers for exons I-IV described in Nakayama et al. (1994) were used in conjunction with the right primer mentioned above. The condition for the PCR amplification was the same as above except that 30 cycles of amplification was used with the annealing temperature of 52 °C. Southern blotting and autoradiography were performed the same as above.

2.4.3. RT-PCR for trkB

For PCR amplification of trkB, we used 5'-TGCTGTGGTGGTGCCTGCTGTG-3' as left primer and 5'-GTTCTCTCCTACCAAGCAGTTCCGG-3' as right primer corresponding to 1266-1290 and 2201-2225 of the rat trkB sequence published by Middlemas et al. (1991). These primers include the tyrosine kinase domain and are therefore specifically amplifying full length trkB transcripts. The PCR condition was the same as BDNF PCR amplification except that the annealing temperature was 47°C. 21 cycles (facial) or 25 cycles (rubral) of PCR amplification was within the linear range of amplification as evidenced by densitometric analysis or phosphoimaging and thus used
routinely for serial dilution series. Southern blotting, autoradiography and densitometric analysis were done the same as described for BDNF PCR analysis except that exposure time for autoradiography was 8-10 hours.

2.4.4. RT-PCR for trkC

The trkC primers have been described previously (Offenhauser et al., 1995), and were designed to bracket the potential insertion site within the tyrosine kinase domain in order to reveal different trkC insertion isoforms. After 30 amplification cycles, trkC PCR products were run on a 5% polyacrylamide gel stained with ethidium bromide and visualized under UV light and photographed. The PCR of input cDNA serial dilutions followed by Southern blotting were within the linear range as determined by phosphoimaging (Molecular Dynamics) when 25 amplification cycles was used for trkB and 30 amplification cycles for trkC (non-inserted trkC isoform at 299bp; data not shown) respectively. These 50mers were also used in trkB or trkC in situ hybridization study (see ISH section) and had little homology to other trk receptors.

2.4.5. RT-PCR for cyclophilin (control)

Cyclophilin was a constitutively expressed gene and cyclophilin PCR was included to ensure the equal amount of input cDNA was evaluated. The left primer corresponds to bases 44-65; 5'-TGGTCAACCCACCGTGTTCTT-3'. The right primer was complementary to bases 393-414; 5'-GTTCCTGACTCACCGACCTACCG-3'. This primer pair was previously described in Mearow et al. (1993). The PCR condition was the same as the BDNF amplification condition except that 17 cycles of PCR amplification was used with annealing temperature of 52°C. Southern blotting and autoradiography was performed the same as BDNF PCR analysis.
2.5. Western Blotting for BDNF

Axotomized and contralateral control facial nuclei were microdissected as described above. The proximal and distal stumps of the axotomized facial nerve were also dissected as well as the contralateral control nerve; both nerves were frozen on dry ice. These were subsequently homogenized in the sample buffer containing 0.2% SDS, 0.1% glycerol, 60mM Tris (pH6.8) and 1mM PMSF, 2mM EDTA. 30 μg protein from each sample was separated on 15% SDS-PAGE gels as described previously (Hincke and Nairn, 1992). Subsequently, the proteins were electro-blotted to PVDF (Biorad, 0.2 μm) overnight in alkaline transfer buffer containing 150mM Tris (pH 10.2) and 20% methanol at 40 V, 4 °C. The membranes were washed in PBS containing 0.1% tween-20 (TPBS) and blocked for 1 hour with 5 % BSA in TPBS (pH 7.0). The membranes were washed and incubated 1 hour with anti-BDNF (Santa Cruz, # sc-546) diluted by 1:10,000 in TPBS. The enhanced chemiluminescence method (Amersham) was utilized to reveal immunoreactive bands. A dilution series of recombinant human BDNF (generous gift of Regeneron Pharmaceuticals Inc.) from 0.5 ng to 10 ng was run on the same gel/membrane. For controls, the anti-BDNF antibody was preabsorbed with the 50x excess peptide used for immunization (Santa Cruz # sc-546p) or with 5-10μg of rhBDNF: The antibody was incubated with either the control peptide or rhBDNF at 4 °C for 3-4 hours and the mixtures were centrifuged for 30 minutes at 15,000 rpm at 4 °C and supernatant was used for immunoblotting (Hincke et al., 1994). These preabsorptions resulted in deletion of the signals. The specificity of this antibody for BDNF and not for the other neurotrophins has also been recently demonstrated by the manufacturer (Santa Cruz).
2.6. Immunocytochemistry (ICC)

2.6.1. Immunocytochemical assessment of the neurotrophin distribution after neurotrophin infusion

Perfusion-fixed sections containing red nuclei were immunostained using antibodies for NGF, BDNF, NT-3 or NT-4/5. The specificity of the antibodies for their respective neurotrophin has been described previously (Anderson et al, 1995, Alderson et al, 1996). The concentrations of the primary antibodies were adjusted so that they exhibited equivalent levels of detection of the homologous neurotrophin on slot blots (0.5-1.0 ng/8 mm²); 1:7500 for the turkey anti-BDNF antibody, 1:5000 for the turkey anti-NT-3 antibody, 1:15,000 for the goat anti-NGF antibody, and 1:10000 for the chicken anti-NT-4/5 antibody. No cross-reactivity with heterologous neurotrophins was apparent on slot blots or in perfusion-fixed midbrain sections taken from animals which had been injected with 1 µg of the neurotrophins (data not shown). Primary antibody omission or pre-absorption with the appropriate, but not related neurotrophins, also abolished specific immunostaining (data not shown). Primary antibodies bound to tissue were localized using an appropriate biotinylated secondary antibody (1:1500) and the avidin-biotin-peroxidase complex (Vectastain, Vector Laboratories).

2.6.2. ICC for GAP-43

Perfusion-fixed spinal cord segments were removed from C1-C2 levels (C3 lesion-only group, n=3) or from T8-T9 levels (T10 lesion-only group, or T10 lesion with BDNF treatment group as described above, n=3 in each group). They were cross-sectioned at 25µm and were either mounted on slides or kept in 30% glycerol in 0.01M PBS and stored at -80°C or 4°C until use. After several washes in 0.01M PBS, the
sections were treated with a blocking solution containing 0.1% Triton-x100, 2% rabbit serum in 0.01M PBS and incubated with the mouse monoclonal anti-GAP-43 antibody (9-1E12, provided by Dr. David Shreyer, Saskatoon, Canada) at a dilution of 1: 10,000 in 0.01 M PBS at 4°C overnight. After 3 X 10 minutes washes in 0.01M PBS, a biotinylated goat anti-mouse IgG antibody at 1:1000 dilution (Jackson laboratory, PA) was applied and incubated for 2 hours at room temperature. The signal was visualized by the avidin-biotin-peroxidase complex (Vectastain, Vector Laboratories).

In order to verify nature of GAP-43 positive axons, rubrospinal axons were anterogradely labeled by injecting 0.5 µl of 20% FluoroRuby using glass microcapillaries (Molecular Probes, Eugene, ROD1817) at the time of lateral funiculotomy. The stereotaxic coordinates were -6.3 mm from Bregma, 1.3 mm lateral from the midline and 6.5 mm deep from the pia surface. Seven days after cervical lesion and anterograde labeling, perfusion-fixed spinal cord segments were removed from C1-C2 levels and GAP-43 immunocytochemistry was performed as above except that a fluorescein-labelled sheep-anti-mouse antibody was used (Sigma C2181). There was no bleed-through from FluoroRuby filled axons, thus all GAP-43 positive axons were green, not orange.

2.7. Statistics

The mean BDNF or trkB grain counts/cell obtained from axotomized FMNs or RSNs was compared to that of contralateral control neurons using the Students' t-test. The cell size data for RSN after cervical axotomy as well as GAP-43 and Tα1-tubulin ISH quantification data were normalized and expressed as percent of, or multiples of contralateral values. When either a normality test (Kolmogorov-Smirnov) or equal variance test failed, Kruskal-Wallis' one way ANOVA on ranks (non-parametric test)
was used to test the differences in median values among the different treatment groups. In order to identify the groups which differed significantly from the control group (no pump or vehicle-treated group), Dunn's multiple comparison procedure was performed at the significance level of $P<0.05$. When both the normality test and equal variance test passed, post hoc Student-Newman-Keuls multiple comparison procedures were applied at the significance level of $P<0.05$. 
CHAPTER 3: Expression of BDNF and trkB in Axotomized FMNs and RSNs

3.1. Overview

In this chapter, I tested the hypothesis that axotomized FMNs receive trophic support in an autocrine/paracrine manner after axonal injury. Further, I hypothesized that this type of trophic support is absent or attenuated in axotomized RSNs after cervical injury. Using in situ hybridization, I found that axotomized FMNs transiently increased the expression of BDNF, which peaked around 1 day and returned to the contralateral control level 7 days after axotomy. The western blots for BDNF showed an increase in a BDNF-like immunoreactive band after axotomy, reflecting the observed increase in BDNF message at the protein level. Moreover, the expression of trkB, the cognate receptor for BDNF, also increased, peaking around 4 days after axotomy and slowly declining thereafter. These ISH findings were corroborated by the RT-PCR results, confirming the increased expression of BDNF and trkB in the facial motonucleus after axotomy. In contrast, only a small number of axotomized RSNs showed increased BDNF ISH signals 3 days after cervical axotomy. While trkB expression declined to 70% of contralateral control level 7 days after axotomy.

Taken together, these results support the overall hypothesis that availability of trophic support after axonal injury differs between axotomized FMNs and axotomized RSNs.

3.2. Introduction

Neurotrophic factors play an important role in the development, maintenance and plasticity of the nervous system (for review see Davies, 1994; Lindholm et al., 1994; Lindsay et al., 1994; Sendtner et al., 1994; Henderson, 1996; Lewin and Barde, 1996).
Observations on the first-discovered member of the neurotrophin family, NGF, have led to the "classical neurotrophic hypothesis" that the target of a developing neuron supplies a limited quantity of a factor and competition for this target-derived factor regulates survival of innervating neurons (for review see Korsching, 1993). It is generally believed that axotomy of a neuron interferes with target-derived neurotrophic support. Most mature motoneurons survive axotomy (for review see Lowrie and Vrbova, 1992), suggesting at some point in development, they become independent of putative target-derived trophic factors. Since it is unlikely that they become completely independent of trophic support, sources other than the target may be able to substitute.

In the case of motoneurons, the importance of a glial source of trophic support was demonstrated in mice carrying a disruption of the CNTF gene. CNTF is normally expressed by myelinating Schwann cells (Dobrea et al., 1992; Friedman et al., 1992; Rende et al., 1992; Sendtner et al., 1992b; Seniuk et al., 1992). In CNTF deficient mice, motoneurons develop normally, but a significant percentage fail to survive as the animals grow older (Masu et al., 1993). After axonal injury, CNTF expression decreases in Schwann cells of the distal nerve stump (Friedman et al., 1992; Rabinovsky et al., 1992; Sendtner et al., 1992b; Seniuk et al., 1992). However, retrograde transport of CNTF to the axotomized motoneuron soma is transiently increased (Curtis et al., 1993) probably due to release of CNTF from injured Schwann cells.

After nerve transection, the reactive Schwann cells of the distal stump begin to produce a battery of trophic factors, including NGF, BDNF and NT-4 (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993). However, motoneurons survive a transection of their nerve, which does not preserve the basal membrane continuity between the proximal and distal stump. Thus, this type of injury, in contrast to crush injury, separates motoneuron cell bodies from the reactive Schwann cells of the distal stump, leaving them with possible trophic support from the proximal stump. Since the proximal...
stump contains some reactive Schwann cells, it was suggested that they provide some
trophic support (Heumann et al., 1987). However, this attractive hypothesis remains
unproved. In the case of NGF, only relatively low amounts of NGF appear to reach the
axotomized neuron from the proximal stump (Raivich et al., 1991). Moreover, the
expression of BDNF mRNA in the proximal nerve stump is low when compared to the
distal stump 17 days after nerve transection (Meyer et al., 1992).

Another possible non-target-derived source of trophic support is via the
axotomized motoneuron themselves or from their immediate CNS environment in an
autocrine/paracrine fashion. A variety of neurons express the mRNAs for both
neurotrophic factors and their receptors during development and adulthood (Ernfors et al.,
1990 a, b; Merlio et al., 1992; Schechter son and Bothwell, 1992; Miranda et al., 1993;
Kokaia et al., 1993; Escandon et al., 1994; for review see Korsching, 1993). Motoneurons
have been shown to express trkB mRNA (Yan et al., 1993, Koliatsos et al. 1993) and take
up and retrogradely transport ^{125}I-labeled BDNF (DiStefano et al., 1992; Yan et al., 1992,
Clatterbuck et al., 1994). Moreover, the axotomized facial and spinal motoneurons of
neonatal rat were responsive to exogenous BDNF as evidenced by the transient rescue of
these neurons from cell death (Sendner et al., 1992a; Yan et al., 1992; Henderson et al.,
1993).

Taken together, I hypothesize that axotomized FMNs receive trophic support from
themselves (autocrine) or from their immediate CNS environment (paracrine) after facial
nerve transection. In particular, the observations in motoneurons, i.e. trkB expression,
retrograde transport of BDNF and responsiveness to exogenous BDNF in neonatal animal
provided a rationale to focus on the expression of BDNF and its receptor, trkB in
axotomized FMNs of the adult rat.

As outlined in the General Introduction, axonal injury in PNS and CNS neurons
results in contrasting outcomes. For example, axotomized FMNs survive and even
increase their soma size after facial nerve transection (Tetzlaff et al., 1984). On the contrary, RSNs undergo severe atrophy during the second week after cervical axotomy of the spinal cord (Egan et al., 1977, Barron et al., 1989; Tetzlaff et al., 1991).

Little is known about the sources of trophic support for CNS neurons. Evidence in favor of target-derived trophic support has been provided for developing and mature CNS neuronal systems. For example, ablation of hippocampal neurons, a target for basal forebrain cholinergic neurons, in the first two weeks after birth results in massive loss of these cholinergic neurons (Burke et al., 1994; Cooper et al., 1996). In neonatal rats, axotomy of the sciatic nerve results in severe reduction of the number of ventral motoneurons, leading to a secondary loss of developing corticospinal and RSNs (Obouhova et al., 1994). In the adult rodent, cortical degeneration due to devascularizations leads to severe atrophy of the neurons in the nucleus basalis magnocellularis (Figueiredo et al., 1996). These studies are consistent with the concept of target-derived trophic support for CNS neurons during development and adulthood.

Potential sources of trophic support after axotomy of a CNS neuron are the targets of axon collaterals, the glial environment of the proximal axon or the neuron itself. Since adult RSNs undergo massive shrinkage after cervical axotomy it appears that the remaining trophic support from the potential sources is limited compared to FMNs. Therefore, I hypothesize that RSNs lack autocrine/paracrine trophic support after axotomy. My initial ISH observation that intact RSNs expressed trkB mRNA, like FMNs, provided the rationale to focus on the expression of BDNF and trkB in RSNs after cervical injury of the spinal cord.
3.3. Results

3.3.1: Axotomized FMNs Increase the Expression of BDNF and trkB after Facial Nerve Transection

*In situ hybridization (ISH) for BDNF and trkB*

Using a 50-mer oligonucleotide probe for BDNF mRNA, ISH revealed very low signals over uninjured FMNs. Similarly, very low levels of BDNF ISH signals were found over FMNs contralateral to the axotomy side even after 8 weeks of autoradiographic exposure (Fig. 1 A). After axonal injury, an increased BDNF ISH signal was observed over axotomized FMNs (Fig. 1 B). Quantification of the autoradiographic silver grains revealed an elevated level of BDNF ISH signals as early as 8 hours post-injury (P<0.05, t-test). One day following axotomy, approximately 50% of the FMNs showed increased ISH signal for BDNF mRNA, with the majority of them indicating increased BDNF gene expression within 2 days post-injury (P<0.01; t-test) (Fig. 2 B). This increase in BDNF ISH signal was transient and started to decline 4 days following injury (Fig. 2 C). By 7 and 14 days after axotomy, no difference in BDNF signal was detected in comparison to the uninjured contralateral side (Fig. 2 D). No apparent glial signal was seen. ISH using the sense probe showed only background levels over axotomized FMNs at any time point (data not shown).

TrkB ISH was performed using a 45mer oligonucleotide probe complementary to the sequences obtained from the cytoplasmic tyrosine kinase domain; thus, this probe specifically recognized the mRNA species coding the full-length trkB receptor. Normal FMNs as well as those contralateral to the axotomized FMNs, showed distinct signals for the full length trkB mRNA after 3 weeks of autoradiographic exposure (Fig. 1 C). No
apparent glial signal was observed. After axotomy, trkB ISH signal within the injured FMNs increased and was most evident 4 days after injury (Fig. 1 D). The grain counts per cell started to increase on day 2 (P<0.05; t-test) (Fig. 3 A) and were significantly elevated by 4 (P<0.01; t-test) and 7 days (P<0.05; t-test) after injury (Fig. 3 B, C). Fourteen and 21 days after facial nerve transection, grain counts for trkB ISH signal were still elevated between 1.5-3 times the level over contralateral control FMNs (P<0.05; t-test) (Fig. 3 D).

**RT-PCR for BDNF and trkB**

Total RNA was extracted from experimental axotomized and contralateral control facial nuclei and reverse transcribed to cDNA. The serial dilutions of the cDNA were amplified by 17 or 21 cycles of PCR. The products were then Southern blotted, hybridized and visualized by autoradiography. This procedure resulted in amplifications that were linear over several dilution steps with regression coefficients greater than $r^2>0.97$. Since this procedure was quantitative within the range of one order of magnitude, relative comparisons between the axotomized and contralateral control facial nucleus could be made.

An increased expression of BDNF mRNA was seen as early as 8 hours after axotomy (Fig. 4 A). This remained elevated for several days and gradually declined thereafter (Fig. 4 B-E), consistent with the ISH data described above. Densitometric quantification of the BDNF PCR product revealed a 2-fold increase in slope for the axotomized side within 8 hours, while no change was seen at 3 hours (data not shown). The slopes of the serial dilutions were between 2 and 4 fold higher on the axotomized side 1 or 2 days after injury (Fig. 5 B, C, F). They subsequently declined 4 days after axotomy and were no longer different from contralateral control side 7 days after axotomy (Fig. 5 D, E).
Consistent with the ISH data, trkB mRNA expression after axotomy, as measured by RT-PCR, did not begin to increase until the second day (Fig. 4 A-C) and was elevated at all time points observed thereafter (Fig. 4 D, E). Control amplifications of cyclophilin mRNA did not change during the first days after injury and verified that equivalent amounts of cDNA had been amplified and loaded. However, probably due to an increased number of proliferating microglial cells within the axotomized facial nucleus, cyclophilin increased slightly on days 4 and 7.

Using a serial dilution BDNF RT-PCR, I also compared the relative difference in the expression levels of BDNF in the distal stump of the facial nerve, a possible source of BDNF after axotomy, and in the facial nucleus. My preliminary result indicated that the distal stump of the transected facial nerve 14 days post-axotomy (reported to be at the maximal expression level, Meyer et al., 1992) displayed a 1.5-2 fold higher BDNF expression compared to the axotomized facial nucleus 1 day after injury (data not shown). My preliminary data indicated further that the proximal nerve stump had very low levels of BDNF mRNA expression 7 and 14 days after axotomy, which is consistent with the finding of Meyer et al. (1992).

**Western blotting for BDNF**

Western blots of axotomized and contralateral facial nuclei as well as proximal and distal stumps of facial nerves showed a distinct BDNF immunoreactive band at an apparent molecular weight around 13 kDa (Fig. 6 A, arrow). Preincubation of the BDNF antibody with rhBDNF or the peptide used for immunization (Santa Cruz # sc-546p) completely abolished this immunoreactive band and that of a cross-reactive band around 43 kDa (data not shown). This 43 kDa immunoreactive band is larger than the expected size of the BDNF precursor protein at around 33 kDa (Heymack and Shooter, 1995). Thus, it most likely represents a crossreactive protein of unknown nature. Based on the
correct molecular weight, absorption controls, specificity of the antibody for BDNF (without crossreactivity to the other neurotrophins), and recognition of the rhBDNF standard, we refer to this 13 kDa immunoreactive band as BDNF protein. Our blots showed a distinct increase in BDNF protein within the axotomized facial nucleus as early as 1 day after axotomy (Fig. 6 A). This BDNF protein increase peaked around 5 to 7 days after injury with levels in the range of 5-10 ng per 30 µg total protein, which was consistently higher than contralateral control facial motor nucleus levels of BDNF expression of around 2-5 ng per 30 µg total protein (Fig. 6 A). The increase in BDNF protein was still sustained 12-14 days after axotomy (Fig. 6 A), beyond the period of BDNF mRNA increase (Figs. 4 E and 5 E).

The BDNF protein expression in the axotomized facial nucleus at 7 days was comparable to the level of BDNF protein found in the distal and proximal stumps of the facial nerve at 14 days after nerve transection (Fig. 6 B). Surprisingly, a significant level of BDNF protein, but barely detectable level of mRNA (see above), was found in the proximal stump of the facial nerve on 3, 7 and 14 days after axotomy (data not shown). The source of this BDNF in the proximal stump is unknown. Studies are underway to examine possible orthograde transport from the axotomized FMNs.

Differential expression of BDNF exons

The BDNF gene consists of 4 short 5’ exons (I-IV) and a fifth 3’ exon encoding the prepro-BDNF protein (Timmusk et al. 1993). BDNF transcripts contain one of these short exons spliced to exon V. Tissue specific and stimulus specific expression appears to be controlled by at least four promoter regions upstream of exons I-IV (Timmusk et al., 1993; Metsis et al., 1993; Nakayama et al., 1994). In order to better understand for the regulation of the increase in BDNF mRNA after axotomy, we studied the differential expression of
these BDNF exons. As outlined in the method section, I used 5' primers to exons I to IV in combination with a 3'-primer on exon V. Amplifications with 30 cycles of PCR followed by Southern blotting revealed increases in all four BDNF mRNA classes. However, the increase in BDNF mRNA containing exons IV and III were more pronounced than the increase associated with exons I and II (Fig. 7 B-E). Since the primer pair taken from exon V only (used in the experiments above, Fig 4) hybridizes with all BDNF mRNA species PCR amplification using 30 cycles reached saturation. Thus, the increase in BDNF expression in axotomized facial motonucleus was less evident in this case (Fig. 7 A) compared to the results obtained with 21 cycles of amplification (Fig. 4).

3.3.2. Axotomized RSNs Fail to Increase the Expression of BDNF and trkB after Cervical Injury

ISH for BDNF and trkB

Uninjured contralateral RSNs showed low levels of BDNF ISH signals and only exceptional RSNs, i.e. 1-2 cells, displayed increased levels of BDNF expression 3 days after cervical axotomy (Fig. 8 A, B). This minor BDNF increase in few axotomized RSNs was consistent in three animals examined.

Full-length trkB ISH signals was seen in both contralateral as well as axotomized RSNs 7 days post-axotomy (Fig 8 C, D). However, quantification of trkB ISH signals revealed that 7 days after axotomy, the mean ISH signals/cell was only 69.7% (±4.37) of contralateral control (Fig. 8, Scatterplot).
**RT-PCR for BDNF and trkB**

Total RNA was extracted from microdissected red nuclei and the expression of BDNF and trkB in the red nuclei was confirmed by serial dilution RT-PCR (amplification of 25, 12.5, 6.75, 3.4 ng cDNA). The PCR products were visualized by Southern blot using BDNF or trkB probe internal to the PCR primers.

There was only a minor increase (<2 fold) in BDNF mRNA expression in axotomized side of red nuclei compared to the contralateral control side 2 days after axotomy (Fig. 9), corroborating a minor increase contributed from few axotomized RSNs displaying high levels of BDNF ISH signals. Seven days after injury, the BDNF mRNA level in axotomized side of red nuclei was comparable to that of contralateral sides (Fig.9).

A decrease in trkB mRNA expression is already detectable on the axotomized side 2 days after injury (Fig. 9). A further decline in trkB mRNA level was observed 7 days after axotomy (Fig. 9) in agreement with the decreased trkB ISH signals in axotomized RSNs. This analysis also confirmed the ISH observation that the trkB expression was decreased in axotomized RSNs compared to the contralateral, intact RSNs (Fig. 8 C, D).

**3.4. Discussion**

**3.4.1. Chapter Summary**

In this chapter, I have shown that axotomized FMNs increased the expression of BDNF mRNA as early as 8 hours after axotomy and sustain this expression at levels around 2-4 fold contralateral for several days. Increased BDNF protein expression is seen in western blots within one day after injury and maintained for at least 2 weeks. This increase in BDNF mRNA and protein expression is followed by increased expression of trkB mRNA coding for the BDNF receptor, which starts 2 days after axotomy and persists
for 2-3 weeks. In contrast to axotomized FMNs, BDNF ISH revealed that only exceptional RSNs increase the mRNA expression of BDNF 3 days after cervical axotomy. Moreover, trkB ISH signals decreased to 70% of contralateral control 7 days after axotomy. RT-PCR for BDNF as well as trkB confirmed these findings.

3.4.2. BDNF and trkB mRNA Expression in Axotomized Peripheral Neurons

These findings of increased trkB mRNA expression in axotomized FMNs extends the study of Piehl et al. (1994), who reported a 2-fold increase in trkB mRNA in spinal motoneurons 3 days after axotomy. My findings are also consistent with the report of Koliatsos et al. (1994) who demonstrated the expression of full-length trkB and BDNF mRNA in PCR amplifications of total RNA extracted from the normal rat facial nucleus. My data on BDNF mRNA also complement northern blot analysis of dorsal root ganglia where a 2-fold increase in mRNA expression for BDNF was observed after axotomy (Ernfors et al., 1993; Sebert and Shooter, 1993). This increase was evident 12 hours after crush injury and lasted for at least 3 weeks (Sebert and Shooter, 1993). Using ISH, Ernfors et al. (1993) demonstrated further that this upregulation was reflected by intense BDNF expression in the "occasional medium-sized neuron" five days after crush injury of the sciatic nerve. This is in contrast to my findings in FMNs where the majority of the axotomized FMNs increase the expression of BDNF mRNA for only the first 4 days.

The increase in BDNF mRNA after axotomy is followed by the predicted increase in BDNF protein. The latter peaked around 5 to 7 days post-axotomy and was still observed after 2 weeks, well after BDNF mRNA expression had returned to control levels, suggesting a long half-life for the BDNF immunoreactive protein. BDNF mRNA in the proximal stump is barely detectable 7 and 14 days after axotomy (data not shown), which is consistent with the report by Meyer et al. (1992). Therefore, it is unlikely that the
increased BDNF protein seen in axotomized FMNs during the second week is derived from non-neuronal cells of the proximal stump.

3.4.3. Regulation and Exon Usage of BDNF Gene after Axotomy

The mechanism of BDNF mRNA induction after axotomy is unknown. In vivo, BDNF mRNA expression has been shown to be inducible by a wide variety of depolarizing events such as seizures, kindling, spreading depression, application of kainic acid and cholinergic agonists as well as a GABA-A antagonist (Ballarin et al., 1991; Ernfors et al., 1991; Kokaia et al., 1993; Wetmore et al., 1994). These BDNF mRNA increases occur rapidly within 1-3 hours and typically do not last beyond 8 hours.

In contrast, after axotomy no increase was seen within 3 hours suggesting that the BDNF increase is not triggered by an early depolarization due to axotomy. This notion is further supported by the fact that another activity-dependent marker of depolarization, c-fos, is not increased after facial nerve axotomy (Jones et al., 1991; Haas et al., 1993). The later and more prolonged increase in BDNF mRNA after axotomy is more likely regulated by the retrograde axonal transport of a positive or negative signal.

The BDNF gene consists of 4 short 5' exons (I-IV) and a fifth 3' exon encoding the prepro-BDNF protein (Timmusk et al. 1993). BDNF transcripts contain one of these short exons spliced to exon V. Tissue specific and stimulus specific expression appears to be controlled by at least four promoter regions upstream of exons I-IV (Timmusk et al., 1993; Metsis et al., 1993; Nakayama et al., 1994). While the expression of exon I, exon II and exon III BDNF mRNA is increased in the hippocampus and cortex after kainic acid injections no changes in exon IV have been observed (Metsis et al. 1993; Nakayama et al., 1994). All three exons are increased after single seizures and kindling in the dentate gyrus while in CA1 of the hippocampus the increase is largely confined to exon III (Kokaia et al. 1994). In contrast, global ischaemia solely induced increases of exon III in the
dentate gyrus while hypoglycemic coma increased exon III and, moderately, exon I (Kokaia et al., 1994). Again, no increase of exon IV was seen with any of these insults in the presence of a significant baseline expression. Exon IV appears to be present in heart and in cultures of astrocytes (Nakayama et al., 1994), and increased in the distal nerve stump undergoing Wallerian degeneration (Funakoshi et al., 1993). In the axotomized facial nucleus, we observed an increased expression of exons IV and III, and to a lesser extent, exons I and II. This represents a novel pattern of BDNF promoter usage suggesting that different signals are responsible for the increase in BDNF mRNA after axotomy. We can not rule out some minor glial contribution from reactive astrocytes since the low levels of ISH signal over glia cells preclude an unambiguous identification of glial BDNF mRNA expression.

3.4.4. Functional Importance of BDNF and trkB in Axotomized Neurons

An importance of TrkB signaling for survival of axotomized immature FMNs has recently been reported in TrkB knockout mice. Homozygous TrkB−/− mice die within a few weeks after birth, however, survival and neuronal cell size of FMNs seem to be comparable to those of wild type mice (Kline et al., 1993). However, when facial nerve was transected on postnatal day 5 (p5) a number of surviving FMNs in these mutant mice on P10 is only 25% of contralateral controls and is one half of the numbers in the wild type counterparts (Alcantara et al., 1997). Moreover, neuronal cell size of those surviving FMNs in mutant mice was substantially smaller, suggesting a role of TrkB signaling for axotomized FMNs.

The increased expression of both BDNF and full-length trkB mRNA by axotomized motoneurons supports the hypothesis that BDNF acts on its receptor, TrkB in an autocrine/paracrine fashion, providing trophic support after axotomy and target deprivation. Autocrine/paracrine actions of BDNF for neuronal survival have been demonstrated in
cultures of dorsal root ganglion cells (Acheson et al., 1995) and cortical neurons (Ghosh et al., 1994). The survival of these neurons were significantly decreased by treatment with BDNF antisense oligonucleotides or with anti-BDNF antibodies, respectively. Since it is unlikely that these antibodies penetrated into the cortical neurons endogenous BDNF was most likely secreted into the culture media and acted on the auto-receptors or on the neighboring cells. It remains to be shown whether increased amounts of BDNF protein are secreted from FMNs after axotomy.

A small increase in BDNF expression by injured motoneurons may have a significant biological effect since it is in a strategically favored location. For example, in compartmented cultures, the effect of NGF to induce tyrosine hydroxylase is about one order of magnitude more potent when administered directly onto neuronal cell bodies of cultured superior cervical ganglion cells as opposed to their axonal compartment (Toma et al., 1993). In addition, the increase in trkB expression after axotomy might enhance the responsiveness of axotomized motoneurons to BDNF.

Thus far, little is known about trophic factor production after CNS injury. Following hemisection of the spinal cord, an increased level of truncated trkB receptor as well as p75LNTR expression was observed in non-neuronal cells associated with the scar formation around the injury site (Frisen et al., 1992; 1993). However, ISH studies showed only a minor increase in NGF expression and no increase in trkA, BDNF, NT-3 and NT-4 mRNA levels. The cellular localization of these trophic factors was unclear (Frisen et al., 1992; 1993). Other studies have reported an increased expression of CNTF, acidic and basic FGF, and TGF-β mainly after CNS injury. Again, precise cellular sources of these factors remain to be shown (Frautschy et al., 1991; Logan et al., 1992; Ip et al., 1993; Koshinaga et al., 1993; Follesa et al., 1994).

My findings further support the concept that axotomized CNS neurons receive limited trophic support after axonal injury. Unlike axotomized FMNs, axotomized RSNs
showed only a minor increase of BDNF expression. Moreover, the trkB mRNA level declined to 70% of contralateral. These observations are consistent with the report of Jelsma et al., (1993) that the full-length trkB mRNA level in retinal ganglion cells dramatically decreases after optic nerve transection.

In summary, upregulation of BDNF mRNA within axotomized FMNs and the production of BDNF protein within the facial motonucleus argues for autocrine/paracrine trophic support for injured FMNs. In contrast, trophic support for axotomized RSNs seems rather inadequate; only the exceptional axotomized RSNs increased BDNF expression and the expression of trkB in these neurons declined.

The presence of trkB receptors in axotomized RSNs, however, predicted their responsiveness to BDNF and provided a rational for the application of BDNF to axotomized RSNs. This issue is a primary focus of chapters 4 and 5 in this thesis.
Fig. 1  In situ hybridization with $^{35}$S labeled oligonucleotides complementary to BDNF (A, B) and to trkB (C, D) followed by radioautography. (B) Facial motoneurons one day after axotomy. Note the increased BDNF ISH signal compared to the contralateral facial motoneurons in (A). (D) Facial motoneurons 4 days after axotomy. Note the increased trkB ISH signal compared to the contralateral motoneurons in (C). Scale bar 20 μm.
Fig. 2 Scatterplots of the quantified silver grains resulting from the BDNF in situ hybridization. The counts per facial motoneuron are plotted against the cytoplasmic volume, allowing the visualization of change in ISH signal and changes in cell size. The filled symbols (●) represent the axotomized and the open symbol (○) the contralateral facial motoneurons. (A) Note the increased signal in some motoneurons by day 1. (B) Most axotomized motoneurons show increased BDNF ISH signals by day 2. (C) The ISH signals are still elevated in some cells on day 4. (D) Only few cells showed elevated levels of BDNF on day 7.
Fig. 3 Scatterplots of the trkB in situ hybridization grain quantification. The filled symbols (●) represent the axotomized and the open symbols (○) stand for the contralateral facial motoneurons. (A) Some facial motoneurons start to show increased trkB ISH signals 2 days after axotomy. (B) Four days after injury, a majority of the motoneurons have increased their levels of trkB expression. (C) This increased is still seen 7 days after axotomy. (D) The mean ± SEM is plotted from individual experiments versus survival time in days after axotomy (see text).
Fig. 4 Serial dilutions of RT-PCR amplifications for BDNF, trkB and Cyclophilin (Cy) visualized by Southern blotting followed by radioautography. Dilutions of amplified cDNA are equivalent to 50, 25, 12.5, 6.25, and 3.125 ng of total RNA extracted from 2 pooled facial nuclei. Amplifications from the contralateral facial nuclei are displayed on the left and from the axotomized facial nuclei on the right. The survival time after axotomy is at (A) 8 hours; (B) 1 day; (C) 2 days; (D) 4 days and (E) 7 days. Note the increased BDNF expression in (A,B,C and D) and the increased trkB expression in (D and E) after axotomy.
Fig. 5  Quantification of the serial dilution RT-PCR amplifications of the BDNF message from axotomized (filled symbols (+)) and contralateral (open symbols (○)) facial nuclei. 21 cycles of PCR amplification are linear over dilution series of 50, 25, 12.5, 6.25 and 3.12 ng of input cDNA concentration. Note the steeper slopes of the linearized regression lines from the axotomized facial nuclei which are already apparent at 8 hours (A), 1 day (B), and 2 days (C). A weak increase is still seen in 4 days (D) but no longer in (E) 7 days after axotomy. The time course of these slopes is plotted in (F).
Fig. 6 (A) Western blots for BDNF in facial nuclei at 1, 3, 5, 7, 12 and 14 days after axotomy. An immunoreactive band of expected apparent size of 13 kDa (→) is revealed in addition to another cross-reactive band of unknown nature at 43 KDa. Only the former shows an increase on the axotomizee side (a) compared to its contralateral (c) counterpart. This increase in BDNF immunoreactive band is evident as early as 1 day, reaches a maximum at 5 to 7 days and is still seen 2 weeks after axonal injury. Based on the serial dilution of rhBDNF protein, which is run in parallel, the BDNF level in axotomized facial nuclei is estimated between 5-10 ng per 30 µg total protein loaded. (B) Western blot for BDNF in the axotomized (a) and contralateral (c) facial nuclei 7 days after axotomy. Note the increase in BDNF immunoreactive band (→) after axotomy. On the right, BDNF expression in the contralateral facial nerve (n) as well as in the distal (d) and proximal (p) stump of the facial nerve 14 days after transection.
Fig. 7  Differential expression of BDNF exons after axonal injury. (A) The lanes marked (V) represent the PCR products using the primer pair located on on exon V and have been used in the study above (Fig. 4). At 30 cycles of amplification with this primer pair, the increased BDNF expression is no longer evident due to saturation (21 cycles are used for Figs. 4). Amplifications with exon specific primers reveal that the contralateral (c) facial nuclei show significant level of BDNF mRNA containing exons I and III, but few of exons II and IV. After axotomy (a), expression of exons IV and III shows marked increase, while exons I and II increase only slightly.
Fig. 8 In situ hybridization for BDNF (a, b) and trkB (c, d). A few exceptional axotomized RSNs express BDNF (a) 3 days after axotomy. Note the decreased trkB ISH signal in axotomized RSNs compared to contralaterals (d) Quantification of trkB ISH signals reveal that trkB expression declines to 70% of contralateral level; open symbols represent contralateral RSNs and filled symbols represent axotomized RSNs 7 days after cervical injury. Scale bar = 20 μm.
Fig. 9  Serial dilution RT-PCR for BDNF and TrkB in axotomized ("a") and contralateral red nuclei ("c"). Only a minor increase in BDNF expression is observed in axotomized red nuclei compared to contralateral. TrkB expression decreases within 2 days following axotomy and declines further by 7 days ("a").
CHAPTER 4: Response of Axotomized RSNs to Neurotrophin Application

4.1. Overview

This chapter demonstrates that application of BDNF or NT-4/5, fully prevented rubrospinal atrophy and also sustained the increased expression of GAP-43 and Tα1-tubulin in RSNs after cervical axotomy. Infusion of BDNF was also found to increase the numbers of RSNs regenerated into a sciatic nerve implanted into the cervical spinal cord injury site of the rubrospinal tract.

In the second part of this chapter, I report that application of BDNF to RSNs after axotomy at the thoracic level of the spinal cord stimulates the expression of GAP-43 and Tα1-tubulin. These RAGs do not increase after thoracic axotomy alone, which correlates the failure of RSNs regenerate into PN transplants grafted into the thoracic spinal cord. It was possible to overcome this regeneration failure presumably by stimulation of RAGs through BDNF application. The present study supports the concept that the application of a specific neurotrophin to the vicinity of an axotomized CNS neuron can stimulate the expression of RAGs and enhance its propensity to regenerate.

4.2. Introduction

As outlined in the General Introduction, I hypothesize that CNS regeneration is hampered by neuronal death and/or atrophy and by inadequate cell body responses after axonal injury. While a "close" (cervical) axotomy of RSNs does not result in acute cell death, it is followed by a marked reduction in cell size in the second week. The latter is accompanied by a decline in the expression of RAGs, which are initially elevated during the first week (Tetzlaff et al., 1991). After a "distant" (low thoracic) axotomy of RSNs, however, neuronal atrophy is minimal and RAG expression is not
increased.

RAGs are a group of genes consistently increased in axotomized PNS neurons (e.g. GAP-43 and α1-tubulin), which generally regenerate successfully. A correlation has also been found between regenerative propensity of CNS neurons and RAG expression (see details in 1.4.2.). RSNs regenerate into a PN grafted at the cervical level of the spinal cord, but not at the thoracic level (Richardson et al., 1984; Tetzlaff et al., 1994), correlating with the transient expression of GAP-43 and tubulins after cervical axotomy (Tetzlaff et al., 1991), and not at all after thoracic axotomy (Tetzlaff et al., 1994). The observation that only a small percentage (1-2%) of RSNs regenerate into the permissive environment of cervical PN graft is likely related to the severe atrophy that occurs during the second week after axotomy. Hence I predict that stimulation of RAGs should increase the number of regenerating RSNs after cervical axotomy and overcome the complete regeneration failure after thoracic axotomy.

Chapter 4 has shown that RSNs, unlike axotomized FMNs, failed to increase expression of BDNF and trkB after cervical axotomy. This finding supports the hypothesis that axotomized CNS neurons receive limited trophic support after axotomy. In this chapter, I attempted to test the hypotheses that application of BDNF into the vicinity of axotomized RSNs, thereby mimicking autocrine/paracrine trophic support 1) prevents neuronal atrophy, 2) stimulates GAP-43 and α1-tubulin expression and 3) ultimately promotes their regenerative capacity. In addition, the expression of other Trk receptors (i.e. TrkA and TrkC) in RSNs was also examined to assess possible responsiveness to other members of the neurotrophins after cervical axotomy. A cervical and a thoracic axotomy paradigm of RSNs was used and rationale is provided in the following section.
4.3. Rationale for Experimental Paradigms

Since RSNs undergo massive atrophy during the second week after cervical axotomy and display a concomitant decline in RAG expression (Egan et al., 1977; Tetzlaff et al., 1991) neurotrophins were applied into the vicinity of the axotomized RSNs cell bodies using an osmotic minipump from days 7 to 14 post-lesion (for details of surgical procedures, see method section). On day 14, cell profile sizes of axotomized as well as contralateral control RSNs was measured and the expression of GAP-43 and Tα1-tubulin in these neurons was examined by ISH.

Since only the trkB ligands, BDNF and NT-4/5 were successful in the cervical experiments, and NT-4/5 was no longer available in sufficient quantity for in vivo study Only BDNF was used in the thoracic paradigm.

Since thoracic axotomy of RSNs does not lead to increased RAG expression an osmotic minipump containing BDNF was implanted at the time of transection of the lateral funiculus i.e. day 0. After 7 days of BDNF application, GAP-43 and Tα1-tubulin expression in axotomized as well as contralateral control RSNs was examined by ISH.

Since reactive Schwann cells of distal PN stumps are known to produce a variety of trophic factors (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993) and express growth permissive adhesion molecules (for review see Carbonetto and David; 1993). I used predegenerated PN transplants. Predegeneration of the PN graft has previously been shown to enhance regeneration of the central DRG axons. (Oudega et al., 1994) In brief, for my study, the sciatic nerve was cut at the obturator tendon and allowed to undergo Wallerian degeneration prior to its implantation into the spinal cord injury sites. (for details see 2.1.4.) Ten weeks later, FG was applied at the distal end of the PN graft to retrogradely label those RSNs neurons that had extended their axons into the graft.
4.4. Results

4.4.1. Cervical Axotomy Paradigm

Neurotrophin receptor expression

Chapter 3 demonstrated that the expression of full-length trkB mRNA was seen in virtually all uninjured RSNs. This expression was decreased to 70% of the contralateral level 7 days after axotomy (Fig. 8, 9) and declined further thereafter as these neurons became atrophic during the second week post-axotomy (data not shown). The expression of the other trk receptors in RSNs 7 days after axotomy was studied by ISH in order to predict the possible responsiveness of RSNs to the other members of the neurotrophin family. TrkA ISH signal was not detectable, either in unlesioned or axotomized RSNs (Fig. 10 a, b). However, interpeduncular neurons in the same sections, as well as cholinergic basal forebrain neurons (Figueiredo et al., 1995) showed strong expression of trkA, ruling out technical problems (data not shown).

TrkC isoforms having 14, 25 or 39 amino acids insertions in the kinase domain are commonly present in neural tissues (Tsoulfas et al., 1993; Valenzuela et al., 1993). Since these isoforms appear to be limited in their signaling capability (Guiton et al., 1995; Tsoulfas et al., 1996), we used an oligonucleotide probe bridging the insertion site to study the expression of the full-length, non-inserted trkC receptor. Weak expression of non-inserted trkC mRNA was detected in uninjured and axotomized RSNs only after prolonged autoradiographic exposure (6-8 weeks) (Fig. 10 d,c). Similar exposure times produced strong hybridization signals in corticospinal neurons (Giehl and Tetzlaff, 1996) and in FMNs (K.L. Fernandes, N.R. Kobayashi and W. Tetzlaff, unpublished observations). In order to further study the expression of trkC isoforms, we used trkC
PCR primers bracketing the insertion site within the tyrosine kinase domain (Offenhauser et al., 1995). RT-PCR results revealed that both axotomized and contralateral RSNs expressed the non-inserted (299bp) as well as the known inserted isoforms (341bp, 374bp, 416bp) of the trkC receptor (Fig. 10 g). The non-inserted and 14 amino acid insert forms of trkC were the predominant types expressed in uninjured RSNs, and there was no apparent change in isoform composition 7 days after axotomy (Fig. 10 g). The ISH analysis of the p75 LNTR showed no signal in unlesioned RSNs (Fig. 10 f). However, at 7 days, but not 3 weeks post-axotomy, we found detectable p75LNTR mRNA expression in the occasional axotomized RSNs (less than 10%, Fig. 10 e). Taken together, these observations suggest that axotomized RSNs may be responsive to BDNF and NT-4/5, cognate ligands for the trkB receptor; and to NT-3, which preferentially binds to the trkC receptor.

Distribution of infused neurotrophins

The receptor expression profiles of RSNs provided the rationale for the application of the neurotrophins. Since acute axotomy-induced atrophy and concomitant decline in RAG expression occur in axotomized RSNs during the second week (Tetzlaff et al., 1991) neurotrophins were infused between days 7 to 14 post-axotomy. Fig. 11 shows a diagram of an infusion cannula inserted into the vicinity of the red nucleus. The cannula is connected to an osmotic minipump via silastic tubing to apply 500 ng/μl/hr of the appropriate neurotrophin. In order to assess the extent of factor distribution within the target tissue, midbrain sections were stained with antibodies to the various neurotrophins at the end of the 7 day infusion period. Immunostaining for rhNGF, rhNT-3 and rhNT-4/5 (Fig. 12, a, c, d) revealed that these factors diffused over most of the midbrain tegmentum ipsilateral to the side of infusion, filling a sphere of tissue
approximately 4 mm in diameter. In contrast, the diffusion of rhBDNF (Fig. 12, b) was confined to an area within 1 to 1.5 mm of the cannula. These finding are consistent with the study of Anderson et al. (1995). Given the relatively limited diffusion of rhBDNF, for all of the factors we limited our analysis to those cases in which the cannulae were located within 0.5-1.0 mm of the lateral margin of the red nucleus. Cannula placements <0.5 mm from the nucleus were excluded to rule out dendritic damage and non specific effects of trauma.

Effects of neurotrophin infusion on RSNs size: BDNF and NT-4.5 prevent atrophy of RSNs after cervical axotomy

Consistent with earlier findings (Egan et al. 1977), atrophy of RSNs was prominent 14 days post-axotomy. I found the median of cell profile size of axotomized untreated RSNs decreased to 62.8 % (25th-75th pct.: 59.6-66.6 %; n=12) of their uninjured contralateral counterparts. The infusion of BDNF or NT-4/5 completely prevented this axotomy-induced reduction in atrophy (Fig. 13 b vs. c; d vs. e), whereas infusion of NGF, NT-3 or vehicle alone (Fig. 13 a vs. b) did not affect the size of the axotomized RSNs. Interestingly, axotomized RSNs in BDNF- or NT-4/5-treated animals continued to exhibit classic signs of retrograde reaction to axotomy, including chromatolysis and a pronounced eccentricity of the nucleus. Analysis of variance on ranks (Kruskal-Wallis' test) revealed significant differences (P<0.0001) in the median cell profile size expressed as percentage of contralateral between the treatment groups. The median percentage of the BDNF- (105.2%; 25th-75th pct.: 92.3-129.1%; n= 9) and the NT-4/5- (107.0%; 25th-75th pct: 96.8-111.3%; n=7) treated groups were significantly different (P<0.05; Dunn's test) from the median percentage of the vehicle treated group (68.8%; 25th-75th pct.: 62.4-75.3%; n=15). Neither infusion of NGF (72.1%; 25th-75th pct.: 67.6-75.5%; n=6) (Fig. 13 g vs. h) nor NT-3 (84.6%; 25th-75th...
pct.: 65.6-90.3% (n=9) (Fig. 13 i vs. j) increased the size of axotomized RSNs compared to vehicle treatment.

In order to gain more insight into the cell size changes after axotomy with or without neurotrophin treatment, the cell profile size measurements were taken from the equivalent level of the red nucleus from different groups (Table 1). This confirms that there were no differences in mean cell profile sizes of contralateral RSNs among these groups and that BDNF and NT-4 treated axotomized RSNs were significantly larger than the axotomized RSNs of all other treatment groups (P<0.05, Newman-Keuls' test). The axotomized RSNs treated with BDNF and NT-4/5 displayed cell profiles sizes that were not different from their contralateral counterparts, while the axotomized RSNs of all other groups were significantly smaller (P<0.01; except for NT-3 treatment, P<0.05). The cell profile sizes of the untreated intact RSNs were comparable to the study by Mori et al. (1997).

Interestingly the effect of the BDNF application lasted beyond the 7 day period of infusion. In BDNF treated animals, the median size of axotomized RSNs was 79% and 83% of the contralateral, intact RSNs on day 21 and 28, respectively (7 and 14 days after cessation of the BDNF treatment). The corresponding values from vehicle controls were 60% and 56% indicating a continuing decline in cell size over time (data not shown). As mentioned above, these data are based on infusions through cannulae positioned within 0.5-1.0 mm from the lateral border of the red nucleus. BDNF infusion had no effect on neuronal soma size if the cannula was placed further away, reflecting the limited diffusibility of this factor (see above).

*Effects of neurotrophin infusion on RAG expression: BDNF prevents decline in GAP-43 and Tα1-tubulin expression*

Since infusion of BDNF and NT-4/5 into the vicinity of axotomized RSNs fully
prevented their atrophy (Fig. 13; Table 1), I wished to determine whether infusion of neurotrophins might also prevent the decline in GAP-43 and Tα1-tubulin mRNA expression typically observed during the second week after axotomy (Tetzlaff et al., 1991). The histogram of GAP-43 ISH quantification obtained from representative animals (marked by arrows in Fig. 16b) showed that on day 14 post-axotomy, only a subpopulation (50%) of axotomized RSNs treated with vehicle displayed increases in GAP-43 hybridization, while the remainder showed signals near intact, control levels, (Fig. 14a; 16a). In contrast, when treated with BDNF or NT-4/5 the majority (>90%) of axotomized RSNs expressed increased levels of GAP-43 mRNA (Fig. 14b, c; 16a). Furthermore, vehicle-infused and no pump animals exhibited only a 2-3 fold mean increase in GAP-43 mRNA expression, whereas treatment with BDNF or NT-4/5 typically produced mean increases of 4-7 fold (Fig. 16b). In 3 cases, the increases were much higher (9-13 fold). GAP-43 expression in NGF- and NT3-treated animals was similar to that seen in controls. The differences among treatment groups were statistically significant (P<0.01, Kruskal-Wallis). Subsequent groupwise comparisons (Dunn's test) revealed that the median increase in GAP-43 ISH signal in the BDNF-treated (5.3x, 25th-75th pct: 5.1-6.5x; n=6) and the NT-4/5-treated (7.6x, 25th-75th pct: 5.1-9.8x; n=4) groups were significantly different from that of the vehicle control group (2.4x, 25th-75th pct: 2.3-2.8x; n=5) (P<0.05). In contrast, neither treatment with NGF (3.0x, 25th-75th pct: 2.2-4.3x; n=4) nor with NT-3 (2.7x, 25th-75th pct: 2.3-3.2x; n=5) produced increases in GAP-43 expression that were significantly different from that of the vehicle control.

Axotomized RSNs also exhibited higher Tα1-tubulin expression in BDNF-treated and NT-4/5-treated animals compared to vehicle treated controls (Fig. 15 a-c). The histogram of representative animals (marked by arrows in Fig. 16d) illustrated that by 14
days after axotomy, about 60% of the RSNs displayed T\(\alpha\)1-tubulin ISH signals at levels below their contralateral counterparts while less than 20% showed increased level of expression (Fig. 16c). In marked contrast, T\(\alpha\)1-tubulin expression was higher on the lesioned side in more than half of the RSNs treated with BDNF or NT-4/5 (Fig. 16c). Mean T\(\alpha\)1-tubulin ISH signals, expressed as multiples of contralateral values, ranged from 0.4x to 1.1x in both ‘no pump’ and vehicle control groups (Fig. 16d). In BDNF and NT-4/5 treated animals, values ranged between 1.3x and 2.3x. Infusion of NGF or NT-3 produced mean values which did not differ from that of controls (ranging from 0.4x to 1.4x). These differences among groups were statistically significant (P<0.01, Kruskal-Wallis), and subsequent groupwise comparisons demonstrated that the median increases in T\(\alpha\)1-ISH signal of the BDNF- (1.6x, 25th-75th pct:1.3-1.8x; n=6) and of the NT-4/5- (1.6x, 25th-75th pct: 1.3-1.3x; n=4) treated groups were significantly different (P<0.05) from that of the vehicle treated control group (0.67x, 25th-75th pct: 0.57-0.76x; n=6). Neither treatment with NGF (0.75x, 25th-75th pct: 0.69-0.98x; n=4) nor with NT-3 (0.87x, 25th-75th pct: 0.65-1.1x; n=5) resulted in median T\(\alpha\)1-tubulin ISH signals different from those of the vehicle treated controls.

Stimulation of rubrospinal regeneration into peripheral nerve transplants

(This part of the study was performed in collaboration with Dr. D.-P. Fan in our laboratory).

Pre-degenerated sciatic nerve segments of 30-40 mm length were inserted into C4 lesions of the rubrospinal tract and regeneration was assessed by retrograde labeling with FG 2 months later. Typically these experiments resulted in a mean of 43 ± 9.3 regenerating neurons in control rats (n= 6, Fig. 17c, open symbols). Animals receiving BDNF infusions into the vicinity of the red nucleus, however, had a mean
number of $131 \pm 19.7$ regenerating RSNs ($n=6$, Fig. 17c, filled symbols) which was significantly different from the untreated controls ($P<0.01$; t-test). Representative sections through the red nucleus for both groups (marked by arrows in Fig. 17c) are shown in Fig. 17a and b.

4.4.2 Thoracic Axotomy Paradigm

Effect of BDNF infusion on GAP-43 and $T\alpha$1-tubulin expression in normal and axotomized RSNs

Since infusion of BDNF into the vicinity of RSNs sustained high levels of GAP-43 and $T\alpha$1-tubulin expression in RSNs after cervical axotomy I tested whether BDNF application stimulated the expression of these genes after thoracic axotomy. Seven days after axotomy, ISH signals for GAP-43 in uninjured contralateral RSNs were undetectable and remained at very low levels in untreated or vehicle-treated RSNs (Fig. 18 a, b). In contrast, BDNF application robustly stimulated the mRNA expression of GAP-43 in majority of axotomized RSNs after thoracic injury (Fig 18 c). Quantification of ISH signals revealed that the BDNF-treated group produced mean increases of 3-3.5 fold while the lesion-only or vehicle-treated group showed only up to a 1.6 fold increase (Fig. 20a). The median differences among these groups were statistically significant ($P<0.0001$, Kruskal-Wallis test). Subsequent pairwise comparisons revealed that the mean increase in GAP-43 ISH signal in the BDNF-treated group ($3.2x\pm0.11$; $n=4$) was significantly different from lesion-only ($1.2x\pm0.08$; $n=3$) as well as vehicle-treated ($1.4x\pm0.12$; $n=4$) groups ($P<0.05$; Newman-Keuls method).

A decrease in $T\alpha$1-tubulin ISH signals was observed in untreated or vehicle-treated RSNs 7 days after thoracic axotomy compared to the contralateral RSNs (Fig. 19).
The mean $\alpha_1$-tubulin ISH signals in axotomized RSNs of no pump or vehicle-treated animals ranged between 0.7-0.9x and 0.6-0.9x, respectively (Fig. 20b). In contrast, BDNF application markedly stimulated the expression of $\alpha_1$-tubulin and produced 1.4-2.3 fold increases in the mean $\alpha_1$-tubulin ISH signals (Figs. 19c; 20b). These differences were statistically significant ($p<0.001$, Kruskal-Wallis) and subsequent pairwise comparison revealed that the mean increases in $\alpha_1$-ISH signal of the BDNF-treated group ($1.9x \pm 0.18$; $n=4$) were significantly different from no pump ($0.77x \pm 0.09$; $n=3$) or vehicle-treated groups ($0.80x \pm 0.09$; $n=4$) ($p<0.05$; Newman-Keuls method).

Interestingly, BDNF application to intact RSNs strongly stimulated $\alpha_1$-tubulin mRNA expression (Fig. 21a-d), it had no effect on GAP-43 mRNA expression (Fig. 21e-h), suggesting the differential regulation of these genes by BDNF.

**GAP-43 immunocytochemistry (ICC)**

It is well documented that the expression of GAP-43 in regenerating PNS neurons is robustly upregulated and GAP-43 content in fast axonal transport markedly increases (For review see Bisby and Tetzlaff, 1992). In fact, GAP-43 is routed into the axonal transport system so that GAP-43-like immunoreactivity is barely detectable in cytoplasms (W. Tetzlaff unpublished observation). Therefore, I performed GAP-43 ICC in the rubrospinal tract at 2 segments rostral to the cervical lesion site. As mentioned earlier, cervically axotomized RSNs increased GAP-43 mRNA expression 7 days post-lesion (Tetzlaff et al., 1991). This increase in expression was accompanied by a pronounced GAP-43 like immunoreactivity in the rubrospinal axons at the C1 level of the spinal cord ipsilateral to the lesion side while those on the contralateral side displayed much weaker immunoreactivity (Fig. 22c vs. d). The identity of the rubrospinal axons
was confirmed by anterograde labeling with a fluorescent tracer, FluoroRuby, which was injected into the vicinity of red nucleus. GAP-43-like immunoreactivity was detected using an FITC labeled secondary antibody, revealing double labeled axons (Fig.22 a, b, arrows).

Even after thoracic axotomy, BDNF infusion produced strong GAP-43-like immunoreactivity in the rubrospinal axons ipsilateral to the lesion site was weak in the rubrospinal axons at T8 of the cord (Fig. 22 e, f). Whereas GAP-43 like immunoreactivity was barely detectable in rubrospinal axons ipsilateral to the lesion side after vehicle treatment or in contralateral rubrospinal axons (Fig. 22 g, h). These results were in agreement with GAP-43 ISH findings (Fig. 18).

*Promotion of rubrospinal regeneration into peripheral nerve transplants*

(This part of the study was done in collaboration with Dr. D.-P. Fan in our laboratory)

In the cervical axotomy paradigm of this chapter, I showed that BDNF application maintained the high levels of GAP-43 and Tα1-tubulin expression in axotomized RSNs and increased numbers of axotomized RSNs regenerating into a PN grafted at the cervical level. Here, I tested the hypothesis that stimulation of GAP-43 and Tα1-tubulin expression in thoracically axotomized RSNs by BDNF enhances the propensity of these neurons to regenerate into PN grafted at the low thoracic level. Ten weeks after the lesion and transplantation, regenerating RSNs were identified by retrograde labelling with FG applied at the grafted nerve end. As in previous studies (Richardson et al., 1984; Tetzlaff et al., 1994), no regenerating RSNs was found in the no-pump or vehicle-treated control groups (Fig. 23 b). Small numbers of FG positive RSNs, ranging between 5-28 cells, were found in the BDNF-treated group (Fig. 23 a, b arrow), indicating successful regrowth at least 10-15 millimeters into the PN graft.
4.5. Discussion

4.5.1. Chapter Summary

I have reported in chapter 1 that intact and axotomized RSNs express the full-length trkB receptor. In this chapter, the expression of other trk receptors were examined in intact and axotomized RSNs. TrkA mRNA expression was not detected in the intact red nucleus though, like p75, it was expressed in a few cells following axotomy at the cervical level of the spinal cord. Full-length trkC, including isoforms bearing amino acid inserts in the kinase domain, were expressed at only very low levels in intact or axotomized RSNs.

In accordance with the observed pattern of receptor expression, infusion of the TrkB ligands, BDNF or NT-4/5, during the second week following spinal cord transection fully prevented the atrophy of axotomized RSNs which remained chromatolytic. Moreover this infusion also maintained the axotomy-induced increase in GAP-43 and Tα1-tubulin mRNA expression. In contrast, NGF and NT-3 treatment were without effect. In a subsequent experiment, BDNF treatment produced a several fold increase in the number of axotomized RSNs regenerating into PN grafts implanted into the cervical transection site. Taken together, these findings support the hypothesis that neuronal atrophy and the concomitant failure of injured cells to maintain expression of regeneration associated genes are important factors which limit the regenerative capacity of axotomized CNS neurons. Furthermore, these effects of axotomy can be attenuated by application of appropriate trophic factors, thereby enhancing the capacity of the injured cell to sustain regrowth of its axon.

Interestingly, the prevention of reduction in cell size after axotomy by BDNF and
NT-4/5 did not include a normalization of the neuronal morphology which remained chromatolytic. Since the cell size could be maintained by the application of trophic factors, I feel justified to use the term atrophy, which implies the lack of some trophic support. It is difficult to evaluate the persisting chromatolytic response after neurotrophin application. Various degrees of chromatolysis are seen in both regenerating and non-regenerating neurons, as well as within the same neuronal phenotypes (for review see Lieberman, 1971; Goldstein et al., 1987). Thus, the significance of a chromatolytic response for the regenerative success of a neuron is incompletely understood and I therefore focused on the more prominent representatives of regeneration associated genes.

4.5.2. Receptor Expression and Effects of Neurotrophins on Neuronal Atrophy

The expression of neurotrophin receptors in uninjured RSNs is reminiscent of spinal cord and brainstem motoneurons, which also predominantly express full-length trkB receptors (Koliatsos et al., 1994; Piehl et al., 1994; Chapter 3). However, RSNs and lower motoneurons respond differently to injury. Axotomy produces an increase in trkB expression as well as de novo expression of the p75 neurotrophin receptor in motoneurons (Piehl et al., 1994; chapter 3). In contrast, trkB expression decreased in axotomized RSNs and p75 expression became detectable in only a small number of cells. The decline in trkB mRNA expression was prevented in axotomized RSNs by BDNF infusion (N.R. Kobayashi and W. Tetzlaff unpublished observation), suggesting that RSNs remain responsive to BDNF and NT-4/5, which was consistent with the observed prevention of their axotomy induced atrophy.

The effect of NT-3 on the atrophy of axotomized RSNs did not reach statistical significance. This is reminiscent of the moderate effect of NT-3 in contrast to BDNF on survival of axotomized facial motoneurons of newborn rats (Sendtner et al., 1992;
Koliatsos et al. 1993; Yan et al., 1993). Moreover, NT-3 application to axotomized motoneurons of adult rats has little effect on cell size (Fernandes et al., 1995; Tuszynski et al., 1996). I am confident that this marginal effect of NT-3 is not attributable to technical deficiencies as immunostainings for NT-3 demonstrated good penetration of this factor through the relevant tissue. In addition, in parallel experiments infusions of NT-3 fully prevent the axotomy-induced cell death of adult corticospinal neurons (Giehl and Tetzlaff, 1996). In this context, it is important to note that trkC isoforms with insertions in the kinase domain are limited in their down-stream signaling capacities (Tsoulfas et al., 1993, 1996; Guiton et al, 1995). In contrast to RSNs, corticospinal neurons express high levels of full-length trkC, and the non-inserted isoform is predominant (N.R. Kobayashi and W. Tetzlaff, unpublished observation). Therefore, the marginal effect of NT-3 on axotomized RSNs may be attributable, not only to the lower level of expression of full-length trkC, but also to co-expression at comparable levels of isoforms carrying amino acid insertions in their kinase domains. While in the present study, axotomized RSNs in the adult rat did not appear to be responsive to NT-3, axotomized RSNs are rescued from cell death by application of exogenous NT-3 in newborn rats (Diener and Bregman, 1994). Presently, it is unknown whether this difference might be due to developmental differences in the pattern of trkC expression in RSNs. It should also be noted that axotomized corticospinal neurons rescued by application of NT-3 remain atrophic, in contrast to those rescued by treatment with BDNF (Giehl and Tetzlaff, 1996). Therefore, it is possible that NT-3 may be a survival factor for RSNs, even in adulthood, but not influence cell size. This also implies that different signaling pathways are activated by stimulation of the TrkB and TrkC receptors, even when they are expressed contemporaneously in the same cell.
4.5.3. Effect of the Neurotrophins on Regeneration Associated Gene Expression

After cervical axotomy, BDNF and NT-4/5, but not NGF, NT-3 or vehicle maintained the increased levels of GAP-43 and Tα1-tubulin mRNA expression in axotomized RSNs. This differential responsiveness to the neurotrophins is consistent with the pattern of trk receptor expression. In essence, the expression of full-length trkB receptors would provide means for a direct stimulation of RSNs by BDNF or NT-4/5.

After thoracic axotomy, BDNF treatment also markedly increased the expression of GAP-43 and Tα1-tubulin. Likewise, BDNF has been reported to stimulate GAP-43 and Tα1-tubulin expression in axotomized facial motoneurons which express trkB (Fernandes et al., 1995), and NGF regulates the expression of these same genes in neurons of the peripheral nervous system which express trkA (Verge et al. 1990; Miller et al., 1994; Mohiuddin et al., 1995). In another study, however, BDNF stimulates the expression of GAP-43, but not Tα1-tubulin, in axotomized retinal ganglion cells (Fournier and McKerracher, 1997). Thus, regulation of regeneration associated genes by BDNF is context dependent and distinct in different neuronal systems.

I do not know, however, how directly the expression of GAP-43 and Tα1-tubulin is controlled by BDNF or NT-4/5 activated signaling pathways. I cannot rule out the possibility that the neurotrophins indirectly maintain the expression of these genes via a pleiotrophic effect.

Interestingly, I found that BDNF had no effect on the baseline expression of GAP-43 in intact RSNs. mRNA expression of GAP-43 shows little change after thoracic axotomy only, but can be markedly stimulated after BDNF treatment. Taken together, it is tempting to speculate that induction of GAP-43 appears to require signals associated with axotomy (Vanselow et al., 1994), which may remove a negative transcription regulator (Chiaramello et al., 1996; Kinney et al., 1996; Weber and Skene, 1997) and
BDNF-mediated signaling may either stabilize the transcriptional machinery or turnover of the message. In contrast, BDNF infusion increased \( \tau \alpha 1 \)-tubulin expression in intact RSNs as well as in axotomized RSNs, indicating that GAP-43 and \( \tau \alpha 1 \)-tubulin are not strictly co-regulated.

The differential regulation of GAP-43 and \( \tau \alpha 1 \)-tubulin is further evidenced by the varying effects of NT-3 on these two genes in axotomized corticospinal neurons (Giehl et al., 1995), retinal ganglion cells (Kittlerova et al. 1996) and dorsal root ganglion cells (Mohiuddin et al., 1995; Gratto and Verge, 1996). Differential responses of these neuronal types to axotomy and distinct modes and doses of applied NT-3 may be partly responsible for these diverse outcomes. In addition, as discussed above, both the absolute and relative levels of \( \text{trkC} \) isoforms carrying insertions in the tyrosine kinase domain may differ in these models, contributing to the apparent discrepancies.

Moreover, although NT-3 binds most avidly to \( \text{trkC} \), it can also bind to \( \text{trkA} \) or \( \text{trkB} \), so that different signaling cascades may be activated by NT-3 on cells expressing distinct complements of \( \text{trk} \) receptors (Davies et al., 1995; Ryden and Ibanez, 1996). Further complexity is added by emerging evidence that the different neurotrophins may elicit distinct downstream responses, even if their actions are mediated through the same cognate receptor (Belliveau et al. 1997). These data underline the necessity of analyzing the specific effects of the different neurotrophins in each neuronal system of interest, rather than relying on inference.

### 4.5.4. Neurotrophins and CNS regeneration

Several studies have demonstrated that local application of neurotrophins can enhance regenerative sprouting of a variety of CNS axons (Schnell et al. 1994; Tuszyński et al., 1994; Xu et al., 1995; Oudega and Hagg, 1996; Grill et al., 1997; Ye
In these experimental paradigms, neurotrophins are applied to the vicinity of the axons and may exert local trophic and/or tropic effects. This stands in contrast to the model introduced in this thesis, where application of neurotrophins to the parent cell bodies enhances their regenerative propensity even after injury at greater distances.

I hypothesize that this effect is mediated through the stimulation of RAG. Tα1-tubulin and GAP-43 are highly expressed during axonal outgrowth in development and are re-expressed in regenerating PNS neurons (Skene and Willard 1981; Miller et al., 1987,1989; Skene, 1989; Tetzlaff et al., 1989). Increased tubulin expression after axotomy is believed to play a role in the replacement of the lost axoskeleton (for review see Bisby and Tetzlaff 1992). GAP-43 is concentrated at the axonal growth cone where it seems to play an important role in the transduction of growth cone guidance signals (for review see Benowitz and Routtenberg, 1997). In vitro, GAP-43 conveys upon neurons a greater propensity to grow (Aigner and Caroni, 1995).

This is consistent with the close correlation between GAP-43 expression and successful regeneration of CNS neurons into PN transplants (Campbell et al., 1992; Schaden et al., 1994; Tetzlaff et al, 1994; Ng et al., 1995; Vaudano et al., 1995). Ordinarily, only a very small fraction of axotomized RSNs typically regenerate into nerve grafts after cervical axotomy and not at all after lesions at greater distance i.e. low thoracic (Rishardson et al., 1984; Houle, 1991; Tetzlaff et al., 1994). This appears to be attributable to the abortion or lack of RAG expression. I show here that the stimulation of GAP-43 and Tα1-tubulin expression by application of BDNF is correlated with an increased number of RSNs regenerating into PN implants.

There are possibilities that other growth associated proteins, e.g. CAP-23 (Widmer and Caroni, 1990) or microtubule associated proteins (Fawcett et al., 1994;
Nothias et al., 1995), might play a cooperative role in this process (Caroni et al., 1995). In any event, the present study supports the concept that the application of a specific neurotrophin to the vicinity of an axotomized CNS neuron can stimulate the expression of RAG and enhance its propensity to regenerate.
Fig. 10 Expression of trkA, trkC and p75 receptors in RSNs. trkA ISH signal is undetectable in axotomized as well as contralateral RSNs (a, b). Expression of full-length non-inserted trkC in RSNs (c) at 7 days after axotomy and in contralateral RSNs (d). Only a few axotomized RSNs upregulate the expression of p75 (e) in addition, intact contralateral RSNs have low baseline expression of p75 (f). 700x magnification, scale bar=20μm. RT-PCR (30 cycles, ethidium bromide staining) for trkC isoforms in axotomized ("a") and contralateral ("c") red nuclei (g). Note the predominant expression of the non-inserted trkC isoform (299bp) as well as the isoforms with 14 amino acid (341bp) and the weak expression of the isoforms with 25 (374 bp) and 35 (416 bp) amino acids insertions.
Fig. 11  Schematic diagram showing the midbrain at the level of the red nucleus and the cervical spinal cord. The approximate insertion site of the application cannula connected to an osmotic minipump containing either vehicle alone, or neurotrophins is illustrated in the coronal midbrain section. The hatched area in the spinal cord indicates the extent of transection at the cervical level (C3) which includes the rubrospinal tract. Scale bar = 5mm
Fig. 12  Immunohistochemistry for NGF (a), BDNF (b), NT-3 (c) and NT-4/5 (d) infused lateral to the red nucleus. NGF immunostaining (a) reveals good tissue penetration of rhNGF covering almost an entire half of the midbrain in the coronal plane. In contrast, rhBDNF (b) diffusion is limited around the center of the application needle within approximately 1 mm of the cannula. Penetration of rhNT-3 (c) and rhNT-4/5 (d) is comparable to that of rhNGF as shown by their respective immunostaining. 10x magnification, scale bar=1.5 mm.
Fig. 13 Cresyl violet staining of vehicle or neurotrophin-treated RSNs 14 days after axotomy (a, c, e) and their contralateral counterparts (b, d, f). Note the severe atrophy of vehicle treated RSNs (a versus b). This atrophy is fully prevented in axotomized RSNs treated with BDNF (c) or NT-4/5 (e), displaying the cell profile sizes comparable to the contralateral RSNs (d, f). NGF and NT-3 have no effect on neuronal atrophy of RSNs 14 days after cervical axotomy (g - j). Note that the axotomized and neurotrophin treated RSNs are chromatolytic. 400X magnification, scale bar = 50µm.
Axotomized
Vehicle

Contralateral

BDNF

NT-4
Axotomized

NGF

NT-3

Contralateral

\( ^a \)
Table 1  Mean cell profile sizes of axotomized as well as contralateral to axotomized RSNs 14 days post-injury with or without neurotrophin treatment. There is no significant difference in the contralateral profile sizes among different animal groups. The cell profile sizes of axotomized RSNs treated with BDNF or NT-4/5 are significantly larger than all the other groups (*P<0.05; Newman-Kuels' test) while NGF or NT-3 treatment results in the cell profile sizes not different from the control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Contralateral</th>
<th>Axotomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pump (n=6)</td>
<td>444.6±36.5</td>
<td>297.2±20.4</td>
</tr>
<tr>
<td>Vehicle (n=5)</td>
<td>449.6±29.1</td>
<td>307.2±33.6</td>
</tr>
<tr>
<td>BDNF (n=6)</td>
<td>494.3±15.8</td>
<td>545.6±59.7*</td>
</tr>
<tr>
<td>NT4/5 (n=4)</td>
<td>517.5±12.8</td>
<td>603.1±35.2*</td>
</tr>
<tr>
<td>NGF (n=4)</td>
<td>437.3±30.8</td>
<td>338.1±17.3</td>
</tr>
<tr>
<td>NT-3 (n=5)</td>
<td>495.3±39.5</td>
<td>368.2±41.3</td>
</tr>
</tbody>
</table>
Fig. 14  GAP-43 ISH in axotomized RSNs treated with vehicle, BDNF or NT-4/5. The FG-labeled, axotomized RSNs are visualized under fluorescent illumination, superimposed with autoradiographic silver grains representing the ISH signals in darkfield illumination. Note that only a subpopulation of axotomized RSNs displays GAP-43 ISH signal with vehicle treatment (a), in contrast, the majority of axotomized RSNs treated with BDNF (b) or NT-4/5 (c) express high levels of GAP-43 mRNA. 360X magnification, scale bar = 40μm.
GAP-43

Vehicle

BDNF

NT-4
Fig. 15 Tα1-tubulin ISH in axotomized RSNs treated with vehicle, BDNF or NT-4/5. The FG-labeled, axotomized RSNs are visualized under fluorescent illumination, superimposed with autoradiographic silver grains representing the ISH signals in darkfield illumination. An increase in Tα1-tubulin expression is observed in axotomized RSNs treated with BDNF (a) or NT-4/5 (b) compared with those RSNs treated with vehicle only (c). 360X magnification, scale bar= 40μm.
Fig. 16 Histograms of the percentage of cells displaying GAP-43 (a) or Tα1-tubulin (c) expression in multiples of their contralateral expression level, obtained from representative animals infused with vehicle, BDNF or NT-4/5 (arrows in b and d). The labels of X-axis (multiples of contralateral) indicate an upper limit value of the bin category. Note the apparent shift to the right (increased expression) for both genes in the numbers of RSNs treated with BDNF or NT-4/5 compared with those treated with vehicle. Each symbol in (b) and (d) represents the mean (±SEM) of ISH signals/cell normalized to that of contralateral RSNs, i.e. expressed as multiples of contralateral derived from an individual animal. Dashed lines indicate the expression level of contralateral (=1). Note the increased expression of GAP-43 (b) as well as Tα1-tubulin (d) in the animal groups treated with BDNF or NT-4/5 compared with the vehicle or no pump control groups.
Fig. 17 Photomicrographs of FG-labeled RSNs regenerated into a peripheral nerve transplant obtained from an animal without treatment (a; c, arrow) and of a BDNF-treated animal (b; c, arrow). (c) shows the numbers of FG-labeled, i.e. regenerated, RSNs of individual animals without treatment (open symbols) and with BDNF treatment (filled symbols). Note a several fold increase in the number of FG-positive neurons in BDNF treated animals compared with the animals without treatment (p<0.01, t-test). 160x magnification, scale bar = 100 μm.
no pump + transplant

BDNF + transplant

\[ \text{no pump + transplant} \]

\[ \text{BDNF + transplant} \]

\[ \text{\# of FG-labeled neurons} \]

\[ \text{C} \quad \text{no pump} \quad \text{BDNF} \]

105
Fig. 18 GAP-43 ISH in axotomized RSNs of untreated (no pump), vehicle-treated or BDNF-treated animal groups. The FG-labeled, axotomized RSNs are visualized under fluorescent illumination, superimposed with autoradiographic silver grains representing the ISH signals in darkfield illumination. Note that the majority of axotomized RSNs treated with BDNF display a marked increase in GAP-43 expression (c). Whereas only a few axotomized RSNs are positive for GAP-43 ISH signals in untreated or vehicle-treated control groups. Scale bar = 40 μm
Fig. 19 Tα1-tubulin ISH in axotomized RSNs of untreated (no pump) (a), vehicle-treated (b) or BDNF-treated (c) animal groups. The FG-labeled, axotomized RSNs are visualized under fluorescent illumination, superimposed with autoradiographic silver grains representing the ISH signals in darkfield illumination. Note that BDNF application (c) markedly increases the expression of Tα1-tubulin in the majority of axotomized RSNs compared to that of the untreated or vehicle-treated control groups. Scale bar = 40 μm.
Tα1-tubulin

No pump

Vehicle

BDNF

a

b

c
Fig. 20  Quantification of GAP-43 (a) and Tα1-tubulin ISH signals. Each symbol represents the mean (±SEM) of ISH signals/cell, expressed as multiples of contralateral obtained from an individual animal. Dashed lines indicate the expression level of contralateral (i.e. 1). Note the 3-fold increase in GAP-43 expression in BDNF-treated group while less than 2 fold-increase of GAP-43 ISH signals is observed in the control groups. Tα1-tubulin expression declines to the levels below contralateral in the control groups whereas BDNF treatment markedly increase the expression of Tα1-tubulin.
GAP-43

Treatment

Tα1-tubulin

Treatment
Fig. 21 Tα1-tubulin and GAP-43 mRNA expression in intact RSNs treated with BDNF for 7 days. BDNF application alone without axotomy stimulates the expression of Tα1-tubulin (a, c vs. b, d), while no change in GAP-43 expression is observed in RSNs of the adjacent section after BDNF treatment (e, g vs. f, h).

Scale bar = 100 μm.
Fig. 22 Immunocytochemistry for GAP-43 in the rubrospinal tract approximately 2 segments rostral to the spinal cord transection site. Rubrospinal axons are anterogradely labeled with a fluorescent tracer, FluoroRuby (a) and are transected at the C3 level of the spinal cord. Seven days after cervical lesion, fluorescent immunocytochemistry reveals strong GAP-43-like immunoreactivity (a, b, arrows) in rubrospinal axons ipsilateral to the injury site. Scale bar = 10 μm. Rubrospinal axons contralateral to the lesion sites at C3 (d) or at T10 (f, h) show weak GAP-43-like immunoreactivity. After a cervical axotomy or thoracic axotomy, BDNF treatment produce a significant increase in GAP-43 like immunoreactivity ipsilateral to the lesion sites (c, e). Thoracic axotomy with vehicle treatment does not result in any apparent changes in GAP-43 like immunoreactivity (g). Scale bar = 20 μm.
Fig. 23 (a) A photomicrograph of FG-labeled RSNs regenerated into a peripheral nerve transplant from an animal treated with BDNF (arrow in b). (b) A number of FG-labeled RSNs i.e. regrow their axons into PN grafted at T10 of the spinal cord. After vehicle treatment, no FG-labeled RSNs are found, while BDNF treatment for 7 days after thoracic axotomy promotes some regeneration of RSNs into PN transplants.
CHAPTER 5: Chronically Injured RSNs Remain Responsive to BDNF

5.1. Overview

Chapter 4 has shown that infusion of BDNF prevents neuronal atrophy and the decline in GAP-43 and Tα1-tubulin expression. This chapter tests the hypothesis that application of BDNF reverses the atrophy of axotomized RSNs once it already has occurred i.e. several months after cervical injury. Furthermore, the effect of BDNF infusion on the expression of GAP-43 and Tα1-tubulin is examined in these chronically axotomized RSNs. Axotomized RSNs are left untreated for 7 or 23 weeks after cervical lesion and BDNF is infused for 7 days during the 8th or 24th week prior to the analyses. BDNF treatment partially reverses the neuronal atrophy of chronically axotomized RSNs, which are significantly larger than those of the untreated or vehicle-treated groups. Moreover, after application of BDNF, the expression of Tα1-tubulin is stimulated in the majority of chronically axotomized RSNs and some of these neurons also show GAP-43 expression. These observations suggest that axotomized RSNs remain responsive to BDNF even in the chronically injured state.

5.2. Introduction

As mentioned in the general introduction, after cervical axotomy of RSNs the expression of GAP-43 and Tα1-tubulin is upregulated, however, it declines during the second week as these neurons undergo severe atrophy (Tetzlaff et. al, 1991). Chapter 4 demonstrated that BDNF and NT-4/5 prevented atrophy of the axotomized RSNs and promoted their regenerative propensity in the acute cervical axonal injury paradigm i.e. when applied during the second week. In this chapter, I have analyzed whether this approach is viable in chronically injured RSNs. This is of clinical interest since
therapeutic interventions for spinal cord injuries often have to be delayed due to complicating factors and due to the fact that the actual extent of the axonal damage is initially unclear. Axonal regeneration into PN grafts placed into the CNS has been studied for chronically injured retinal ganglion cells, central axons of DRGs and brain stem spinal neurons. (Thanos and Vanselow, 1989; Houle, 1991; Houle et al., 1994). In general, declining numbers of regenerating neurons were observed with increasing delay of PN grafting (Thanos and Vanselow, 1989; Houle et al., 1994; Houle and Ye, 1997). It could be hypothesized that this is related to a decline in the expression of RAGs. Therefore, this chapter tests the hypothesis that application of BDNF reverses atrophy of chronically injured RSNs and stimulates the expression of GAP-43 and Tα1-tubulin after long term cervical injury.

5.3. Rationale for Experimental Paradigm

The acute cervical axotomy paradigm used in chapter 4 provided a model to examine whether application of appropriate trophic support allows injured RSNs to maintain their cell body reaction crucial for regeneration. In order to address the responsiveness of axotomized RSNs to BDNF after long-term axonal injury, axotomized RSNs are left untreated and allow to undergo atrophy for 7 weeks or 23 weeks. Using osmotic minipumps, BDNF is infused into the vicinity of the red nucleus for 7 days during the 8th or 24th week as in the acute experimental paradigm (for details of surgical procedures, see Chapter2: Materials and Methods). Subsequently, cell profile sizes are measured and GAP-43 and Tα1-tubulin ISH was performed.
5.4. Results

5.4.1. BDNF Partially Reverses Atrophy of Axotomized RSNs after Chronic Cervical Lesion

Chapter 4 showed that 14 days after cervical axotomy, the untreated or vehicle-treated RSNs underwent considerable atrophy to the median cell profile sizes of 62.8% and 68.8% compared to their contralateral counterparts. Eight weeks after axotomy, this neuronal atrophy was slightly more pronounced; the median cell profile size of vehicle-treated RSNs was 58.2% (25\textsuperscript{th}-75\textsuperscript{th} pct: 53.6-62.5 %; n= 5) of the contralateral controls (Fig. 24 a vs. b). The extent of atrophy reported here was comparable to the recent study by Mori et al. (1997) using a similar histological technique. The infusion of BDNF for 7 days during the 8\textsuperscript{th} week post-lesion partially reversed this atrophy and produced a median cell profile size of 81.2% (25\textsuperscript{th}-75\textsuperscript{th} pct: 73.0-90.1; n=5) (Fig. 24 c, d). Mann-Whitney rank sum test revealed a significant difference between the BDNF-treated and vehicle-treated groups (P<0.01).

BDNF treatment was still effective to partially reverse the atrophy of RSNs 24 weeks after cervical axotomy (85.5 %; 25\textsuperscript{th}-75\textsuperscript{th} pct: 81.4%-95.8%; n=5). Neuronal atrophy of vehicle-treated RSNs at 24 weeks post-lesion appeared unchanged (59.8%; 25\textsuperscript{th}-75\textsuperscript{th} pct: 51.7-65.0%; n=6) (P<0.01) (Fig. 25). Furthermore, my preliminary observation indicated that even 52 weeks after axotomy, BDNF infusion had marginal effect on neuronal atrophy of axotomized RSNs. The mean cell profile size in 3 individual animals were found to be 58.8%, 68.5% and 64.4% of contralateral (Fig. 26 e vs. f). At this time point, neuronal atrophy had progressed further in control animals showing mean cell profile sizes of 35.5% and 55.5% after vehicle treatment (Fig. 26 c vs. d) and 27.5% and 35.7% without treatment (Fig.26 a vs. b). However, these data
were based on those RSN which were clearly identifiable after cresylviolet staining (Fig. 26a, arrows). Hence, very small profiles which appear ambiguous in their glial or neuronal nature are most likely missed in this analysis (Fig. 26a, arrowheads).

In support of this speculation, the number of profiles detectable in the untreated and vehicle-treated RSNs appeared less than those treated with BDNF (Fig. 26a, c, e). Subsequently, RSNs cell counts were taken based on the presence of a clearly visible nucleus in a cresylviolet stained neuronal profile and using the criterion that the cell profile size was at least twice as big as an average glial cell profile. My preliminary data show that only 45% of the RSNs were countable 1 year after cervical injury without treatment while 75% of the chronically axotomized RSNs were identifiable when treated with BDNF during the last week prior to analysis.

5.4.2. BDNF Stimulates Ta1-tubulin and GAP-43 Expression in Chronically Axotomized RSNs

BDNF infusion partially reversed neuronal atrophy in chronically axotomized RSNs. Subsequently, I tested the hypothesis that BDNF stimulates the expression of Ta1-tubulin. Eight weeks after cervical axotomy, Ta1-tubulin expression in axotomized RSNs declined below the contralateral level (Fig. 27a vs.b). However, BDNF infusion stimulated the expression of Ta1-tubulin in the majority of axotomized RSNs (Fig. 27e vs. e). BDNF also had a pronounced effect on Ta1-tubulin expression 24 weeks after injury (Fig. 28a vs. c) while the majority of vehicle-treated RSNs showed Ta1-tubulin ISH signals below the level of contralateral (Fig. 28a vs. b). My preliminary ISH results indicated that Ta1-tubulin expression was stimulated with BDNF treatment even 52 weeks after cervical injury (Fig. 29e vs. f) when compared to the untreated or vehicle treated controls (Fig. 29a-d).
The effect of BDNF infusion on GAP-43 expression was examined in these chronically axotomized RSNs. Previously Tetzlaff et al., (1991) reported that only very few atrophic RSNs express increased levels of GAP-43 7 weeks after cervical injury. This is consistent with my observation that 8 and 24 weeks after cervical injury, vehicle treated RSNs did not express GAP-43 with the exception of 1 or 2 cells per section of red nucleus (this reflects less than 5-10%) (Fig. 30, 31). After infusion of BDNF during 8th and 24th week respectively, more numbers of RSNs displayed increased level of GAP-43 expression (Fig. 30, 31).

5.5. Discussion

This chapter demonstrated that the infusion of BDNF partially reversed neuronal atrophy and stimulated RAG expression in chronically axotomized RSNs, suggesting that these neurons are still responsive to BDNF application long after their axonal injury.

5.5.1. Assessment of Neuronal Atrophy: Limitations and Considerations

The validity of the data presented here should be considered in light of the low numbers of animals at the extended time point and methodological constraints measuring cell profile sizes and cell death in chronically injured atrophic RSNs. The conventional histological staining methods (e.g. cresylviolet staining) cannot distinguish the very atrophic RSNs from neighboring glial cells. Hence my atrophy data very likely represent a conservative underestimate of the degree of cell size reduction since the very atrophic neurons escaped measurement. This is also supported by my preliminary cell counts which revealed more profiles after BDNF treatment. The latter are not due to de novo generation of neurons, but most likely to reflect partial recovery of cell soma.
sizes which now become identifiable under the criteria mentioned above. However, the validity of cell counts in chronically injured neurons is debatable.

Several approaches to address this problem have been attempted, but they appear to have drawbacks. Retrograde labeling of RSNs with a fluorescent tracer (e.g. FG) prior to lesion has been used to assess neuronal atrophy and cell death after spinal cord injury (Goshgarian et al., 1983; Feringa et al., 1988; McBride et al., 1989; Mori et al., 1997). The limitation for this technique is that perineuronal glial cells may pick up the tracer when they phagocytize debris of degenerated neurons. In addition, some retrograde tracers (e.g. Fast Blue) can be passed over from the labeled neurons to surrounding glial cells even without neuronal death (Tetzlaff et al., unpublished observation). This makes it difficult to distinguish the shrunken cells from these false positive glial cells.

Alternatively, immunostaining for neuron specific markers such as neurofilaments or neurotransmitter-related enzymes is often used to distinguish neurons from glial cells (e.g. Choline acetyltransferase (ChAT) for cholinergic neurons). However, the extent of expression of these neuronal markers by injured neurons is uncertain. For this reason, counting ChAT-positive neurons initially has overestimated the cell death of basal forebrain cholinergic neurons after fimbria-fornix transection (Williams et al., 1986; Kromer et al., 1987) as the expression of enzyme is downregulated after axotomy (Williams et al., 1986; Kromer et al., 1987). Subsequent NGF application produces reexpression of ChAT clearly demonstrating that these neurons have survived axotomy, but become negative for the marker (Fischer and Bjorklund, 1991; Sofroniew et al., 1993). This is somewhat reminiscent of my finding that BDNF treatment reveals a larger number of clearly identifiable RSNs after chronic injury, leading to a conclusion that available techniques are insufficient to provide reliable cell death and atrophy analyses. Therefore, the data obtained from untreated or vehicle
treated RSNs represent an underestimation of the extent of neuronal atrophy after chronic axotomy and thus the difference between cell profile sizes between BDNF-treated and control RSNs appears to be less pronounced.

Cell death of RSNs has been investigated after spinal cord transection using fluorescent prelabeling of RSNs prior to lesion (Goshgarian et al., 1983; McBride et al., 1989; Mori et al., 1997). Mori and colleagues (1997) attempt to attenuate the cell death and atrophy of cervically axotomied RSNs by inserting a piece of embryonic spinal cord into the lesion cavity at the time of injury. This approach has previously been shown to rescue axotomized RSNs in neonates (Bregman and Reier, 1986), suggesting that the graft substituted a target-derived trophic support for these neurons. In adults, Mori et al. (1997) report that transplants attenuate cell death of chronically axotomized RSNs without preventing neuronal atrophy of the remaining axotomized RSNs. However, in the light of the difficulties to assess cell death in chronic atrophic state and the reversal of atrophy by BDNF shown in this chapter, their data could be interpreted differently i.e. the embryonic transplant prevents neuronal atrophy, hence more neurons are countable. It remains to be shown whether in other models of chronic CNS neuronal injury the extent of neuronal death had actually been overestimated as well. The delayed application of the appropriate trophic factor used in this study may be a viable solution to this issue.

5.5.2. Axonal Regeneration Requires Cell Body Response of Injured Neurons

The molecular mechanisms involved in sprouting and regeneration are thought to be distinct and trophic support required for sprouting and regeneration can be different. Sprouting has been shown in intact as well as axotomized axons and it has been proposed that this is a local event at the nerve ending which may not require a parental cell body response. For example, sprouting of motoneuron axons from the neuromuscular endplate into denervated muscle occurs without apparent cell body
response (Brown et al., 1996). Moreover, collateral sprouting of intact nociceptive sensory fibers after partial denervation of the skin has shown to be NGF dependent (Diamond et al., 1987; 1992b), while regeneration of axotomized sensory neurons is not (Diamond et al., 1987; 1992a).

Furthermore, differential effect of neurotrophins on sprouting and axotomy-induced cell body response is observed when NT-3 is applied to the injury site of the corticospinal tract collateral sprouting is stimulated from lesioned corticospinal axons (Schnell et al., 1994). However, direct application of NT-3 into the vicinity of corticospinal neurons is not followed by increased expression of GAP-43, a representitative marker for cell body response to axotomy (Giehl et al. 1995). Conversely, BDNF application into the injured corticospinal tract does not elicit a local sprouting response, but when applied to the axotomized corticospinal neurons produce pronounced expression of GAP-43 (Giehl et al., 1995).

While my thesis study was underway, the growth potential of chronically injured RSNs after cervical axotomy was examined by Houle and colleagues (1997). These authors assessed RSN regeneration into PN transplants after application of BDNF or CNTF to the site of injury (Ye and Houle, 1997). These trophic factors were applied twice, i.e. 28 and 31 days after injury, prior to the insertion of PN graft on day 35 and the wall of the wound cavity was surgically removed each time. This procedure most likely involves resection of rubrospinal axons. On day 65, Ye and Houle (1997) found an average of 37 and 43 RSNs regenerated into PN grafts after treatment with BDNF or CNTF respectively while no regeneration was seen after PBS treatment. It can be speculated that the local trophic factor application by Ye and Houle (1997) enhanced sprouting of the injured rubrospinal axons. These may thereby gain access to the permissive environment of the PN graft which sustains RAG expression in the RSNs (D.-P. Fan and W. Tetzlaff, unpublished observation) and thus facilitates regeneration.
It remains to be shown, whether these regenerating RSNs belong to the small number of chronically axotomized RSNs which express RAGs as late as 7-8 weeks after injury (see also Figs. 27, 30). Alternatively, these regenerating RSNs re-increase RAG expression in response to the application of trophic factors and subsequently have regenerated.

In conclusion, this chapter demonstrates that the delayed application of trophic factors to the chronically injured neuronal cell body may be a feasible approach to "reinitiate" the growth state of the injured CNS neurons. The partial effect of BDNF on neuronal atrophy and RAG expression may be related to the duration of treatment (i.e. 7 days). Future experiments will show whether the prolonged BDNF treatment improves the growth state of chronically injured RSNs. Furthermore, the combination of different modes of trophic factor application (i.e. to the vicinity of axotomized RSNs and to the lesion site) may promote greater numbers of chronically injured RSNs to regenerate.
Fig. 24 Cresylviolet staining of RSNs 8 weeks after cervical axotomy. Note that axotomized RSNs underwent severe atrophy (a) compared to the contralateral (b). BDNF application during the 8th week for a period of 7 days reverses this atrophy to cell profile sizes comparable to the contralateral (c vs. d). Scale bar = 50 μm.
8 weeks

Axotomized Vehicle

Contralateral

BDNF

a

b

c

d
Fig. 25  Cresylviolet staining of RSNs 24 weeks after cervical axotomy. Axotomized RSNs remain responsive to BDNF application during the 24th week after chronic injury and their atrophy is reversed to cell profile sizes comparable to the contralateral (c vs. d) while RSNs treated with vehicle have undergone considerable atrophy (a vs. b). Scale bar = 50 μm.
24 weeks

Axotomized Contralateral

Vehicle

BDNF

a b c d
Fig. 26 Cresylviolet staining of RSNs 52 weeks after cervical axotomy. Note that only a small number of axotomized RSNs are identifiable as neurons with clearly visible nuclei (a, c, arrowheads) in untreated or vehicle-treated controls (a, c, vs. b, d). Identity of small "cells" (a, c, arrows) are indistinguishable from neighboring glial cells. BDNF application had a partial reversal effect on neuronal atrophy, revealing more neurons than previously thought (e vs.f). Scale bar = 50 μm.
52 weeks

Axotomized

Contralateral

No pump

Vehicle

BDNF

a

b

c
d

e

f
Fig. 27  Tα1-tubulin mRNA expression in RSNs 8 weeks after cervical axotomy. Tα1-tubulin expression declines in vehicle treated RSNs labeled with FG (c) compared to the contralateral (a vs. b). On the other hand, BDNF treatment during the 8th week for 7 days stimulates Tα1-tubulin expression in axotomized RSNs labeled with FG 8 weeks after cervical axotomy (d vs. e, f). Scale bar = 40 μm.
8 weeks

Vehicle

Contralateral

a

BDNF

d

Axotomized

b

e

Axotomized

c

f
Fig. 28  \( T_{\alpha 1} \)-tubulin mRNA expression in RSNs 24 weeks after cervical axotomy. Only minor populations of axotomized RSNs treated with vehicle display \( T_{\alpha 1} \)-tubulin ISH signals (a vs. b) while the majority of BDNF treated RSNs show \( T_{\alpha 1} \)-tubulin expression comparable to the contralateral (c vs. d). Scale bar = 40 \( \mu \text{m} \).
24 weeks

Axotomized       Contralateral

Vehicle

a

BDNF

c

b
d
Fig. 29  Tα1-tubulin mRNA expression in RSNs 52 weeks after cervical axotomy. Tα1-tubulin expression is barely detectable in the no pump and vehicle treated controls (a vs. b; c vs. d). Tα1-tubulin mRNA expression is markedly induced in axotomized RSNs after BDNF treatment during 51st week for 7 days ( e vs. f). Scale bar = 40 μm.
52 weeks

Axotomized

No pump

Vehicle

BDNF

Contralateral

a

b

c

d

e

f
Fig. 30  GAP-43 mRNA expression 8 weeks after cervical axotomy. A small number of FG labeled axotomized RSNs remain positive for GAP-43 ISH signals after vehicle treatment (a vs. b, c). After BDNF treatment, a greater number of FG-labeled axotomized RSNs appear to express GAP-43 (d vs. e, f). Scale bar = 40 μm.
8 weeks

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Fig. 31  GAP-43 mRNA expression 24 weeks after cervical axotomy. Occasionally, a few vehicle treated control RSNs maintain GAP-43 expression even 24 weeks after axotomy, however the BDNF treatment produces an increase in ISH signal and numbers of GAP-43 positive RSNs. Scale bar = 40 μm.
24 weeks

Axotomized Contralateral

Vehicle

BDNF

a b c d
CHAPTER 6: OVERALL SUMMARY AND GENERAL DISCUSSION

6.1. Overall Summary

This thesis focused on two of the major problems in CNS regeneration, the atrophy of the axotomized neuron and its failure to express RAGs. My overall hypothesis was that differences in the availability of trophic support for axotomized PNS neurons versus CNS neurons were essential to these problems. Chapter 3 supported this hypothesis by demonstrating that the expression of BDNF and its receptor, trkB increased in axotomized FMNs. This increase was absent in axotomized RSNs after cervical axotomy. My finding of trkB expression in axotomized RSNs subsequently led to the working hypothesis that these neurons might be responsive to the exogenous application of BDNF and NT-4/5. Chapter 4 showed that after acute cervical axotomy, neuronal atrophy as well as the previously described decline in GAP-43 and Tα1-tubulin expression was fully prevented by infusion of either BDNF or NT4/5. Moreover, BDNF stimulated the expression of Tα1-tubulin and produced an apparent induction of GAP-43 expression after low thoracic injury, which without BDNF treatment was insufficient to induce RAG expression. Ultimately, BDNF increased the numbers of regenerating RSNs into PN grafts transplanted into the cervical as well as the thoracic level of the cord. Taken together, chapter 4 supported the hypothesis that treatment of axotomized RSNs with an appropriate trophic factor promotes their capacity for axonal regrowth. Finally, chapter 5 addressed the responsiveness of chronically injured RSNs to a delayed application of BDNF infused over the period of 7 days. Neuronal atrophy was partially reversed and the expression of GAP-43 and Tα1-tubulin was re-stimulated even 52 weeks after cervical axotomy. These observations suggest that chronically axotomized RSNs maintain their responsiveness to BDNF long after axonal injury.
The findings presented in this thesis emphasize a new conceptual approach for treatment of CNS injuries: Providing an appropriate trophic factor to the vicinity of the axotomized neuronal cell body can overcome the atrophy and failure of the injured neuron to express RAGs and stimulate its regenerative propensity. Hopefully these insights will contribute to the development of therapies for CNS regeneration.

6.2. General Discussion

6.2.1. Axotomized PNS and CNS Neurons Differ in Their Expression of Trophic Factors

As outlined in chapter 3 axotomized FMNs increase the expression of BDNF and its receptor, trkB giving rise to the hypothesis that they receive autocrine/paracrine trophic support. Although this autocrine/paracrine trophic support has not been experimentally proven in this thesis several lines of evidence are in favor of this concept. For example, BDNF can act in an autocrine manner on cultured DRG (Acheson et al., 1995) and is expressed in a subpopulation of axotomized DRGs (Seber and Shooter, 1993; Ernfors et al., 1993).

Further, autocrine/paracrine BDNF actions have been shown for cortical neurons in vitro (Gosh et al., 1994) and inhibitions of endogenous BDNF by anti-BDNF-antibody infusions in vivo aggravates axotomy induced death of corticospinal neurons but has no effect on the survival of uninjured corticospinal neurons (Giehl et al., 1997). The latter can also be rescued from axotomy-induced death by exogenous BDNF application suggesting that the expression of endogenous BDNF is either not sufficient or not effectively reaching all injured neurons. A subpopulation of injured corticospinal neurons
expresses BDNF, however it is not known yet whether this expression increases after axotomy and whether the BDNF-expressing neurons constitute the subpopulation that actually survives the axotomy due to autocrine support, implying that the dying population has insufficient paracrine support.

Little change in BDNF mRNA expression after cervical axotomy of RSNs further supports the concept that the autocrine/paracrine support to axotomized CNS neurons may be attenuated or missing. RSNs express trkB and are responsive to exogenous BDNF application. Interestingly, the application of BDNF, but not vehicle, produced a massive increase in BDNF expression in axotomized RSNs, implying a possibility that this treatment initiated an autocrine/paracrine loop (N.R. Kobayashi and W. Tetzlaff unpublished observation). My experiments do not distinguish a direct effect of the applied BDNF from a possible indirect effect of the endogenous BDNF. Whether this newly synthesized BDNF is in a different cellular compartment and thereby reaches different part of the neuron, i.e. the axon, is unclear.

Further evidence for the differences in trophic factor expression between axotomized CNS and PNS neurons stems from investigations of FGF-2 expression. Increased mRNA and immunoreactivity for FGF-2 has been recently reported in axotomized DRG (Ji et al., 1995) and in FMNs after facial nerve (K. Stilwell, N.R. Kobayashi and W. Tetzlaff unpublished observation). In addition, the expression of the high affinity FGF receptor increases in axotomized FMNs, suggesting an additional trophic support for these neurons (K. Stilwell, N.R. Kobayashi and W. Tetzlaff, unpublished observation). In contrast, FGF-2 mRNA expression in RSNs is barely detectable and fails to increase after axotomy (K. Stilwell, N.R. Kobayashi and W. Tetzlaff, unpublished observation). The precise function of these factors in axotomized DRG neurons and FMNs has not been determined, however, these observations
support the concept that axotomized CNS neurons contribute little to their own trophic support after axonal injury.

The expression of other trophic factors is likely to be increased in axotomized PNS neurons, e.g. interleukin-6 (Murphy et al., 1995), yet this possibility has not been investigated in axotomized CNS neurons.

6.2.2. Gene Expression and Growth Potential of Neurons after Axonal Injury

The molecular mechanisms underlying axonal sprouting are distinct from those involved in axonal elongation during neural regeneration, as evidenced by different dependency on trophic factors and de novo gene expression. In vivo axonal sprouting of intact DRG to denervated skin appears to be dependent on NGF while axonal regeneration of axotomized DRG is not (Diamond et al., 1992 a, b). Recently, Smith and Skene (1997) has shown that mature sensory neurons in vitro are competent to initiate neurite extension and branching without induction of transcription, indicating that the complement of proteins constitutively expressed is sufficient for a process similar to sprouting in vivo. However, when the peripheral branch of DRG neurons are axotomized prior to dissociation and plating in vitro, these neurons are capable of long and rapid extension of neurites at a rate similar to in vivo regeneration, and terminal branching of neurites is much less frequent than in naive DRG cultures. In addition, this distinct growth mode is transcription dependent, implying that axotomy induced changes in gene expression are required prior to the rapid elongation of neurites. These observations are consistent with the hypothesis that axonal regeneration requires de novo expression of specific subsets of genes. This is originally emphasized in experiments by Richardson and Issa (1984) demonstrating that the central axons of DRG regenerate into PN transplants only if the peripheral axon is axotomized.
Subsequent work by others demonstrated that peripheral but not central axotomy of the DRG elicited a pronounced cell body response (Greenberg and Lasek, 1988; Schreyer and Skene, 1992; Chong et al., 1994).

GAP-43 and Tα1-tubulin have been characterized as the most prominent markers of the neuronal response to injury. Although their precise role in both axonal sprouting and axonal elongation remains to be shown the correlation between GAP-43 expression by axotomized neurons and successful regeneration has been documented in a large numbers of studies as mentioned earlier (for detail see section 1.4.2.). This thesis has demonstrated that BDNF application into the vicinity of cell bodies of axotomized RSNs increases the numbers of regenerating RSNs into PN grafts. I propose that this effect is mediated by the stimulation of gene expression necessary for regeneration, including GAP-43 and Tα1-tubulin expression.

GAP-43 and Tα1-tubulin expression is only transiently upregulated in RSNs after cervical injury without apparent increase in BDNF expression. BDNF application to the intact RSNs does not have apparent effect on GAP-43 expression in these neurons. However, GAP-43 expression in RSNs after thoracic axotomy is seen only after BDNF treatment. These observations suggest that BDNF is not directly involved in the induction of GAP-43. However, they support the view that some trophic factors are crucial modifiers of the neuronal response to injury. This in agreement with a recent report by Berry et al., (1996) that explant of PN grafted into the vitreous body of the eye promotes the regeneration of retinal ganglion cells into the distal segment of the optic nerve. The study emphasize the importance of trophic factor supply near injured neuronal cell body for robust growth capacity, even overcoming an inhibitory nature of CNS environment. It remains to be shown whether the increase in endogenous
expression of BDNF and other factors (e.g. FGF-2) in axotomized FMNs directly influences the pattern of gene expression required for regeneration.

Understanding the significance of endogenous trophic factor expression by axotomized neurons may be a critical step towards the development of a therapeutic strategy to enhance the cell body response to axonal injury. It appears that exogenous BDNF application "switched on" the growth program of injured RSNs. However, the exogenous application has several drawbacks and limitations such as mechanical damage by the insertion of cannula, limited diffusion and limited half life of peptide growth factor, and apparent requirement for continuous application. One could envision that these experimental problems could be circumvented by the transgenic or vector mediated neuronal overexpression of the appropriate trophic factor. A choice of promoter is crucial for a stable expression of the transgene in neurons. The Tα1-tubulin promoter, for example is suitable for this application since it is inducible by axotomy, maintained active for several weeks (Gloster et al., 1994; Wu et al., 1997), and most importantly, BDNF stimulates Tα1-tubulin expression (F.D. Miller, personal communication). This approach should mimic and enhance the production of endogenous BDNF and provide the BDNF trophic support similar to axotomized FMNs.

PN grafts, a permissive environment for axonal growth, has been used to assess growth potential of axotomized RSNs in this thesis. In order to promote regeneration within the CNS, stimulating the neuronal cell body response may not be sufficient and the inhibitory nature of CNS environment has to be overcome simultaneously. A variety of different strategies have attempted to bridge the injury site and bypass this hostile environment: genetically engineered cells overexpressing different types of trophic factors (Tuzynski et al., 1994; 1996; Grill et al., 1997), Schwann cell seeded tubes (Xu et al., 1995; 1997), transplantation of fetal spinal cord (Bregman,
1988; Reire et al., 1988, Bregman et al., 1997), transplantation of PN bridges plus trophic factor application (Chen et al., 1996), suspension of “growth permissive” cells e.g. Schwann cells (Paino et al., 1994; Martin et al., 1996) or olfactory ensheathing cells (Li et al., 1997).

Some molecules associated with oligodendrocytes and astrocytes are found to be inhibitory to axonal growth (Schwab and Caroni, 1988; McKeon et al., 1991; Bovolenta et al., 1993; McKerracher et al., 1994; Mukhpadyay et al., 1994; Keirstead et al., 1992, 1995). Attempts have been made to attenuate the inhibitory nature of CNS environment. For example, Keirstead et al. (1992; 1995) has reported that myelin disruption using anti-galactocerebroside antibody and serum complement proteins promotes regeneration of axotomized brainstem neurons within the spinal cord of the hatchling chick beyond the onset of myelination. Immunological neutralization of neurite inhibitory molecules by the IN-1 antibody has allowed small numbers of axotomized corticospinal axons to regenerate a few millimeters within the thoracic spinal cord and allowed partial reflex and locomotor functional recovery (Schnell et al., 1994; Bregman et al., 1995). These studies support the concept that the inhibitory nature of the CNS neurons can be experimentally attenuated, yet, the number of regenerating axons has been relatively small in each of these studies.

It appears that successful regeneration within the CNS will require a combination of multiple interventions, i.e. stimulation of the neuronal cell body response as well as the neutralization of the inhibitory environment of the CNS. This thesis contributed a novel concept for the stimulation of the neuronal cell body response to axotomy. Hopefully this knowledge can be further exploited for the design of CNS repair strategies.
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180


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