MECHANISM AND CONSEQUENCE OF P75 SIGNALLING

DISSERTATION

Presented in Partial Fulfillment of the Requirement for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Anthony W. Harrington, B.S.

The Ohio State University
2005

Dissertation Committee:
Professor Sung Ok Yoon, Adviser Approved by
Professor Tsonwin Hai
Professor John Oberdick
Professor Mike Zhu Adviser
Ohio State Biochemistry Graduate Program
ABSTRACT

The neurotrophin receptor p75 is responsible for a wide variety of signaling essential for proper development of the nervous system including cell survival and cell death. The first identified role of p75 was in augmenting Trk dependent survival and differentiation. However, p75 can also signal independently of Trks to promote apoptosis. The goal of this thesis was to understand the mechanism of p75-mediated cell death and to determine the in vivo consequences of p75 signaling. P75 is known to be induced in neurons and glia following injury. We therefore examined the outcome of this p75 induction in vivo. P75 was found to play a pro-apoptotic role following axotomy injury in the brain and spinal cord. Our analyses of neurotrophin expression after injury revealed that NGF is induced primarily as the precursor form, ProNGF. ProNGF was responsible for activating p75 following injury. In terms of the signaling pathway activated by p75, cJun N-terminal Kinase (JNK) was known to be involved. We found that activation of a small G protein Rac is necessary for JNK activation and apoptosis. P75 can also activate Rho in cooperation with the Nogo Receptor, leading to inhibition of axon growth. We identified a duel RhoGEF, Kalirin 9, as a p75 interacting protein. Kalirin 9 binding to p75 is regulated by NgR, and its activity GDP/GTP exchange
activity is required for Rho activation by NgR and p75. A transgenic mouse expressing a dominant negative Kalirin 9 is able to suppress injury-mediated Rho activation suggesting Kalirin 9 is the exchange factor responsible for Rho activation by p75 and NgR after spinal cord injury. These findings demonstrate the important roles for the small G proteins Rac and Rho in p75 signaling.
ACKNOWLEDGMENTS

I am indebted to my adviser, Sung Ok Yoon, who made this thesis possible. I thank her for the personal and scientific growth I’ve experienced in my years as a graduate student as well as for the training, support, advice and patience I’ve received.

I wish to thank all my colleagues who contributed to the work presented in this thesis, especially Chhavy Tep for her work with the Kalirin 9 transgenic mouse.

I thank our scientific collaborators especially Babara Hempstead, Klaus Giehl, Zhigang He, Michael Beattie and Jacqueline Bresnahan.
VITA

1998-present  Ph.D. Candidate, The Ohio State University, The Ohio State
Biochemistry  Program

1994-1998  B.S. Biochemistry, Virginia Polytechnic Institute and State University

Publications


Fields of Study

Major Field: Biochemistry
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iv</td>
</tr>
<tr>
<td>Vita</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>Chapter 1: Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>3</td>
</tr>
<tr>
<td>Results</td>
<td>11</td>
</tr>
<tr>
<td>Discussion</td>
<td>21</td>
</tr>
<tr>
<td>References</td>
<td>27</td>
</tr>
<tr>
<td>Figures</td>
<td>38</td>
</tr>
<tr>
<td>Chapter 2: ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>47</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>50</td>
</tr>
<tr>
<td>Results</td>
<td>57</td>
</tr>
<tr>
<td>Discussion</td>
<td>68</td>
</tr>
<tr>
<td>References</td>
<td>73</td>
</tr>
<tr>
<td>Figures</td>
<td>84</td>
</tr>
<tr>
<td>Chapter 3: Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>92</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>93</td>
</tr>
<tr>
<td>Results</td>
<td>97</td>
</tr>
<tr>
<td>Discussion</td>
<td>103</td>
</tr>
<tr>
<td>References</td>
<td>106</td>
</tr>
<tr>
<td>Figures</td>
<td>109</td>
</tr>
<tr>
<td>Chapter 4: Kalirin9 is a specific GEF for myelin inhibitory molecules</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>112</td>
</tr>
</tbody>
</table>
Materials and Methods………………………………………………..………114
Results………………………………………………………………..……….119
Discussion…………………………………………………………….....……130
References…………………………………………………………….....……135
Figures……………………………………………………………………143

Conclusion………………………………………………………………150
References………………………………………………………………159

Appendix A- Phosphatidylinositol 3-kinase is necessary but not sufficient to inhibit p75-mediated activation of the JNK pathway and apoptosis……………………………………………………………………163

Appendix B- Activation of Cdk5 kinase activity by p75 is involved in NGF dependent apoptosis……………………………………………………………………195

Appendix C- Altered Rac and Rho levels in the cerebellum of p75 knockout mice……………………………………………………………………198

Unified Bibliography……………………………………………………………………201
LIST OF FIGURES

1.1 Oligodendrocytes from p75 null mice fail to die upon NGF treatment...........38
1.2 The signaling ability of p75 is required for NGF-dependent apoptosis in oligodendrocytes.................................................................39
1.3 P75 activates JNK1 and 3...............................................................41
1.4 P75 activates JNK3 following depletion of JNK1 and 2......................42
1.5 JNK activation is necessary for NGF-dependent apoptosis in oligodendrocytes.................................................................43
1.6 P75 activates Rac1 in a NGF-dependent manner.................................44
1.7 Trk activation intercepts p75-mediated JNK activity at or upstream of Rac GTPase...............................................................45
1.8 Rac1 is the upstream regulator of the p75-mediated JNK pathway........46
2.1 Spinal cord injury leads to a loss of oligodendrocytes.........................84
2.2 Spinal cord injury-specific induction of p75 among oligodendrocytes.....85
2.3 p75+ oligodendrocytes are positive for active caspase 3.....................86
2.4 Mouse oligodendrocytes fail to die in culture in the absence of p75.......87
2.5 Increased survival and decreased apoptosis of oligodendrocytes after spinal cord injury in p75−/− mice.......................................................88
2.6 Spinal cord injury-specific induction of pro-NGF.................................89
2.7 Pro-NGF present in injured mouse spinal cord extracts is active in p75-mediated apoptosis of oligodendrocytes in culture.................................90
3.1. P75 is induced by cortical axotomy and is required for axotomy-induced death of corticospinal neurons

3.2. A NGF gene product is required for the induction of cell death of axotomized CSN

3.3. ProNGF is induced after internal capsule lesion, and released as a biologically active form into the CSF

3.4. ProNGF binds p75 in vivo and its binding to p75 is responsible for axotomy-induced death of CSN in vivo

4.1. Kalirin9 binds p75 and is coexpressed with p75 in vivo

4.2. NgR regulates Kalirin9 binding to p75

4.3. Activated NgR induces RhoA to bind Kalirin9

4.4. Kalirin9 is functionally involved in p75-NgR signaling

4.5. Kalirin9 is involved in RhoA activation after spinal cord injury

4.6. RhoA activation after spinal cord injury is attenuated in the mutant Kalirin9 transgenic mice

A1.1. Neurotrophins activate PI-3 kinase in oligodendrocytes

A1.2. PI-3 kinase is necessary to prevent p75-mediated JNK activation and cell death

A1.3. PI-3 kinase is not sufficient to prevent p75-mediated JNK activation or cell death

A1.4. Ras is sufficient to prevent p75-mediated apoptosis

A1.5. Erk plays a role in suppressing p75-mediated apoptosis

A1.6. RasV12 prevents NGF-dependent activation of JNK, yet increases basal JNK activity

A2.1. Cdk5 activation by p75 contributes to apoptosis

A3.1. Altered Rac and Rho activity in the cerebellum of p75 null mice
CHAPTER 1

INTRODUCTION

NGF belongs to a family of neurotrophins whose primary role is the promotion of neuronal survival and differentiation. NGF exerts its role by activating two distinct types of receptors, TrkA, a receptor tyrosine kinase and p75, a member of TNF receptor family. The role of TrkA is undisputed as a survival promoting receptor. The role of p75, on the other hand, still remains controversial, in part because it is associated with the promotion of both apoptosis and survival.

In the sympathetic system, p75 is involved in the death of superior cervical ganglion neurons during development, but whether p75 also plays a similar role in the adult is still unclear (Bamji et al., 1998), (Brennan et al., 1999). In the central nervous system, the role of p75 similarly remains controversial, since the absence of p75 resulted contradictorily in an increase, a decrease, and no change in the total number of basal forebrain neurons (Yeo et al., 1997), (Peterson et al., 1999), (Ward and Hagg, 1999). The difficulty of discerning the role of p75 in these systems may continue, given the complexity of p75 action in the presence of resident Trk A. One consistent piece of

evidence emerging in the literature from these data is that p75 can induce apoptosis when activated by a neurotrophin in the absence of the Trk specific to that neurotrophin (Dechant and Barde, 1997). Conversely, when co-expressed with Trk, p75 augments Trk function.

These dichotomous roles for p75 are reflected in the signaling pathways it activates. For its role in survival, p75 is known to activate NF-κB (Carter et al., 1996). For its role in apoptosis, it has been shown to activate c-jun N-terminal (JNK) kinase (Casaccia-Bonnefil et al., 1996), (Yoon, 1998), and caspases (Gu et al., 1999), and to induce ceramide production (Dobrowsky et al., 1994). Of these apoptotic pathways, the JNK pathway has been shown to be necessary (Yoon, 1998), but the mechanisms whereby p75 activates the JNK pathway are largely unknown.

In this report, we present data demonstrating that p75 activates Rac GTPase, which in turn activates JNK in a NGF-dependent manner. This activation is required for p75-mediated apoptosis. As an experimental system for studying the apoptotic pathway of p75 without the interference of TrkA, we chose to utilize primary oligodendrocytes. In oligodendrocytes, NGF induces cell death and p75 activates all the known apoptotic pathways, such as JNK, caspases, and ceramide production (Casaccia-Bonnefil et al., 1996), (Gu et al., 1999). Using oligodendrocytes, we demonstrate that activation of Rac is prolonged by NGF, but not by BDNF or NT3. This prolonged activation of Rac correlates with the ability of these neurotrophins to induce apoptosis, suggesting that the kinetics of Rac activation may determine the fate of a cell.
MATERIALS AND METHODS

Primary Rat cortical Oligodendrocyte Culture

Primary oligodendrocyte cultures were obtained as previously described (Yoon, 1998), except that the cells were subjected to an immunopanning procedure using Ran2 antibody to remove astrocytes/microglia following an overnight shakeoff. The purity of cultures was determined based on staining with antibodies against galactocerebroside (O1), myelin basic protein (MBP), and glial fibrillary acidic protein (GFAP). For staining procedures, please see below. In these cultures, 47% of the cells were O1+/MBP+, 17% were O1+/MBP-, 28% were O1-/MBP+, and 6% were GFAP+. Of the O1+ cells, 88% were p75+, of the MBP+ cells, 80% were p75+, and none of the GFAP+ cells was p75+.

Primary Mouse Oligodendrocyte Culture from the cortex

The p75 knockout mice that carried the mutation in exon 3 of the p75 gene (Lee et al., 1992) and the wild type mice were obtained from heterozygote mating as littermates. The mice were back-crossed to C57/BL6 for 10 generations to make them congenic. Their genotype was determined by PCR analyses of tail DNA according to Bentley and Lee (Bentley and Lee, 2000). At postnatal days 16-18, the brain was dissected, and a trituturated cell suspension was loaded onto a 36% Percoll gradient, and oligodendrocytes were isolated following centrifugation at 10,000 g according to Lubetzki et al. and Fuss et
al. (Lubetzki et al., 1991) (Fuss et al., 2000). Isolated oligodendrocytes were resuspended in 10% FBS in DMEM, and plated onto poly-D-Lysine coated 4-well slide dishes at 0.1x10^6 per well. The following day, the medium was changed to a differentiation medium with no serum as previously described (Yoon, 1998). The culture was kept for 6 days before NGF was added at 100 ng/ml for the indicated amount of time. In these cultures, 40% of the cells were MBP^+ and 7% were GFAP^+. Of the MBP^+ cells, 70% were p75^+, none of the GFAP^+ cells was p75^+.

**Immunocytochemistry**

The antibodies used for immunocytochemistry were 9651 (anti-75, (Huber and Chao, 1995)), O1 (a generous gift from Dr. Patrick Wood), anti-MBP (Roche Molecular Biochemicals, Indianapolis, IN), and anti-GFAP (Sigma, St. Louis, MO). For double staining of mouse oligodendrocytes for p75 and MBP, mouse oligodendrocytes were first stained live for p75 by incubating them for 1 hr at room temperature with 9651. To visualize the p75 stain, cells were washed twice with the serum-free media, and incubated at 37°C for 1 hr with Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were then fixed, and double-stained for MBP. MBP staining was visualized using an anti-mouse secondary antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR). For double staining of rat oligodendrocytes with 75 and MBP or p75 and GFAP, cells were fixed, and stained for p75 using 9651 without Triton X-100, and subsequently stained for MBP/GFAP following permeabilization with Triton X-100. For double staining of rat oligodendrocytes with O1 and p75, cells were stained live for O1 first, fixed, and stained for p75.

4
Generation of Recombinant Adenoviruses

The DN-JNK2 virus:  The cDNA for a dominant-negative mutant (DN) of JNK2 was isolated from SRα3-DN-JNK2 by digesting it with Hind III and Sma I, and sub-cloned into Track CMV shuttle vector (He et al., 1998) that was digested with Hind III and EcoRV.  The DN-JNK2 mutant contains two point mutations, T183A and Y185F (Kallunki et al., 1994).  The recombinant adenovirus construct was generated in RecA+ bacteria using the Track CMV-DN-JNK2 and pAdEasy 1 according to He et al. (He et al., 1998).  The virus was subsequently generated in 293 cells by transfection and further purified using two rounds of CsCl centrifugation.  The CsCl present in the virus preparation was removed by dialysis.

The DN-p75 virus:  The construct comprises of two domains: the extracellular and transmembrane (TM) domains are from rat p75, and the cytoplasmic domain is from human EGF receptor that was rendered kinase-dead.  In order to join the two chimeric domains in frame without altering any amino acid residues, a Bst B I site was introduced at the junction between the p75 and EGF receptors.  Introduction of the Bst B I site results in the silent mutation of phenylalanine, the last amino acid in the p75 TM domain (TTC to TTt).  The extracellular and TM domains of p75 were isolated by PCR using pcDNA3 HA-p75 as a template (Khursigara et al., 1999).  The sequence for the forward primer was GGGGTACCACCATGTCTGCACTTCTGATC and the sequence for the reverse primer was gcttcgaAAAGCAATATAGGCCAC (the underline represents the silent phenylalanine mutation and the sequences in large caps represent those in rat p75).  The PCR fragment was first cloned into pCR II vector to generate pCRII-p75Ex/TM (Invitrogen, Carlsbad, CA), and sequenced in its entirety for any errors.  The cytoplasmic
domain of human EGF receptor was isolated by PCR using pCMV5-EGF receptor as a template (Yoon et al., 1997). The sequence for the forward primer was agcgttCGAA
GGCGCCACATC and the sequence for the reverse primer was tcaTGCTCCAATAAATTCACCT (the underline represents the Bst B I site and the sequences in italics represent the stop codon introduced at the 3’ end). The PCR fragment was first cloned into pT7Blue 3 (Novagen, Madison, WI), and later mutated at the ATP binding site (lys to phe) using primers CCCGTCGCTATCGCGAATTAAGAGAA and TTCTCTTAATTCCCGATAGCGACGG (the underline represents the lys to ala mutation: AAG to GCG). The fragment was sequenced in its entirety for the presence of point mutations and for any PCR errors. To join the two domains, the cytoplasmic domain of the kinase-dead EGF receptor was first cut with Xho I, blunted with T4 DNA polymerase, and subsequently digested with Bst B I. The digested fragment was ligated into the Bst B I and Eco R V sites in the pCRII-p75Ext/TM. The resulting chimeric molecule has GCT TTT CGA AGC at the junction (the underline represents the silent mutation and the italics represent the sequences from the EGF receptor). The chimera was cloned into Track CMV shuttle vector using Kpn I and Hind III sites. The virus was generated as described for the DN-JNK2.

The N17Rac1 virus: The fragment containing human Rac1 was prepared by PCR using SRα-N17Rac1 as a template. The PCR fragment was cloned into the pCRII vector (Invitrogen, Carlsbad, CA), and sequenced in its entirety for the presence of point mutation (The to Asn) and for any PCR errors. The confirmed N17Rac1 was digested
with Xho I and Hind III and introduced to the Sal I and Hind III sites in the Track CMV vector. The recombinant adenovirus was generated as described for the DN-JNK2 virus.

**Adenovirus Infection**

Mature oligodendrocytes were infected with recombinant adenoviruses at 100-150 PFU/cell. Following 18-24 hrs infection, cells were treated with NGF for 4 hrs and lysates were prepared for Immunoprecipitation/Kinase (IP/K) assays and Western analyses as described below.

**Quantification of apoptotic oligodendrocytes**

**Rat oligodendrocytes:** Following a 4 hr NGF treatment, cells were fixed with 3% paraformaldehyde, stained for TUNEL (TMR red, Boehringer Mannheim, Indianapolis, IN), and mounted with Vectashield containing DAPI to label the nuclei (Vector Labs, Burlingame, CA). Cells expressing the mutant genes were identified by GFP, since all the recombinant adenoviruses express GFP as well as the mutant genes. The apoptotic oligodendrocytes were quantified by counting TUNEL+ cells among GFP+ oligodendrocytes. The quantitation data are from 2-4 independent experiments, each with 200-300 cells counted for a total of 400-1200 cells.

**Mouse oligodendrocytes:** At indicated times after NGF treatment, cells were fixed, and incubated with anti-MBP antibody. Cells were then stained for TUNEL, and processed for visualization of MBP stain using an anti-mouse secondary antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR). For quantification of apoptotic cells after BDNF and NT3 treatment, the number of pyknotic cells among MBP+ cells was quantified. The quantitation data are from 2-6 independent experiments, each with 100-200 cells counted for a total of 200-1200 cells.
**Immunoprecipitation**

To detect the active form of TrkB and TrkC, oligodendrocytes were either untreated or treated with BDNF or NT3 for 5 min, and the resulting lysates (1mg) were subjected to immunoprecipitation using pan-Trk antibody (C14 and B3, Santa Cruz, Santa Cruz, CA). The active forms of the receptors were detected using phospho-Trk-Y490 antibody (Cell Signaling Technology, Beverly, MA).

**JNK Kinase Assay and Western analyses**

The procedures for immunoprecipitation/kinase (IP/K) assays were identical to what was previously described (Yoon, 1998). For IP/K assays with JNK2 and JNK3 antibodies, 100 - 300 µg, and for JNK1 antibodies, 25 - 50 µg of lysates were used. For solid-phase kinase assays, bacterial cell lysates containing GST-c-jun were first bound to glutathione beads in 50mM NaCl, 5mM MgCl2, 0.1mM EDTA, 0.05% Triton X-100, and 20mM HEPES (pH 8.0). Following three washes of the beads in the binding buffer, oligodendrocyte lysates were added for a 2 hr binding at 4°C. This step was to bring down active JNK’s that are known to bind its substrates (Derijard et al., 1994). The subsequent kinase reaction was the same as described previously (Yoon, 1998).

Western analyses were identical to what was described previously (Yoon, 1998).

**Immuno-depletion**

The oligodendrocyte lysates were subjected to immunoprecipitation reactions twice using Affigel beads that were conjugated with anti-JNK1 (G151 from Pharmingen, San Diego, CA) and JNK2 antibodies (Santa Cruz, Santa Cruz, CA). To confirm the extent of depletion, approximately 5 ng of 35S-labeled JNK1 and 5 ng of 35S-labeled JNK2 proteins were added to the oligodendrocyte lysates as tracers prior to immuno-
depletion. Following immuno-depletion, 10% of the total depleted or undepleted lysates were analyzed for the presence or absence of $^{35}$S-labeled JNK1 and JNK2. The rest of the lysates were used for the solid-phase kinase assays.

**In vitro translation of JNK1 and 2 proteins**

The cDNAs of JNK1 and 2 were prepared by PCR and cloned into pCR II vector (Invitrogen, Carlsbad, CA), and subsequently sequenced in its entirety for any PCR errors. The JNK1 and 2 proteins were synthesized *in vitro* in the presence of $^{35}$S-methionine using the T7 transcription/translation coupled system (Promega, Madison, WI). The amount of each protein synthesized was estimated to be approximately 200 ng/reaction at 90% incorporation rate. As a tracer in immuno-depletion, approximately 5 ng of each protein was added to the oligodendrocyte lysates.

**Rac Activity Assay**

For samples loaded with GDP or GTPγS as specificity controls, lysates were incubated at 30°C for 15 minutes with either 100 μM GTPγS or 1 mM GDP in the presence of 10 mM EDTA. Samples were then placed on ice and MgCl₂ was added to a final concentration of 60 mM. GST-PBD Sepharose beads were added to the samples and rotated for 30 minutes at 4°C. The beads were washed and Rac protein was detected using anti-Rac1 antibody (Upstate Biotechnology, Lake Placid, NY) as described (Benard et al., 1999).

For Rac activity assays using oligodendrocyte lysates, oligodendrocytes were either untreated or treated with neurotrophins at 100 ng/ml for 5 min, 1 hr, and 4 hrs. Dishes were then placed on ice and cells washed once with ice-cold PBS. Following removal of PBS, cells were lysed in a lysis buffer, containing 25mM HEPES (pH 7.5),
150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1% NP-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 25 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 2 mM PMSF. Lysates were rotated for 5 minutes at 4°C, and clarified by centrifugation at 14,000 rpm for 5 minutes at 4°C. 10% of the supernatant of each sample was saved for Western analysis as a control for the total Rac protein using anti-Rac 1 antibody (Upstate Biotechnology, Lake Placid, NY), while the remainder was incubated with approximately 30 µg of GST-PBD bound to glutathione Sepharose beads. Lysates were rotated with beads for 1 hour at 4°C and washed 3 times with lysis buffer. Bound Rac-GTP protein was detected by Western analysis using anti-Rac 1 antibody (Upstate Biotechnology, Lake Placid, NY).

**Affinity Cross-Linking**

Affinity cross-linking was performed as described using ¹²⁵I-NT3 as the ligand, except that the full-length p75-NT3 complex was immunoprecipitated with 9992, an anti-p75 antibody, and the mutant p75-NT3 with anti-HA antibody.
RESULTS

**p75 protein is required for NGF-dependent apoptosis in oligodendrocytes**

We were asking whether oligodendrocytes undergo apoptosis in the absence of p75. For this, cortical oligodendrocytes were prepared from 16-18 day old p75 knockout mice and their wild type littermates, and the responses of each to NGF were assessed. It should be stressed here that mouse oligodendrocytes were isolated by Percoll gradient immediately following dissection (Lubetzki et al., 1991), (Fuss et al., 2000), and cultured in differentiation medium for 6 days before the analyses. This procedure differs from rat cultures which are prepared by the shake-off method developed by de Vellis (McCarthy and de Vellis, 1980). In the shake-off method, oligodendrocytes are expanded as a mixed culture during an 8-9 day incubation period, before they are isolated from astrocytes and microglia, and allowed to differentiate for 5-7 days. As shown in Fig. 1A, MBP+ mouse oligodendrocytes express p75 in culture as do their rat counterparts. By the 6th day in culture, the proportion of MBP+ cells reached approximately 40%, and the proportion of p75 expressing cells reached approximately 70% among MBP+ cells. NGF induced apoptosis in these cultures based on TUNEL assays, the extent of which increased from 8-9% at 4 hrs post-NGF to 32-34% at 48 hrs post-NGF (Fig. 1C). In the knockout mouse cultures, there was no increase in the number of TUNEL+ cells for
the entire 48 hr period of NGF treatment (Fig. 1C). A representative picture of apoptotic cells bearing pyknotic nuclei is shown in Fig. 1B. These data therefore indicate that NGF-dependent death of oligodendrocytes requires p75 protein.

The ability to activate the JNK pathway is required for p75-mediated apoptosis in rat oligodendrocytes

NGF-dependent apoptosis in primary rat oligodendrocytes was inhibited when a p75 blocking antibody was used, suggesting that the apoptosis was mediated by NGF binding to p75 (Casaccia-Bonnefil et al., 1996). In order to further confirm that the apoptosis was indeed due to the action of p75, we sought to inhibit the signaling capacity of endogenous p75, using a mutant p75 that lacks its cytoplasmic signaling domain in adenovirus. The mutant p75 contains the extracellular and trans-membrane domains of rat p75, but its cytoplasmic domain was replaced with that of the human EGF receptor (Fig. 2A). The cytoplasmic domain of the EGF receptor was rendered inactive in its tyrosine kinase function by mutating its ATP binding lysine residue to alanine. We expected the resulting construct to compete effectively for binding to NGF against the endogenous p75, but be incapable of its own signaling. To facilitate the detection of the chimeric receptor, HA tag was placed at the N-terminus of rat p75 cDNA following the signal peptide sequence (Khursigara et al., 1999). Since the adenovirus contains GFP under a separate promoter (Fig. 2A), all the infected cells also express GFP as well as the mutant p75 receptor (Fig. 2E).

The mutant receptor was first tested for surface expression and its ability to bind neurotrophins in affinity cross-linking experiments using $^{125}$I-NT3 (Fig. 2B). $^{125}$I-NT3 was chosen instead of $^{125}$I-NGF, since NT3 is more resistant than NGF to
degradation upon iodination. The mutant p75 yielded a NT3-receptor complex of about 130 kDa in molecular weight, consistent with the increase in its size in chimeric form. This result suggests that the mutant p75 is expressed on the cell surface and binds neurotrophins as efficiently as the full-length p75.

The mutant p75 receptor was then introduced via infection into rat oligodendrocytes, and its effect on NGF-dependent apoptosis was assessed. The control cultures were infected with the GFP adenovirus. 24 hrs after infection, cells were treated with NGF for 4 hrs at 100 ng/ml, and the extent of apoptosis was measured by counting the number of TUNEL$^+$ oligodendrocytes among GFP$^+$ infected cells. A representative picture is shown in Fig. 2E. In cultures infected with the control GFP virus, the proportion of TUNEL$^+$ cells increased 6-8 fold following a 4 hr NGF treatment. In the presence of the mutant p75, however, the number of TUNEL$^+$ oligodendrocytes remained at the basal level of 3-4%, (Fig. 2C). This result suggests that the mutant p75 receptor inhibited the action of endogenous p75 in rat oligodendrocytes.

We have previously reported that inhibition of JNK activity by CEP-1347 resulted in rescue of rat oligodendrocytes from NGF-dependent apoptosis (Yoon, 1998). Since the mutant p75 receptor blocked NGF-dependent apoptosis, we asked whether the JNK activity was altered in the presence of the mutant p75. For this, cells were infected for 24 hrs either with the control GFP or the mutant p75 receptor adenovirus, and the resulting lysates were subjected to a solid-phase JNK kinase assay to measure the total JNK activation ((Derijard et al., 1994); Fig. 2D). In control cultures, NGF addition led to an increase in total JNK activity, while activation of JNK was no
longer observed in the presence of the mutant p75 (Fig. 2D). These data therefore indicate that not only the binding of p75 to NGF, but also the ability of p75 to signal and activate JNK are necessary for its action in inducing apoptosis in oligodendrocytes.

**p75 activates JNK 1 and 3 in oligodendrocytes**

JNK activity has been shown to increase as PC12 cells differentiate (Eilers et al., 1998), and to remain elevated following axotomy of adult dorsal root ganglion neurons (Kenney and Kocsis, 1998). Similarly, cerebellar granule neurons exhibit a high basal level of JNK activity (Coffey et al., 2000). These data suggest that JNK activity may be involved in axonal outgrowth or regeneration. In contrast, JNK activities have also been linked to apoptosis of a variety of neurons: The death of motor and sympathetic neurons as well as PC12 cells was prevented when JNK activity was inhibited (Xia et al., 1995), (Maroney et al., 1998), (Maroney et al., 1999), (Eilers et al., 1998).

This apparent dichotomy may be due to distinct actions among different JNK isoforms. An example is found in a recent report in which only JNK3, and not JNK1 or 2, was implicated in arsenite-induced apoptosis (Namgung and Xia, 2000). We therefore sought to identify the types of JNK activated by p75 in oligodendrocytes, which undergo apoptosis in a NGF-dependent manner. To address this question, the specificity of various JNK antibodies was first tested in 293 cells. 293 cells were transfected with HA-tagged JNK1 or 2, or Flag-tagged 3, and these JNK proteins were immunoprecipitated with commercial antibodies against JNK1, 2 or 3. The presence of each transfected JNK protein in the immunoprecipitates was later detected using either HA or Flag antibodies in Western analyses. The JNK3 antibody (Upstate Biotechnology, Lake Placid, NY)
immunoprecipitated both JNK1 and 3 efficiently (Fig. 3A). JNK2 antibody (Santa Cruz, Santa Cruz, CA) immunoprecipitated JNK1 and 2, but not JNK3. For JNK1, we tested two different antibodies: C17 polyclonal (Santa Cruz, Santa Cruz, CA) and G151 monoclonal antibodies (Pharmingen, San Diego, CA). The C17 JNK1 antibody brought down JNK1 and 3, but not JNK2 and G151 JNK1 antibody immunoprecipitated JNK1 and 2, but not JNK3. These results are tabulated in Fig. 3B.

Each of these antibodies was then used in immunoprecipitation/kinase (IP/K) assays using lysates from oligodendrocytes that were treated with NGF for 4 hrs at 100 ng/ml (Fig. 3C). p75 activated JNK1 based on G151 and C17 antibody data, but it did not activate JNK2 robustly. There appeared to be activation of JNK3 by p75 based on anti-JNK3 data, but the data were not conclusive, since JNK3 antibody detected both JNK1 and 3 efficiently (Fig. 3A). We therefore performed immuno-depletion with oligodendrocyte lysates using G151 JNK1 antibody, and tested whether p75 still activated JNK upon NGF treatment. To monitor the extent of depletion, $^{35}$S-JNK1 and 2 were added to the lysates as tracers prior to depletion. As shown in the upper panel of Fig. 4, there was a robust JNK activation following depletion of JNK1 and 2 from oligodendrocyte extracts. Based on the tracers, there was very little JNK1 and 2 protein left following depletion (Fig. 4, lower panel), suggesting that the JNK activation observed after immuno-depletion represents activation of JNK3. We therefore conclude that p75 activates JNK1 and 3 in oligodendrocytes.
Activation of JNK is required for p75-mediated apoptosis

We have previously used an inhibitor of the JNK pathway, CEP-1347, to demonstrate that JNK activity was necessary for the death of oligodendrocytes (Yoon, 1998). Similarly, the mutant p75 receptor that is incapable of signaling via the JNK pathway inhibited NGF-dependent apoptosis in oligodendrocytes. With CEP-1347, however, the possibility exists that the drug may have affected other pathways that were not measured. With the mutant p75, it is also likely that another pathway that is required for survival, such as the NF-kB pathway, was concurrently blocked. Therefore, we tested whether direct inhibition of endogenous JNK could lead to suppression of p75-mediated apoptosis by introducing a dominant-negative (DN) JNK into oligodendrocytes. Since there is a greater than 70% sequence homology among the three JNK isoforms, it is likely that a DN mutant of one isoform will inhibit all three isoforms. We chose DN-JNK2, because it is regulated similarly to the other JNK’s, but has a higher affinity to c-jun than JNK1, and JNK2 activates c-jun promoter while JNK1 does not (Kallunki et al., 1994). The DN mutant contains two point mutations, T183A and Y185F, at the phosphorylation sites that are required for its activity (Kallunki et al., 1994).

The DN-JNK2 virus was first tested for its action against JNK1 and JNK3, as well as for the total JNK activity in rat primary oligodendrocytes, by infecting the cells with either the GFP control or the DN-JNK2 virus. 24 hrs after infection, cells were treated with NGF for 4 hrs at 100 ng/ml, and the lysates were subjected to a solid-phase JNK kinase assay to measure the total JNK activation (Fig. 5A, top panel). NGF addition led to an increase in the total JNK activity in the control GFP virus infected cells, while there
was no increase in the DN-JNK2 infected cells. The DN-JNK2 was also effective against individual JNK1 and JNK3, as demonstrated in IP/K assays, using either JNK1 or JNK3 antibodies (Fig. 5A, middle two panels). These results confirm that the mutant JNK2 was effective in inhibiting NGF-dependent JNK activation.

To test whether the DN-JNK2 could rescue the death of oligodendrocytes, cell viability was determined by counting the number of TUNEL$^+$ cells among the GFP$^+$ oligodendrocytes. When NGF was added at 100ng/ml for 4 hrs, the number of TUNEL$^+$ cells among GFP$^+$ oligodendrocytes increased 4-5 fold in the GFP virus infected cells, while it did not increase in the DN-JNK2 virus infected cells (Fig. 5B). Therefore, these results together with the data from CEP-1347 and the data with the mutant p75 receptor indicate that JNK activation is indeed necessary for NGF-dependent apoptosis in oligodendrocytes.

**Rac1 is an upstream regulator in p75-mediated JNK activation**

P75 has been shown to activate the JNK pathway in several cell types other than oligodendrocytes. These include superior cervical ganglion (SCG) neurons (Bamji et al., 1998) and the Schwannoma cell line (Gentry et al., 2000). In SCG neurons (Mazzoni et al., 1999) and oligodendrocytes (Fig. 5), JNK activation is causally linked to p75-mediated apoptosis. The observation that p75 activates JNK in several different systems may imply that JNK activation by p75 is a central feature in p75 signaling. The mechanism by which p75 activates JNK is still unknown. Although nine different molecules are known to interact with p75 to date, none of them has been linked to the JNK pathway (Khursigara et al., 1999), (Ye et al., 1999), (Casademunt et al., 1999) (Chittka and Chao, 1999), (Yamashita et al., 1999), (Irie et al., 1999), (Salehi et al.,
2000). We therefore sought to find an upstream regulator that may be most proximal to the receptor. A candidate molecule is Rac, a GTPase belonging to the Rho family of small G proteins. Rac has been shown to function as the upstream regulator in the JNK pathway in cell lines as well as in neurons (Coso, 1995), (Minden et al., 1995), (Bazenet et al., 1998). In addition, p75 was recently reported to interact with another member of the Rho family of small G proteins, RhoA (Yamashita et al., 1999). Binding of p75 to NGF led to suppression of Rho activity in 293 cells (Yamashita et al., 1999).

We first tested whether p75 modulates Rac activity in oligodendrocytes in a NGF-dependent manner, using the GST-PBD or p21 binding domain of PAK 1. PAK 1 binds Rac only when Rac is bound to GTP via PBD. The activated, GTP-bound Rac can then be detected in Western analyses following pulldown assays using the GST-PBD (Benard et al., 1999). The specificity of this assay was tested using unhydrolyzable GTP analog, GTP-γS, or GDP. As shown in Fig. 6A, the GST-PBD pulled down only the GTP-γS-bound Rac and not the GDP-bound Rac. Using the assay, we examined whether p75 can modulate Rac activity in oligodendrocytes. In oligodendrocytes, NGF induced Rac activation starting 5 min after NGF addition at 100 ng/ml, and its activation lasted for 4 hr post-NGF treatment (Fig. 6B). In contrast to NGF, BDNF failed to activate Rac, while NT3 addition led to transient activation of Rac. We next asked whether the different temporal pattern of activation of Rac observed with the different neurotrophins correlates with their ability to activate JNK. NGF addition led to robust JNK activation starting 1 hr post-NGF, lasting up to 4 hrs, while BDNF did not during a 4 hr treatment (Fig. 7A). There was a small increase in
JNK activity after a 4 hr NT3 treatment, but this activation was not sufficient to lead to apoptosis, since only NGF could induce apoptosis in oligodendrocytes, while BDNF or NT3 could not (Fig. 7B). These results suggest that prolonged Rac activation by p75 may be one of the key steps required for NGF-dependent apoptosis.

It was reported recently that NGF induced Rac activation in PC12 cells (Yasui et al., 2001), (Yamaguchi et al., 2001). In those studies, Rac activation subsided 3 min after NGF treatment. These results suggest that in rat oligodendrocyte cultures, transience or absence of Rac activation, with NT3 or BDNF respectively, is due to concomitant activation of resident TrkB and TrkC. The rat oligodendrocytes expressed TrkB and TrkC, since BDNF and NT3 treatment resulted in tyrosine phosphorylation of the receptors, based on Western analyses with the phospho-TrkY490 antibody (Fig. 7C). We have previously reported that co-activation of TrkA and p75 following introduction of ectopic TrkA into rat oligodendrocytes led to suppression of JNK (Yoon, 1998). Our data here with BDNF and NT3, in addition to the reports by Yasui et al. (Yasui et al., 2001) and Yamaguchi et al. (Yamaguchi et al., 2001), indicate that one of the key points where Trk activation intercepts p75 signaling is at or upstream of Rac.

Since temporal activation of Rac correlated with cell death in oligodendrocytes, we next investigated whether Rac activation was required for NGF-dependent apoptosis. For this purpose, we generated an adenovirus carrying the cDNA for the DN-Rac1. The DN-Rac1 contains a mutation at residue 17 (threonine to asparagine). Following infection with either GFP or the DN-Rac1 virus, cell viability was determined by counting the number of TUNEL+ cells among the GFP+ oligodendrocytes following a 4
hr NGF treatment at 100 ng/ml. In the presence of the DN-Rac1, the number of apoptotic oligodendrocytes was reduced compared to the control GFP virus infected cells, following NGF treatment (Fig. 8A). To test whether Rac activation was required for NGF-dependent JNK activity, the lysates from either GFP or the DN-Rac1 virus infected cultures were subjected to solid-phase kinase assays. In oligodendrocytes infected with the control GFP virus, there was activation of JNK upon NGF addition, but in cells infected with the DN-Rac1, JNK activation was no longer observed (Fig. 8B). These data, therefore, indicate that p75 utilizes Rac 1 to activate JNK, and Rac activation is an obligatory step in the NGF-dependent apoptotic machinery activated by p75. This Rac activation is under competitive regulation by Trk signaling, thereby governing cell survival and death.
DISCUSSION

In this report, we present the data that p75 activates Rac GTPase in a NGF-dependent manner. This Rac activation is causally linked to apoptotic action by of p75, since N17Rac1 inhibits NGF-dependent JNK activation, which in turn leads to suppression of apoptosis. Co-activation of Trk modulates p75-mediated Rac activation, thereby identifying Rac GTPase as one of the key molecules whose activity is critical for cell survival and death in neurotrophin signaling. The crucial role of the JNK pathway in p75 signaling is further confirmed by the fact that blocking p75 from signaling via the JNK pathway or suppressing the JNK activity itself led to inhibition of NGF-dependent death. We also report that p75 activates JNK3, an injury-specific JNK, in a system where it induces apoptosis.

**p75 is required for NGF-dependent death of oligodendrocytes in culture**

We demonstrate here that mouse oligodendrocytes expressed p75 as early as the second day in culture, and they did not die in the absence of p75 when NGF was added (Fig. 1). In addition, blocking the signaling ability of p75 in rat oligodendrocytes also resulted in reversal of the death effect of NGF (Fig. 2). These results directly confirm the apoptotic role of p75 in oligodendrocytes. It is not clear, however, whether p75 plays a
similar role in oligodendrocyte biology in vivo. The current literature strongly indicates that p75 is induced among oligodendrocytes by an injury or disease state. For instance, in the white matter plaques of multiple sclerosis (MS) patients, p75 was induced among oligodendrocytes and their precursors (Dowling et al., 1999), (Chang et al., 2000). Direct infliction of injuries to the white matter in the cortex and the spinal cord can also induce p75 expression among oligodendrocytes (Yoon, S.O., Bresnahan, J., and Beattie, M., unpublished data). This correlation between p75 expression in vitro and in vivo suggests that culture conditions can model injury or stress situations these cells encounter in vivo. Our data demonstrating that p75 activates an injury-specific JNK3 in cultured oligodendrocytes also supports this notion (Fig. 4).

What is the consequence of p75 induction in an injury or a disease state? Based on our culture data, one might suggest that p75 plays a pro-apoptotic role, perhaps serving to eliminate diseased cell populations. In support of this notion, Dowling et al. (Dowling et al., 1999) reported that 47% of TUNEL-positive oligodendrocytes expressed p75 in MS lesions. Contrary to these data, Chang et al. (Chang et al., 2000) found no TUNEL-positive cells among NG2+ oligodendrocyte precursors in MS lesions. This data may be interpreted as indicating that p75 plays an opposing, anti-apoptotic role in developing oligodendrocytes when the greater population is injured, and in this way may contribute to regeneration. Alternatively, p75 may still be involved in inducing apoptosis in NG2+ oligodendrocytes, but its expression is not sufficient to induce apoptosis. It is quite possible that induction of p75 is one of many factors required for these cells to undergo apoptosis, but not sufficient to induce death in vivo. In support of this notion, Ladiwala et al. (Ladiwala et al., 1998) have reported that although human
oligodendrocytes do express p75 in culture, they failed to die when NGF was added. NGF did not induce the JNK pathway in human oligodendrocytes, indicating that some component of the p75-mediated JNK signaling pathway is not present in these cells, or its activation was somehow inhibited. Analyses of p75 knockout mice in an injury or disease paradigm will help answer the question of whether the induced expression of p75 among oligodendrocytes in vivo presages their fate as it does in culture.

**P75 activates JNK1 and JNK3**

Our results demonstrate that p75 activates JNK1 in addition to JNK3, and their activation is required for NGF-dependent apoptosis. The involvement of JNK1 in apoptosis of oligodendrocytes is different from that revealed by the data for cortical cultures, where JNK1 was not implicated in arsenite-induced apoptosis (Namgung and Xia, 2000). In cortical neurons, the basal level of JNK1 activity was high, suggesting that JNK1 may be involved in differentiation (Namgung and Xia, 2000). JNK activity has been shown to increase as PC12 cells or cerebellar granule neurons differentiate, although the specific JNK isoform responsible for this increase was not determined (Eilers et al., 1998), (Yao et al., 1997), (Coffey et al., 2000). This dual role of JNK has also been demonstrated in the analyses of JNK1 and 2 double knockout mice. Loss of JNK1 and 2 resulted in increased apoptosis in the forebrain region, while it inhibited apoptosis in the hindbrain area during embryonic development (Kuan et al., 1999). In oligodendrocytes in culture, JNK1 plays a role in inducing cell death, since total JNK activity as well as JNK1 activity was suppressed with the mutant p75, the DN-JNK2, and N17Rac (Fig. 2, 5, and 8).
Although JNK1 and 2 may play diverse roles, it appears that the role of JNK3 is in inducing apoptosis following injuries to the nervous system (Yang et al., 1997), (Namgung and Xia, 2000). It is highly relevant in this regard that p75 activates an injury-specific JNK3 in a system where it induces cell death (Fig. 4). It is well documented in the literature that p75 is robustly induced in neurons, Schwann cells and oligodendrocytes following injuries (Taniuchi et al., 1986), (Roux et al., 1999), (Hayes et al., 1992), (Koliatsos et al., 1991). For instance, p75 has been reported to be induced in dying neurons following a seizure (Roux et al., 1999), ischemia (Park et al., 2000), and in cortical neurons following experimental allergic encephalomyelitis (Nataf et al., 1998), (Calza et al., 1997). It remains to be seen whether JNK3 activation is indeed required for p75-mediated apoptosis \textit{in vivo}.

\textbf{P75 activates Rac GTPase persistently}

We demonstrated that p75 activates Rac GTPase in a NGF-dependent manner (Fig. 6). These results differ from those for RhoA activity, where NGF binding to p75 led to suppression rather than activation (Yamashita et al., 1999). The suppression of Rho A activity by p75 was implicated in the promotion of process outgrowth in ciliary neurons (Yamashita et al., 1999). In oligodendrocytes that undergo apoptosis in a NGF-dependent manner, Rac activation is causally linked to apoptosis (Fig. 8). As a member of the Rho family of small G proteins, Rac plays a role in cytoskeletal reorganization as well as being the upstream regulator in the JNK pathway (Coso, 1995), (Minden et al., 1995). In NGF signaling, Rac activity is distinctly involved in activation of the JNK pathway, as we demonstrated by the effect of DN-Rac1 in
oligodendrocytes. The dual roles for Rac have been more clearly demonstrated by a single point mutation (tyrosine at residue 40 to cysteine) that resulted in the loss of JNK activation, but a continued effect on cytoskeletal reorganization (Lamarche et al., 1996). These results suggest that distinct effector molecules are involved in eliciting these diverse, Rac-mediated effects, although it is not known what regulates the process that determines whether Rac activation would lead to cell death or to cytoskeletal reorganization.

Our data suggest that the kinetics of Rac activation may be a determinant in this process. In oligodendrocytes, NGF induced a long-term Rac activation, lasting up to 4 hrs post-NGF (Fig. 6B). In PC12 cells where NGF activated Rac transiently (Yasui et al., 2001), this activation was linked to neurite extension and differentiation, rather than cell death (Yamaguchi et al., 2001). Similarly to the data from PC12 cells, NT3 activated Rac only transiently in oligodendrocytes that express TrkC (Fig. 6B), and did not induce cell death (Fig. 7B). These results may be interpreted as suggesting that the kinetics of Rac activation determine whether Rac plays a role in apoptosis or in differentiation in neurotrophin signaling. This is reminiscent of the data that prolonged ERK activation correlated with differentiation, while transient ERK activation correlated with proliferation in PC12 cells (Qui and Green, 1992).

Based on the correlation that we observed with NGF, between apoptosis and prolonged Rac activation, we hypothesize that additional effector molecules may be recruited to Rac-GTP at later points in NGF induction. Recruitment of these effector molecules may also be responsible for the differences in temporal regulation of Rac GTPase, perhaps by providing a stabilizing scaffold.
Although NT3 activated Rac transiently and induced JNK, it did not induce apoptosis in oligodendrocytes (Fig. 6 and 7). This NT3 response in oligodendrocytes is similar to the NGF response in PC12 cells. In PC12 cells, NGF induced JNK (Minden et al., 1994), (Eilers et al., 1998) as well as transient Rac activation (Yasui et al., 2001), (Yamaguchi et al., 2001). We interpret these data as suggesting that JNK activation may not be sufficient to induce apoptosis in oligodendrocytes, although it is indeed necessary, since blocking JNK activity prevented NGF-dependent apoptosis. In order to induce apoptosis, p75 may need to activate a pro-apoptotic pathway(s) other than the JNK pathway, or suppress an anti-apoptotic pathway(s). Alternatively, TrkC may activate a pathway that intercepts p75 signaling downstream from JNK in oligodendrocytes.

In conclusion, we report that p75 activates Rac GTPase persistently, and this activation is essential for p75 to induce JNK and apoptosis. Simultaneous activation of Trk counteracts apoptotic action by p75, by modulating the kinetics of p75-mediated Rac activation.
REFERENCES


Bentley CA, Lee KF (2000) p75 is important for axon growth and schwann cell migration during development [In Process Citation]. J Neurosci 20: 7706-7715.


Chittka A, Chao MV (1999) Identification of a zinc finger protein whose subcellular
distribution is regulated by serum and nerve growth factor. Proc Natl Acad Sci U S A 96:
10705-10710.

N-terminal kinase in developmental and stress responses in cerebellar granule neurons [In
Process Citation]. J Neurosci 20: 7602-7613.

Coso OA, Chiarielo, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki,T., and
Gutkind, J.S. (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the

Dechant G, Barde YA (1997) Signalling through the neurotrophin receptor p75NTR.
Curr Opin Neurobiol 7: 413-418.

A protein kinase stimulated by UV light and Ha-ras that binds and phosphorylates the c-

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation
of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265:
1596-1599.


Fig. 1.1 Oligodendrocytes from p75 null mice fail to die upon NGF treatment.

(A) Mouse oligodendrocytes express p75 in culture, as do their rat counterparts. A representative picture of a mouse oligodendrocyte culture taken from the wild type mice at P16 cortex. P75 expression on the cell surface was detected using 9651, anti-p75 antibody (Red), and oligodendrocytes were identified by MBP stain (Green). Scale bar: 8 \( \mu \text{m} \). (B) Oligodendrocytes fail to die in the absence of p75 when NGF is added. Four to six days after plating, mouse oligodendrocytes were treated with 100 ng/ml of NGF for 4 hrs, fixed and stained for MBP. Pyknotic cells among the MBP+ cells are indicated by arrows and also shown at higher magnification in the insert. Scale bar: 20 \( \mu \text{m} \). (C) Quantification of TUNEL+ cells among MBP+ cells. The quantitation data are from 2-4 independent experiments, each with 100-150 cells counted for a total of 200-600 cells.
Fig. 1.2 The signaling ability of p75 is required for NGF-dependent apoptosis in oligodendrocytes.

(A) Schematic diagram of the mutant-p75 lacking the cytoplasmic domain. The cytoplasmic domain of this mutant receptor was replaced with the corresponding domain of the kinase-dead EGF receptor. The arrows indicate two independent CMV promoters, one directing expression of the mutant p75, and the other, of GFP. All the adenoviruses used in this study co-express GFP. B) The mutant-p75 binds $^{125}$I-NT3. Cos cells were infected with the full-length (FL) p75 or the mutant-p75 adenovirus, and subjected to cross-
linking with $^{125}$I-NT3. PC12 cells were used as a positive control (lane 1), and uninfected Cos as a negative control (lane 2). The FL-p75 was immunoprecipitated with 9992 antibody (lane 3), and the mutant-p75 with HA antibody (lane 4). (C) The mutant-p75 protects oligodendrocytes from NGF-dependent apoptosis. Oligodendrocytes were infected with GFP control or the mutant p75 adenovirus in 4 well slide dishes for 24 hrs at 150 PFU/cell. Following 4 hr NGF treatment, cells were stained for TUNEL. The number of TUNEL$^+$ cells was determined among GFP$^+$ cells. The quantitation data are from 3-5 independent experiments, each with 200-300 cells counted for a total of 600-1500 cells. (D) The mutant-p75 inhibits JNK activation in oligodendrocytes. 24 hours after infection with the viruses, oligodendrocytes were treated with NGF at 100 ng/ml for 4 hrs. The changes in JNK activity were measured by solid-phase kinase assays. The presence of the mutant-p75 was detected with anti-HA antibody, and the JNK protein with anti-JNK antibody. (E) A representative picture of oligodendrocytes quantified following infection with adenoviruses and NGF treatment. The cells expressing the mutant p75 were identified by GFP fluorescence, since the virus also expresses GFP as well as the mutant p75 cDNA. The arrows indicate the TUNEL$^+$ cells among GFP$^+$ cells. Scale bars: 20 µm.
Fig. 1.3 P75 activates JNK1 and 3.

(A) The specificity of antibodies used in immunoprecipitation/kinase assays. 293 cells were transfected with HA-JNK1, HA-JNK2, or Flag-JNK3 cDNAs. The lysates from each transfected dish were subjected to immunoprecipitation reactions, using anti-JNK1 (C17, polyclonal; Santa Cruz), anti-JNK1 (G151, monoclonal; Pharmingen), anti-JNK2 (FL; Santa Cruz), and anti-JNK3 (Upstate Biotechnology) antibodies. The immunoprecipitated proteins were detected using either anti-HA (JNK1 and 2) or anti-Flag (JNK3) antibody. (B) A summary of the data presented in Fig. 4A. (C) The lysates from rat oligodendrocytes were subjected to immunoprecipitation/kinase assays using the four antibodies. P75 activates JNK1, based on C17 and G151 antibodies and JNK3, based on C17 and JNK3 antibodies.
Fig. 1.4 P75 activates JNK3 following depletion of JNK1 and 2.

The oligodendrocyte lysates were subjected to immuno-depletion to remove JNK1 and 2 using JNK1 and JNK2 antibodies. The depleted lysates were used in solid-phase kinase assays (upper panel). The efficiency of immuno-depletion is shown in the lower panel. The extent of immuno-depletion of JNK1 and 2 proteins was determined using $^{35}$S-JNK1 and JNK2 that were added together as a tracer to the oligodendrocyte lysates.
**Fig. 1.5** JNK activation is necessary for NGF-dependent apoptosis in oligodendrocytes.

(A) The DN-JNK2 inhibits NGF-dependent activation of JNK in oligodendrocytes. Oligodendrocytes were infected with GFP control or DN-JNK2 adenovirus for 24 hrs at 150 PFU/cell. Infected cells were untreated or treated with NGF for 4 hrs, and the lysates were subjected to solid-phase kinase as well as IP/K assays. The presence of DN-JNK2 is shown in an HA Western, and the JNK protein in a JNK Western. (B) The DN-JNK2 rescues oligodendrocytes from NGF-mediated apoptosis. The quantification procedure was identical to what was described in the legends for Fig. 2.
Fig. 1.6 P75 activates Rac1 in a NGF-dependent manner.

(A) The specificity of the pulldown Rac assay. The lysates from 293 cells were incubated with either 1 mM GDP or 0.1 mM GTPγS and subjected to a pulldown assay using GST-PBD. The bound Rac protein was detected in a Western analysis with anti-Rac1 antibody. (B) NGF addition led to a prolonged activation of Rac1 in oligodendrocytes. Oligodendrocytes were treated for the indicated amount of time with NGF, BDNF, or NT3 at 100ng/ml. The lysates were subjected to Rac activity assays. Note that BDNF does not activate Rac1, while NT3 activates it transiently.
Fig. 1.7 Trk activation intercepts p75-mediated JNK activity at or upstream of Rac GTPase

(A) Temporal course of JNK activation by neurotrophins. Rat oligodendrocytes were treated with neurotrophins for the indicated amount of time at 100 ng/ml. The lysates were subjected to solid-phase kinase assays. (B) Only NGF is capable of inducing cell death among oligodendrocytes. Mouse oligodendrocytes were treated with 100 ng/ml of NGF, BDNF, or NT3 for 4-5 hrs, and the number of pyknotic cells were counted among MBP⁺ cells. The quantitation data are from 3 independent experiments, each with 100-200 cells counted for a total of 300-600 cells. (C) Rat oligodendrocytes express TrkB and TrkC. Rat oligodendrocytes were untreated or treated with 100 ng/ml of BDNF or NT3 for 5 min. The activated receptors were detected using phospho-Trk-Y490 antibody. Active TrkA from PC12 cells was used as a positive control (lanes 4, 5).
Fig. 1.8 Rac1 is the upstream regulator of the p75-mediated JNK pathway.

(A) DN-Rac1 inhibits NGF-dependent JNK activation. Oligodendrocytes were infected with either GFP or the DN-Rac1 for 24 hrs, and either left untreated or treated with 100ng/ml NGF for 4 hrs. The resulting lysates were used in solid-phase kinase assays. (B) The DN-Rac1 protects oligodendrocytes from NGF-dependent apoptosis. The procedure is identical to the one described in the legend for Fig. 2.
CHAPTER 2

INTRODUCTION

It has been recently discovered that unprocessed NGF precursor, pro-NGF, binds p75 preferentially over TrkA, and this selective binding of pro-NGF to p75 leads to apoptotic death of cells that express both TrkA and p75 (Lee et al., 2001). Mature NGF, on the other hand, binds and activates both receptors, which results in promotion of cell survival due to TrkA-mediated survival signal overriding p75-mediated apoptotic signal (Yoon, 1998; Friedman, 2000; Harrington et al., 2002). Before the discovery of an independent function for pro-NGF, p75 was thought to activate its apoptotic program when TrkA was not expressed in a cell. With the finding that pro-NGF can activate p75 regardless of the presence of TrkA, the ratio of pro-NGF to mature NGF now emerges as a critical regulatory factor for the maintenance of the balance between survival and death (Chao and Bothwell, 2002).

In vivo, p75 is often induced by an injury or a disease state among neurons, oligodendrocytes, or Schwann cells. p75 expression was induced in dying neurons following seizure (Roux et al., 1999), ischemia (Park et al., 2000), excitotoxic agents

---

2 : Michael S. Beattie*, Anthony W. Harrington*, Ramee Lee, Ju Young Kim, Sheri L. Boyce, Frank M. Longo, Jacqueline C. Bresnahan, Barbara L. Hempstead, and Sung Ok Yoon *equal contributing authors

(Oh et al., 2000), axotomy (Giehl et al., 2001), and in cortical neurons following experimental allergic encephalomyelitis (Calza et al., 1997; Nataf et al., 1998). In the white matter plaques of multiple sclerosis patients, p75 induction was also observed among oligodendrocytes and their precursors (Chang et al., 2000; Dowling et al., 1999). In neonatal Schwann cells, p75 has been shown to play a death-inducing role following axotomy (Syroid et al., 2000). In the CNS, however, the consequence of this induced p75 expression has been unclear. Induction of p75 could be one of the first steps that initiate the apoptotic cascade after injury, or it may signify regenerative responses undertaken by the injured system, perhaps in cooperation with resident Trk’s.

It is well established in the literature that neurotrophins are induced, or their level is greatly increased, by pathological conditions that are known to cause induction of p75 (Bengzon et al., 1992; Donovan et al., 1995; Heumann et al., 1987; Widenfalk et al., 2001). The recent discovery of the functionality of pro-NGF leads to the prediction that the presence or absence of pro neurotrophins under injury conditions will determine whether activation of p75 induction will lead to a pro-apoptotic or anti-apoptotic response. For instance, in Alzheimer’s patients, the level of pro-NGF is increased (Fahnestock et al., 2001), suggesting a possible role for p75 in Alzheimer’s disease. Induction of p75 among cortical neurons in Alzheimer’s patients has been reported (Mufson and Kordower, 1992).

In this report, we investigated whether expression of p75 and pro-NGF is responsible for injury-mediated apoptosis of oligodendrocytes in vivo, using spinal cord injury as an injury model. As one of the secondary events triggered by the initial
spinal cord injury, oligodendrocytes undergo apoptosis (Crowe et al., 1997), and the presence of apoptotic oligodendrocytes was shown to correlate closely with lesion extension along fiber tracts undergoing Wallerian degeneration after spinal cord injury (Shuman et al., 1997). The death of oligodendrocytes might contribute to chronic demyelination, resulting in spinal cord dysfunction (Beattie et al., 2000; Blight, 1993; Crowe et al., 1997; Li et al., 1996; Liu et al., 1997; Shuman et al., 1997; Warden et al., 2001). Although the temporal and spatial patterns of oligodendrocyte death have been well characterized in a number of studies (reviewed in (Beattie et al., 2000; Warden et al., 2001)), the mechanisms by which oligodendrocytes die during Wallerian degeneration is unknown.

Here, we report that p75 and pro-NGF are both induced following spinal cord injury. p75 expression was specifically induced among oligodendrocytes, and the majority of p75+ cells was positive for cleaved caspase 3, suggesting that they were undergoing apoptosis. Corollary to these data, the number of cleaved caspase 3+ oligodendrocytes was reduced after spinal cord injury in the absence of p75. Likewise, the absence of p75 permitted survival of oligodendrocytes under conditions that would otherwise lead to apoptosis both in vivo and in vitro. In addition, we demonstrate that pro-NGF present in the extracts from the injured spinal cord is active in inducing apoptosis among oligodendrocytes, while its action can be blocked by pro-NGF-specific, but not pro-BDNF-specific, antibody. Together, these results support the hypothesis that a consequence of p75 expression after injury is, in part, to eliminate damaged cells in the CNS, and its activation is mediated predominantly by pro-NGF.
MATERIALS AND METHODS

Animals used in the study
Mice: Two groups of p75+/+ and p75−/− mice were used for the study. For the injury study, a congenic C57/BL6 line that carries a mutation in exon 3 of the p75 gene (Lee et al., 1992) was purchased from the Jackson Laboratory (Bar Harbor, ME). For the culture study, the p75−/− and p75+/+ mice were obtained from heterozygote mating as littermates. Their genotype was determined by PCR analyses of tail DNA according to Bentley and Lee (Bentley and Lee, 2000).

Rats: Adult female Long-Evans hooded rats were obtained from Simonsen Labs (Los Angeles, CA).

Spinal cord injuries. Mice were anesthetized with isoflurane and the spinal cord was exposed at T10. A Beaver blade was used to produce a dorsal hemisection of the cord, including the dorsal columns and the dorsal part of the lateral funiculus. Contusion injuries in rats were made using the NYU device (Gruner, 1992). Under pentobarbital anesthesia, the spinal cord was exposed at T10 and a 10g weight was dropped from 25 mm onto the dural surface as previously described (Basso et al., 1996). All procedures were approved by the Institutional Laboratory Animal Care and Use Committee and followed the NIH Guidelines for the proper use and care of laboratory animals.
Perfusion
Under deep anesthesia (80 mg/kg ketamine, Fort Dodge Animal Health, Fort Dodge, IA; 10 mg/kg xylazine, Vedco, Inc, St. Joseph, MO), rats and mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde fixative. The spinal cords were removed, and three adjacent blocks were cut from the cords, each being either 3mm long for the mouse cords or 5mm long for the rat cords. In each case, one block was centered on the lesion, and the others were rostral and caudal to that block. The blocks were sectioned at 20 µm thickness on a cryostat.

Immunohistochemistry
Sections were incubated in blocking solution containing 10% goat serum, 10% horse serum, 1% BSA, and 0.3% Triton X-100 in 0.1M PB for 2 hrs at room temperature. For double-staining for p75 and oligodendrocyte cell bodies, the sections were incubated simultaneously with an anti-p75 antibody, 9651 (Huber and Chao, 1995) and CC1 antibody (Bhat et al., 1996; Crowe et al., 1997) in 5% goat serum, 5% horse serum, and 0.1% BSA in 0.1M PB at room temperature overnight. 9651 recognizes the extracellular domain of p75. CC1 antibody recognizes the APC gene product which is expressed in rat oligodendrocyte somata and proximal processes (Bhat et al., 1996). Although CC1 antibody can detect astrocytes, less than 0.5% of cells were positive for both CC1 and GFAP, an astrocytic marker, in our rat and mouse spinal cord tissue. Sections were then incubated with biotinylated anti-rabbit antibody (Vectorlabs) for p75 stain, and an anti-mouse antibody conjugated to Alexa 488 (Molecular Probes) for CC1. P75 staining was visualized using Extravidin Cy3 (Sigma). For double-staining for p75 and active caspase 3, 192 anti-mouse anti-p75 antibody was used simultaneously with active caspase 3 anti-
rabbit antibody (Cell Signaling, Beverly, MA). For 192 immunostaining, a biotinylated anti-mouse secondary and an anti-mouse antibody conjugated to Alexa 488 (Molecular Probes) was used, and for active caspase 3, an anti-rabbit secondary conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA) was used. For double-staining for CC1 and active caspase 3, a biotinylated anti-mouse secondary and an anti-mouse antibody conjugated to Alexa 488 (Molecular Probes) was used for CC1. Active caspase 3 was detected with anti-rabbit secondary conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA). The sections were mounted with Vectashield containing DAPI to label the nuclei (Vector Labs). For confocal microscopy, BioRad MRC 1024 attached to a Nikon Optiphot-2 was used.

For cell counts shown in Figs. 1 and 5A, CC1 staining of rat and mouse tissues were done in the same way as the fluorescence staining, except that the positive staining was visualized using DAB and the Vectastain ABC kit (Vector Labs).

Cell counts:
All the counts were done blind to mouse genotype or lesion condition. Cell counts were made on rat and mouse coronal sections at a series of rostral and caudal locations relative to the contusion or hemisection lesions. Counts and reference volumes were estimated using procedures specified in the Stereologer™ program (Systems Planning and Associates, Inc., Alexandria, VA). For rat contusion injuries, three sections were randomly sampled from 1 mm blocks taken from 13 and 7 mm rostral, and 7 and 13 mm caudal to the lesion epicenter. A pilot study was run to determine the optimal disector size and spacing to allow for counts of at least 100 cells per block. CC1 positive cells were only counted when the cell body and proximal processes were darkly labeled and
were within the inclusive zone of each disector frame. Results are reported as total number of oligodendrocytes, and as density (number per mm$^3$). Data were gathered from rats with 25 mm spinal cord injury surviving for 5 or 8 days (n=4/time point), or 3 or 6 weeks (n=3/time point), and control uninjured rats (n=3). Mouse CC1$^+$ cells were counted in a similar fashion at 8d post-injury, except that the distance from the lesion center sampled was 1.2 mm and 1.8 mm rostral (R1 and R2) and 1.2 mm and 1.8 mm caudal (C1 and C2). The number of mice analyzed was n=6 for p75$^{+/+}$ and n=5 for p75$^{-/-}$. For quantification of CC1$^+$/active caspase 3$^+$ cells in Fig. 5B, p75$^{+/+}$ (n=5) and p75$^{-/-}$ (n=5) mice were analyzed at 5d post-injury, using rostral 4 mm blocks.

**Primary oligodendrocyte cultures**
The p75 knockout and the wild type mice were obtained from heterozygote mating as littermates. For spinal cord oligodendrocytes (Fig. 4), mouse pups at postnatal days 12-14, and for cortical oligodendrocytes (Fig. 7), mouse pups at postnatal days 15-16, were used. Cell suspension obtained from the triturated tissues was loaded onto a 36% Percoll gradient, and oligodendrocytes were isolated following centrifugation at 10,000g (Fuss et al., 2000; Lubetzki et al., 1991). Isolated oligodendrocytes were resuspended in 10% FBS in DMEM, and plated onto poly-D-Lysine coated 4-well slide dishes at 0.1x10$^6$ per well. The following day, the medium was changed to a differentiation medium with no serum as previously described (Yoon, 1998). The culture was kept for 4 days before NGF was added at 100ng/ml for the indicated amount of time. Rat oligodendrocytes were cultured as described (Harrington et al., 2002).

**Quantification of apoptotic oligodendrocytes in culture**
For quantification of apoptotic mouse oligodendrocytes, cells were fixed at indicated times after NGF treatment, and incubated with anti-myelin basic protein (MBP) antibody.
Cells were then stained for TUNEL, and processed for visualization of MBP stain using an anti-mouse secondary antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR).

**Western analyses**
The spinal cords were homogenized in a lysis buffer containing 1% Nonidet P-40, 20mM Tris (pH 8.0), 137mM NaCl, 0.5mM EDTA, 10% glycerol, 10mM Na2P2O7, 10mM NaF, 1µg/ml aprotinin, 10µg/ml leupeptin, 1mM vanadate, and 1mM phenylmethylsulfonyl fluoride. Induction of p75 by spinal cord injury was detected on Western analyses using an anti-rabbit, anti-p75 antibody from Covance (Berkeley, CA). For detection of pro-NGF and mature NGF, anti-mouse anti-NGF from Chemicon International (Temecula, CA) was used, but the same data were obtained with anti-rabbit anti-NGF from Cedarlane (Hornby, Ontario). The samples for neurotrophin Western analyses were prepared in Laemli buffer that was supplemented with 20 mM DTT and 100 mM iodoacetamide to prevent any potential dimeric interaction between mature NGFs. BDNF and NT3 antibodies were from Promega (Madison, WI).

**Immunodepletion**
The lysates were subjected to two rounds of immunoprecipitation using pro-NGF antiserum. The supernatants resulting from immunoprecipitation were analyzed in Western analyses with NGF antibody (Chemicon International, Temecula, CA) to assess the extent of depletion. The lysates taken before immunodepletion was used as undepleted controls in Fig. 6B.

**Generation of recombinant pro-NGF and mature-NGF.**
The cDNA of murine NGF was amplified by RT-PCR and sequenced in both direction for any errors. To improve translation initiation, 11 bases from the mouse untranslated
region of murine NT-3 including the Kozak consensus site was exchanged for the murine NGF sequence. PCR-mutagenesis was performed to add 6 histidine (His) residues at the C-terminus, and residues RR (bp 1008-1013 ) near the C-terminus was mutated to AA to impair cleavage of the His tag. To generate pro-NGF with impaired furin cleavage (pro-NGF), the KR (bp 651-657 ) was mutated AA. After bidirectional sequencing, the constructs were cloned into pcDNA, and stable 293 transfectants expressing pcDNA, pcDNA-pro-NGF, pcDNA-mature-NGF were isolated following G418 treatment. For purification, cells were cultured for 18 hrs in serum free media, and the resulting media were collected after removing cells by centrifugation. His-tagged mature or cleavage resistant pro-NGF was purified using Ni-bead chromatography (Xpress System Protein purification, Invitrogen) as per the manufacturer's instructions using imidazole (350mM) for elution. Medium from cells stably transfected with pcDNA vector alone was harvested and purified in parallel. The concentration of pro-NGF or mature -NGF was estimated by silver stain, using known concentrations of mature NGF (Harlan Bioproducts for science) in parallel.

**Generation of pro-NGF and pro BDNF –specific antibodies**

GST fusion proteins encoding amino acids 23-81 (asp23 to arg81) of human pro-NGF, or amino acids 25-90 (asn25-asp90) of human pro-BDNF were generated in bacteria and purified by chromatography with glutathione-sepharose. Rabbits (using GST-pro-NGF) or chickens (using GST-pro-BDNF) were immunized to generate antisera. Specific antisera were purified by first incubating whole serum with GST to adsorb GST-specific immuno-reactivity, and then by adsorption to, and elution from a glutathione column to which GST-pro-BDNF or GST-pro-NGF had been irreversibly coupled.
Antibody blocking experiments using injured spinal cord extracts

The extracts from injured spinal cord were added at 0.14 µl in volume, which was estimated to give a final pro-NGF concentration of 14 ng/ml based on Western analyses. Extracts from sham-operated spinal cord were used at the same volume. For the dose curves in Fig. 7B, rat oligodendrocytes were treated with column-purified recombinant pro-NGF, column-purified recombinant mature-NGF, injured spinal cord extracts from mice, sham extracts, or vehicle at the indicated concentrations. For the vehicle control for the recombinant NGF’s, the elution buffer containing 350mM imidazole was used. The final concentration of imidazole therefore ranged from 250 µM to 5mM. Following a 24hr incubation period, samples were processed for TUNEL and MBP staining as described. For antibody blocking experiments, either the injured or sham extracts (0.14 µl) were pre-incubated with mature-NGF (5 µl; Chemicon), pro-NGF (5 µl), pro-BDNF (10 µl) antibodies, or pre-immune serum (10 µl) for 2 hours at 4°C. The extract and antibody mix was then added to oligodendrocytes for 24 hrs before they were processed for TUNEL and MBP stain.

Statistical Methods
A two-way ANOVA (site x time) was used for the cell counts in the rat, and a student t-test, in the mice to evaluate the number of surviving and apoptotic oligodendrocytes.
RESULTS

Temporal and spatial correlation between apoptotic profile and actual loss of oligodendrocytes after spinal cord injury

Although the presence of apoptotic oligodendrocytes has been documented after spinal cord injury (Crowe et al., 1997), it has not been determined whether this results in significant cell loss, which could potentially contribute to eventual demyelination. To address this question, we performed thoracic spinal cord contusion lesions on rats and assessed the changes in the number of oligodendrocytes in regions rostral and caudal to the lesion center at 5, 8, 21, and 42 days after the injury. The oligodendrocytes were identified by CC1/APC immunoreactivity (Bhat et al., 1996; Crowe et al., 1997; Rosenberg et al., 1999). At the 5th day post-injury, there was a small reduction in the number of oligodendrocytes in the dorsal columns, but by the 8th day post-injury, the extent of reduction reached 30 - 50% throughout the four sampled regions (Fig. 1). The greatest reduction, which was approximately 50%, was found in the dorsal columns rostral to the injury center. Previously, the number of apoptotic cells was found to be highest in this region (Crowe et al., 1997), indicating a spatial correlation between the number of apoptotic cells and the loss of oligodendrocytes. Temporally, the greatest loss in oligodendrocytes was found at the 8th day post-injury, which also coincides with the time when the number of apoptotic cells reached its peak (Crowe et al., 1997). This
temporal and spatial correlation between the extent of apoptosis and actual cell loss indicates that apoptosis in oligodendrocytes after spinal cord injury leads to the eventual loss of oligodendrocytes.

**Spinal cord injury-specific induction of p75 among oligodendrocytes**

It has been shown that the presence of p75 is required for the NGF-dependent death of oligodendrocytes in culture (Harrington et al., 2002). In addition, p75 expression has been observed in the white matter plaques of multiple sclerosis patients (Chang et al., 2000; Dowling et al., 1999), and near the lesion epicenter following spinal cord injury (Brandoli et al., 2001; Casha et al., 2001; Reynolds et al., 1991). We asked whether spinal cord injury induces p75 expression among oligodendrocytes, and whether its expression correlates with the observed pattern of cell loss in the dorsal columns after spinal cord injury. Contusion injuries were performed on rats, and the presence of p75 was determined at various time points after spinal cord injury by performing Western analyses using 5 mm spinal cord blocks taken from the lesion centers, as well as from regions rostral and caudal to the central samples. P75 expression was not detected in sham operated animals, but first observed at 5d post-injury, continuing to 8d post-injury (Fig. 2A). This temporal expression pattern of p75 correlates closely to the actual loss of oligodendrocytes (compare with Fig. 1). It should be noted that there was very little p75 protein detected in sham operated animals, although sensory neuron projections into the spinal cord are known to contain p75 in their terminals (Richardson and Riopelle, 1984). This may be due to a low level of p75 present in these fibers. Taniuchi et al. have reported that $^{125}$I-NGF binding in the spinal cord was less than 5% of the level found in
the periphery (Taniuchi et al., 1988). Our Western data agree with this observation, and also with a report that demonstrated low levels of p75 in the adult spinal cord, except among motor neurons during development or after axotomy (Ernfors et al., 1989).

Since p75 expression correlates with oligodendrocyte loss temporally, we tested whether oligodendrocytes express p75 upon spinal cord injury by performing immunohistochemistry. Similarly to the Western data, p75+ cells were mainly found at 8d post-injury in the dorsal funiculus, although we also observed some positive cells at 48 hrs post-injury (data not shown). These p75+ cells were positive for CC1/APC, indicating that oligodendrocytes in the dorsal funiculus expressed p75 (Fig. 2B). p75 expression was not observed in sham-operated animals, as was also the case with Western analyses (Fig. 2). Together, these results indicate that p75 expression is induced among oligodendrocytes in a manner specific to spinal cord injury, and its temporal expression profile correlates with actual loss of oligodendrocytes.

**Majority of p75+ oligodendrocytes is apoptotic**

The temporal course of p75 expression correlated with the loss of oligodendrocytes and the apoptotic profile previously reported after spinal cord injury (Crowe et al., 1997). We therefore tested whether p75+ cells were undergoing apoptosis by measuring caspase 3 activation. For this, the sections taken from 8d post-injury were processed for double immunohistochemistry for p75 and active caspase 3. In injured animals, the majority of p75+ cells was also positively stained with active, cleaved caspase 3 antibody (Fig. 3A). In sham operated animals, the number of cells positive for
active caspase 3 was much smaller than in injured animals, and no cells double positive for p75 and active caspase 3 were observed.

A spinal cord injury-mediated increase in caspase 3 activity in oligodendrocytes has been reported, but only up to 2d post-injury (Springer et al., 1999). Since the greatest loss of oligodendrocytes is observed from 5d post-injury, we asked whether caspase 3 was still activated among oligodendrocytes at 8d post-injury. If so, this would suggest that the induction of apoptosis among oligodendrocytes is continuous, rather than initiated at a given point, then ceasing. As shown in Fig. 3B, the majority of oligodendrocytes in the dorsal funiculus was positive for active caspase 3 stain, even at 8d post-injury. The number of oligodendrocytes positive for active caspase 3 was much reduced in sham operated animals. Together with the data for p75 and active caspase 3, these results suggest that p75+ oligodendrocytes undergo apoptosis continuously after spinal cord injury.

**P75 is required for the apoptosis of spinal cord oligodendrocytes in culture**

We next tested whether ligand-dependent activation of p75 is necessary for apoptosis of spinal cord oligodendrocytes. As the first step, we investigated whether the absence of p75 would render spinal cord oligodendrocytes resistant to NGF-dependent apoptosis by culturing the spinal oligodendrocytes from p75+/+ and p75/− mice. In culture, spinal cord oligodendrocytes taken from p12-14 pups expressed p75, as did their cortical counterparts (data not shown; (Harrington et al., 2002)). When NGF purified from the submaxillary gland (Harlan Bioproducts for Science) was added to p75+/+ oligodendrocytes at 100 ng/ml for 4hr to 48hr, the proportion of TUNEL+ and MBP+ cells...
increased to 24% from a basal level of 2% (Fig. 4B). Among p75<sup>+</sup> oligodendrocytes, however, for the entire period of NGF treatment, the proportion of TUNEL<sup>+</sup> and MBP<sup>+</sup> cells remained the same as for those left untreated. A representative picture is shown in Fig. 4A. These data therefore indicate that p75 is required for NGF-dependent apoptosis in spinal cord oligodendrocytes in culture.

**p75 plays a critical role for apoptosis of oligodendrocytes after spinal cord injury**

As the second step, we investigated whether p75 is required for apoptosis of oligodendrocytes in vivo by injuring the spinal cord in p75<sup>+/+</sup> and p75<sup>−/−</sup> mice. After spinal cord injury, mouse oligodendrocytes express p75 in the dorsal column, as their rat counterparts do (Fig. 5A, left panel). For spinal cord injury in mice, dorsal hemisection was chosen as our method instead of contusion, since hemisection also induces axon degeneration and subsequent oligodendrocyte death without the potential complications associated with a temporally-expanding contusion injury in the small mouse spinal cord. Two different approaches were taken to assess the role of p75 following spinal cord hemisection. One was to measure the extent of oligodendrocyte survival (Fig. 5B), and the other was to measure the extent of oligodendrocyte apoptosis (Fig. 5C) in the absence of p75. The extent of oligodendrocyte survival was first determined by estimating the density of the CC1<sup>+</sup> cells per mm<sup>3</sup>, using sections taken from 1.8 mm and 1.2 mm rostral, and 1.2 mm and 1.8 mm caudal to the injury epicenter at the 8<sup>th</sup> day following spinal cord injury (Fig. 5B). Following hemisection, the density of oligodendrocytes in the dorsal columns dropped 51% from 39,600 to 19,500 per mm<sup>3</sup> in p75<sup>+/+</sup> mice, while it
dropped 36% from 42,600 to 27,100 per mm$^3$ in p75$^{-/-}$ mice. This result indicates that the lack of p75 resulted in a 15% increase in oligodendrocyte survival.

As the CC1$^{+}$ cell counts include mainly the healthy surviving cells, it is likely that the density estimation does not fully represent the extent of protection in the absence of p75. For this reason, we examined the number of apoptotic oligodendrocytes in p75$^{+/+}$ and p75$^{-/-}$ mice at 5d post-injury. As a marker for apoptosis, we stained sections with cleaved caspase 3 in addition to CC1. A representative picture is shown in Fig. 5A (right panel). The average number of CC1$^{+}$/cleaved caspase 3$^{+}$ cells in the dorsal columns was 229 per section in p75$^{+/+}$ mice, while it was 155 in p75$^{-/-}$ mice, representing a 32% reduction in the number of apoptotic oligodendrocytes in the absence of p75 (Fig. 5C). The extent of protection assessed by cleaved caspase 3 is higher than the estimation of surviving oligodendrocytes, since the staining of cleaved caspase 3 allows detection of oligodendrocytes that are at an early stage in the apoptotic process. Together, these data indicate that p75 is one of the critical components for inducing apoptosis of oligodendrocytes following spinal cord injury.

Spinal cord injury-mediated production of Pro-NGF is responsible for the death of oligodendrocytes

What is activating p75 to elicit apoptotic programs in vivo? It has been known that NGF expression is strongly induced among astrocytes, activated microglia, and meningeal cells in the spinal cord by spinal cord injury in the rat (Krenz and Weaver, 2000; Widenfalk et al., 2001). Since neurotrophins are known to be present in pro forms in many brain tissues (Chao and Bothwell, 2002), and in the case of pro-NGF, its expression is increased in Alzheimer’s patients (Fahnestock et al., 2001), we decided to
examine the profiles of neurotrophin expression in our spinal cord injury paradigm. BDNF and NT3 were present in pro-forms as reported (Lee et al., 2001), based on the predicted size, but no significant increase was detected in the amount of pro- or mature forms of BDNF or NT3 produced after spinal cord injury (Fig. 6A). The lack of induction of BDNF was further confirmed using pro-BDNF-specific antibody, which produced similar results to those shown in Fig. 6A (data not shown). In contrast, NGF expression was strongly induced in a manner specific to spinal cord injury based on Western analyses using an anti-mature-NGF antibody (Fig. 6A). The size of induced proteins that are detected with anti-mature-NGF antibody (14 kDa, 24-32 kDa) suggests that both mature and pro-NGF are induced in rats, but the extent of induction of pro-NGF is greater than that observed with mature NGF (Fig. 6A). Interestingly, only pro-NGF and not mature NGF was induced following spinal cord injury in mice (Fig. 6A). This may be due to species differences, or it may represent the difference in the type of injury inflicted, that is, contusion lesion in rats vs hemisection in mice.

To confirm that the high-molecular weight bands are indeed pro-NGF, we utilized pro-NGF-specific antibody. Pro-NGF antibody was generated using a 51 amino acid sequence present in the pro domain of NGF (See Experimental Procedures for detail). The specificity of this pro-NGF antibody was tested both in biochemical and biological assays. In Western analyses and in immunodepletion assays, the pro-NGF antibody detects only recombinant, cleavage-resistant pro-NGF, and not recombinant mature-NGF (Fig. 6C and 6D). In a functional assay using oligodendrocytes, the antibody blocks the apoptotic action of recombinant pro-NGF, but not that of recombinant mature-NGF (Fig. 7D). These results confirm that the pro-NGF antibody specifically detects and binds pro-
NGF, but not mature-NGF. When the rat lysates containing both pro-NGF and mature NGF were subjected to immunoprecipitation using this pro-NGF-specific antibody, only high molecular-weight bands of 26-32 Kda were detected, and not the band of 14 Kda (Fig. 6B, upper panel). Similar data were obtained with mouse lysates as well (Fig. 6B, lower panel). In addition, when both the rat and mouse lysates were subjected to immunodepletion using the pro-NGF antibody, these high molecular-weight bands were no longer detected, while the undepleted samples demonstrated immunoreactive species of 26-32 Kda in Western analyses (Fig. 6B). These data therefore confirm that the high molecular-weight bands of 26-32 Kda are pro-NGF.

We next investigated whether pro-NGF present in the spinal cord extracts activates p75. Since only pro-NGF, and not mature-NGF was induced in mice after spinal cord injury, we used mouse extracts obtained after spinal cord injury as a source of pro-NGF in functional assays using p75+/+ and p75−/− mouse oligodendrocytes. When the spinal cord injury extracts were added at 0.14 µl (350 ng total protein added in 1ml media) to p75+/+ mouse oligodendrocytes for 24 hrs, the proportion of TUNEL+ and MBP+ cells reached 17%, while it remained at 4% with extracts from sham-operated mice. With p75−/− mouse oligodendrocytes, the proportion of TUNEL+ and MBP+ cells remained below 4% (Fig. 7A). This 17% value is very close to that obtained on p75+/+ mouse oligodendrocytes using 100 ng/ml (4 nM) of NGF purified from submaxillary gland (Fig. 4B). Based on estimation using purified mature-NGF as a control in Western analysis, 0.14 µl of mouse extracts contains approximately 10-20 ng of pro-NGF (0.15-0.30 nM) in pro-NGF concentration. This represents approximately a 13-26 fold increase
in the action of pro-NGF on p75 compared to mature NGF, which is comparable to that reported for smooth muscle cells (Lee et al., 2001).

We next compared the activity of pro-NGF in the spinal cord extracts to that of recombinant pro-NGF and recombinant mature-NGF in oligodendrocytes. Generation and purification of recombinant, cleavage-resistant pro-NGF and comparably purified mature-NGF were previously described (Lee et al., 2001). Within an estimated concentration range from 0.05 nM to 0.5 nM, pro-NGF in the spinal cord extracts induced apoptosis comparably to recombinant pro-NGF, and more effectively than that observed with recombinant mature-NGF (Fig. 7B). These results therefore suggest that the apoptotic factor present in the injured spinal cord acts via p75, and its dose response suggests that the activity reflects a proneurotrophin.

In order to confirm that the activity was due to pro-NGF, we pre-incubated the injured spinal cord extracts with pre-immune serum, anti-mature-NGF antibody, pro-NGF-specific antibody, or pro BDNF-specific antibody before they were added to oligodendrocytes. The specificity of pro-NGF antibody was discussed earlier. Pro-BDNF-specific antibody was generated using peptide sequences present in the pro domain of BDNF, in a manner similar to pro-NGF antibody (See Experimental Procedures for detail). In Western analyses, pro BDNF antibody detects only pro-BDNF and not mature BDNF (Fig. 6C). As a negative control, parallel cultures were treated with extracts from sham-operated mice, while cells treated with purified NGF from the submaxillary gland (Harlan Bioproducts for Science) were used as a positive control for the antibody action. The extent of apoptosis was assessed after 24 hrs and is expressed in terms of the fold increase observed in TUNEL+ and MBP+ cells over that observed with
samples treated with the individual antibody alone (Fig. 7C). The extent of apoptosis in cells treated with spinal cord injury extracts was significantly attenuated with pro-NGF-specific antibody as well as anti-mature-NGF antibody. Pre-immune serum or pro BDNF-specific antibody did not have any effect.

Pro-NGF-specific antibody was also effective in attenuating the extent of apoptosis of cells treated with NGF. NGF purified from the submaxillary gland has been reported to contain pro-NGF, which we confirmed (Fig. 7C; lower panel). The extent of apoptosis mediated by submaxillary gland derived NGF typically ranges from 5-7 fold above control in oligodendrocytes (Fig. 4; (Harrington et al., 2002)). In the presence of pro-NGF antibody, the extent of apoptosis was reduced to 2 fold above control (Fig. 7C). This result suggests that pro-NGF present in submaxillary gland preparations is largely responsible for its apoptotic action, although it comprises only 2-6% of total NGF. To directly compare the effects of mature-NGF and pro-NGF, recombinant preparations were utilized (Lee et al., 2001), where the mature-NGF preparations contained less than 2% pro-NGF. Utilizing these reagents, mature-NGF can induce apoptosis of oligodendrocytes, but less effectively than pro-NGF: 0.5 ng/ml of recombinant pro-NGF yielded 23% apoptosis, whereas 50 ng/ml recombinant mature-NGF yielded 17% apoptosis (Fig. 7D). These results, together with the observed inability of pro-NGF antisera to reduce the apoptosis induced by mature NGF (Fig 7D) suggests that although both pro-NGF and mature-NGF are each capable of inducing apoptosis, pro-NGF is more active than mature-NGF in inducing oligodendrocyte cell death. Together, these results indicate that pro-NGF present in the spinal cord injury extracts was largely responsible for inducing apoptosis in oligodendrocytes in culture. In addition, these data strongly
suggest that pro-NGF is most likely a factor responsible for inducing apoptosis in vivo after spinal cord injury.
DISCUSSION

In this report, we present data that support a physiological role of pro-NGF in p75-mediated apoptosis after spinal cord injury. The temporal induction of p75 correlates with the loss of oligodendrocytes following spinal cord injury. Consistent with these observations, in p75−/− mice after spinal cord injury, there is a reduction in the number of cleaved caspase 3+ oligodendrocytes, and an increase in the number of surviving oligodendrocytes, indicating a predominantly pro-apoptotic role for p75 activation following injury. We further demonstrated that this apoptotic action of p75 is likely to be mediated by pro-NGF in vivo by showing that pro-NGF in extracts from the injured spinal cord induces apoptosis of oligodendrocytes in culture at an affinity comparable to that exhibited by purified, recombinant, cleavage-resistant pro-NGF. Together, these data provide strong evidence for the apoptotic role of p75 and pro-NGF after injury to the spinal cord.

Pro-apoptotic role of p75 following injury in vivo

Injury-mediated induction of p75 in the CNS has been well documented in the literature (Calza et al., 1997; Koliatsos et al., 1991; Nataf et al., 1998; Park et al., 2000; Reynolds et al., 1991; Roux et al., 1999). The role of p75 under these circumstances, however, had not yet been clearly determined in vivo. The present report provides
direct evidence that p75 plays a pro-apoptotic role in oligodendrocyte cell death after spinal cord injury. Oligodendrocytes in the spinal cord express significant levels of p75 in response to spinal cord injury, and in p75\(-/\) mice, their apoptosis is attenuated, while their survival is enhanced.

The pro-apoptotic role of p75 in oligodendrocytes in vivo was further supported by our spinal cord oligodendrocyte culture data. Like their cortical counterparts, spinal cord oligodendrocytes express p75 in culture, and NGF binding to p75 leads to apoptosis in the absence of TrkA. Spinal cord oligodendrocytes do not express TrkA either in culture or in vivo (data not shown). Although it is not clear whether p75 is expressed in cortical oligodendrocytes in vivo, it has been argued that culture conditions represent a stress situation that models in vivo injury. In support of this contention, p75 was shown to activate an injury-specific JNK3 in cortical oligodendrocyte cultures (Harrington et al., 2002). Our current finding, that injury induces p75 among oligodendrocytes in the spinal cord, and that these cells also express p75 in culture and die upon binding NGF, provides in vivo evidence in support of this contention.

Although the absence of p75 was fully protective of spinal cord oligodendrocytes in culture, its effect in vivo was not complete. These observations suggest that p75 is but one contributing factor in vivo, where other factors and molecules are also involved. Likely candidates include cytokines, such as TNF\(\alpha\), and excitatory amino acids (Beattie et al., 2000; McDonald et al., 1998). The analyses of TNF receptor knockout mice suggest that TNF\(\alpha\) plays a protective role by activating NF-\(\kappa\)B pathways after spinal cord injury (Kim et al., 2001). This result differs from previous reports where TNF\(\alpha\) induced apoptosis of oligodendrocytes in culture (Hisahara et al., 1997; Ladiwala et al.,
1998; Louis et al., 1993). When TNFα was applied to dorsal columns, it failed to induce apoptosis among oligodendrocytes (Schnell et al., 1999). When applied together with sublethal doses of kainic acid, however, TNFα induced rapid, massive cell death in the spinal cord gray matter (Hermann et al., 2001). These results suggest that induction of secondary injury is likely to involve interactions among multiple pathways. In this report, we identify the p75 neurotrophin receptor as a key player in the downstream apoptotic cascade that could lead to functional loss following spinal cord injury.

In culture, p75−/− mouse oligodendrocytes were resistant to the cell killing effect of the injured spinal cord extracts. This seems surprising, since spinal cord injury induces expression of an array of potential apoptotic agents that act on receptors other than p75, as was discussed previously. A possible explanation may be that our culture conditions provide compensatory mechanisms that result in overall protection against such insults. One potential protective reagent is insulin present in our serum free media. Expression of the IGF family of factors has been reported to be induced among reactive astrocytes after spinal cord injury (Hammarberg et al., 1998; Yao et al., 1995). Administration of these factors at the time of demyelinating injuries was known to exert a protective and regenerative effect (Pulford et al., 1999; Sharma et al., 1997; Yao et al., 1995).

Selective up-regulation of NGF and pro-NGF upon injury

In brain extracts, NGF and BDNF were reported present predominantly as pro-forms (Fahnestock et al., 2001; Lee et al., 2001). In the spinal cord, NT3 also exists mainly as 30 kDa pro-NT3 (Fig. 6). Of the three neurotrophins, only NGF was induced after spinal cord injury. Comparing NGF and pro-NGF induced in rats after spinal cord
injury, the level of pro-NGF is at least equivalent to or higher than the level of mature-NGF. The significance of this preferential induction of pro-NGF as compared to mature NGF is not clear with regard to oligodendrocyte survival, as significant levels of TrkA are not expressed either before or after spinal cord injury (data not shown). In the absence of TrkA, either form of NGF should be capable of activating p75 to induce apoptosis, albeit at a different affinity (Fig. 7D). Perhaps, different population of cells express pro-NGF or mature NGF, or the secretion mechanism or the site of release for the two forms may differ (Farhadi et al., 2000). These different factors are likely to affect the accessibility of mature-NGF/pro-NGF to p75+ oligodendrocytes.

The NGF level has been shown to increase both in meningeal layer (Widenfalk et al., 2001) and also among astrocytes and activated microglia (Krenz and Weaver, 2000) after spinal cord injury. Activated microglia are often found juxtaposed to apoptotic oligodendrocytes during Wallerian degeneration (Shuman et al., 1997), suggesting that NGF produced by microglia may activate p75 expressed among adjacent oligodendrocytes. Although it is not yet known which cell types express pro-NGF after spinal cord injury, a scenario analogous to what was reported in the developing retina, where NGF secreted by microglia promoted apoptotic actions of p75 (Frade and Barde, 1998) may apply as well.

In hippocampal cultures, the release of BDNF and NT3 required stimuli such as depolarization (Farhadi et al., 2000). NGF, on the other hand, was constitutively released both in a 32 kDa precursor form as well as a 14 kDa mature form when expressed in hippocampal neurons (Mowla et al., 1999). Following injury, a 32 kDa precursor was the predominant form of NGF present in the spinal cord, at least after
hemisection in mice. Since a 32 kDa precursor can be released from the cell, at least in culture, this result suggests that proteolytic processing that cleaves pro-NGF to mature-NGF may be a critical step in determining the extent of oligodendrocyte death. In culture, pro-NGF is significantly more potent than mature-NGF in inducing apoptosis of oligodendrocytes, suggesting that inhibition of the protease activity responsible for the conversion of pro-NGF to mature-NGF can provide a potential therapeutic means following spinal cord injury. Lee et al. (Lee et al., 2001) have identified matrix metalloproteinase (MMP) 3 and plasmin as a protease that can cleave pro-NGF and BDNF in vitro. MMP 2 and 9 expression increases following spinal cord injury (de Castro et al., 2000), however, pro-NGF was not cleaved by MMP 2 or 9 in vitro (Lee et al., 2001). It is plausible that as yet unidentified MMP’s or other proteases regulate the proteolytic processing of pro-NGF once it is released from the cell.

In conclusion, we report that pro-NGF is induced by spinal cord injury, and it is likely to play a role in inducing apoptosis in vivo by activating p75. Activation of p75 contributes to the demise of oligodendrocytes. The p75 neurotrophin receptor is therefore identified as a key player in the downstream apoptotic cascade in secondary degeneration following spinal cord injury and other CNS injuries.
REFERENCES


Widenfalk, J., Lundstromer, K., Jubran, M., Brene, S., and Olson, L. (2001). Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. Journal of Neuroscience 21, 3457-3475.


Fig. 2.1 Spinal cord injury leads to a loss of oligodendrocytes.

Contusion injury in rats leads to a loss of oligodendrocytes. Estimated total number of oligodendrocytes within the confines of the rat dorsal columns at 7 and 13 mm rostral and caudal to a contusion lesion at spinal level T10 (25 mm NYU device). Counts were made using a stereology program (see methods). An approximately 50% reduction in numbers was seen after 8 and 21 days, with significant reductions continuing to 42 days ($p < 0.05$). Similar results were obtained when measuring the density (number per mm$^3$).
Fig. 2.2 Spinal cord injury-specific induction of p75 among oligodendrocytes.

(A) Injury-specific induction of p75 in rats after contusion injury. A 5 mm spinal cord block was taken from rostral (R), caudal (C) as well as from the injury epicenter (L) at the indicated times after initial injury and processed for anti-p75 Western. The data are from a representative Western blot, and the same pattern was observed from 5 independent sets of samples. The lysates from 293 cells transfected with vector (GFP) or rat-p75 cDNA (HA-p75) were used as a negative or positive control, respectively. The same blot was reprobed for actin as a negative control. (B) Oligodendrocytes express p75 after spinal cord injury. Co-localization of CC1 and p75 in the dorsal column region 13 mm rostral to the injury epicenter, and 8 days after the lesion. The arrows point to CC1⁺/p75⁺ cells. Scale bar, 12.5 µm.
Fig. 2.3 p75<sup>+</sup> oligodendrocytes are positive for active caspase 3.

(A) Co-localization of p75<sup>+</sup> and active caspase 3 in the rostral region at 8d post-spinal cord injury. The arrows point to cells that are positive for both. (B) Co-localization of CC1<sup>+</sup> and active caspase 3 in the rostral region at 8d post-spinal cord injury. The arrows point to cells that are positive for both. Scale bar, 12.5 µm.
Fig. 2.4 Mouse oligodendrocytes fail to die in culture in the absence of p75.

(A) A representative picture of MBP+ cells counted for data in Fig. 4B. After 6d in culture, cells were treated with purified mature-NGF at 100 ng/ml. At the amounts of time indicated in Fig. 4B, cells were fixed, stained for TUNEL, then subsequently for MBP. The arrows point to TUNEL+ cells in the field. Scale bar, 20 µm. (B) Quantification of TUNEL+ MBP+ cells following purified NGF treatment at 100 ng/ml. The quantification data are from 6 independent experiments, with 80 - 120 cells counted in each experiment, for a total of 480 - 720 cells counted.
Fig. 2.5 Increased survival and decreased apoptosis of oligodendrocytes after spinal cord injury in p75<sup>−/−</sup> mice.

(A) Left: Induction of p75 among oligodendrocytes in mice after hemisection. Scale bar, 12.5 µm. Right: Representative pictures of cells stained for cleaved caspase 3 and CC1 at 5d post-injury. The arrows point to cells that are stained for both antibodies. Note the decrease in the number of CC1<sup>+</sup>/cleaved caspase 3<sup>+</sup> cells in p75<sup>−/−</sup> mice. Scale bar, 20 µm. (B) The loss of oligodendrocytes is attenuated in the dorsal funiculus of p75<sup>−/−</sup> mice after dorsal hemisection (p<0.05). The number is presented as the density of oligodendrocytes per mm<sup>3</sup>. Counts were made using a stereology program. (C) Reduction in the number of oligodendrocytes expressing active caspase 3 in p75<sup>−/−</sup> mice after dorsal hemisection (p<0.01). The number represents average cell counts in the dorsal columns per coronal section of the spinal cord.
Fig. 2.6 Spinal cord injury-specific induction of pro-NGF.

(A) High molecular weight NGF is predominantly induced after spinal cord injury. 30 µg of extracts were analyzed at each time point in Western analyses for NGF (Chemicon), BDNF (Promega), and NT3 (Promega). The extracts were prepared from rostral 5mm (rats) or 3mm (mice) blocks from the lesion center, but the pattern of NGF expression did not vary whether the sample was taken caudally or from the lesion center. The arrow indicates the position of recombinant BDNF and NT3 which were used at 250 ng as a control for antibody specificity. The star points to purified NGF being weakly recognized by anti-BDNF antibody. (B) The high molecular weight NGF is pro-NGF. 350 µg of lysates was immunoprecipitated with pro-NGF antibody, and probed with anti-mature NGF antibody (Chemicon). The bands at 26-32Kda disappear almost completely following depletion with pro-NGF antibody, indicating that these bands are pro-NGF (compare lanes dep and undep). Although not shown, mature-NGF is present in undepleted rat samples in longer exposure. (C) Specificity of pro-NGF and pro-BDNF antibodies. Western blot of 50ng of mature-NGF (Harlan Bioproducts for Science); 50ng of mature-BDNF (Promega); 50 µg of lysate from 293 cells stably expressing cleavage resistant pro-NGF; and 250ng of supernatant from 293 cells infected with cleavage-resistant adenoviral pro-BDNF. A set of pro and mature-NGF was probed with anti-pro/mature NGF antibody (Cedarlane) and pro-NGF antibody, and a set of pro and mature-BDNF was probed with anti-mature-BDNF (Santa Cruz) and anti-pro-BDNF antibody. (D) Pro-NGF antibody depletes pro-NGF, but not mature-NGF. Recombinant pro-NGF (lanes 1 and 2) or recombinant pro and mature-NGF (lanes 3 and 4) were subjected to immunodepletion using pro-NGF antibody. Note that pro-NGF antibody depletes only pro-NGF, but not mature-NGF (compare lanes 3 and 4).
(A) p75 is required for apoptosis mediated by the injured spinal cord extracts. Extracts from sham-operated or injured mouse spinal cord extracts were added to either p75+/+ or p75−/− mouse oligodendrocytes. After 24 hrs, cells were processed for TUNEL/MBP stain as described. For quantification of apoptotic cells, 150-200 cells were counted in each of 2-4 independent experiments to yield the total cell count of 300-800. (B) Pro-NGF in the injured spinal cord extracts behaves similarly to recombinant, cleavage-resistant pro-NGF in its affinity to induce apoptosis of oligodendrocytes. The concentration of pro-NGF in the extracts was estimated when added to 1ml media. After 24 hrs, cells were processed for TUNEL/MBP staining as described. For quantification of apoptotic cells, 250-350 cells were counted in each of 3 independent experiments to yield the total cell count of 750-1050. rec pNGF; recombinant, cleavage-resistant pro-NGF, SCI; spinal cord injury extracts, rec mNGF; recombinant mature-NGF. (C) Pro-NGF present in the spinal cord injury extracts is responsible for apoptosis of oligodendrocytes. Upper panel: The data are represented in terms of the fold-increase in the proportion of TUNEL+MBP+ cells compared to that with the samples treated with individual antibody. The death mediated by the spinal cord injury extract is significantly attenuated with Chemicon anti-NGF and pro-NGF antibodies, but not by pre-immune serum or pro-BDNF antibody. The cells treated with purified NGF (Harlan Bioproducts for Science) were used as a positive control. For quantification of apoptotic cells, 250-350 cells were counted in each of 3 independent experiments to yield the total cell count of 750-1050. Lower panel: Purified NGF from Harlan contains pro-NGF. (D) Functional specificity of pro-NGF antibody. Rat oligodendrocytes were treated with 50ng/ml of purified, recombinant mature-NGF or 0.5ng/ml of purified, recombinant, cleavage-resistant pro-NGF in the presence of either preimmune serum or anti-pro-NGF antibody. After 24 hrs, cells were processed for TUNEL/MBP staining as described. For quantification of apoptotic cells, 200-300 cells were counted in each of 3 independent experiments to yield the total cell count of 600-900. Note that both pro-NGF and mature NGF can induce apoptosis, but pro-NGF is at least 50 fold more active than mature-NGF.
INTRODUCTION

Neuronal loss is typically associated with trauma and degenerative or ischemic disorders of the nervous system. The common neurotrophin receptor, p75, has been implicated in such damage-induced death (1, 2), but the identity of the physiological ligand has remained unclear. ProNGF, the precursor of the neurotrophin NGF, was recently suggested as such a ligand. ProNGF was induced along with p75 after spinal cord injury (3). In addition, the recombinant, cleavage-resistant proNGF was shown to bind p75 selectively in vitro (4), and proNGF present in the injured spinal cord lysates induced apoptosis in culture (3). For proNGF to be a true pathophysiological ligand for p75, however, endogenous proNGF must be secreted, then bind and activate p75 in vivo. Furthermore, if proNGF binding to p75 is responsible for cell death after injury, disruption of the interaction between proNGF and p75 may be expected to prevent the death of cells that would otherwise undergo cell death in vivo. Here, these questions were addressed using adult corticospinal neurons (CSN), for which p75 is implicated in death after axotomy (5).

---

3 A. W. Harrington, B. Leiner, C. Bleichschmitt, J. C. Arevalo, R. Lee, K. Mörl, M. Meyer, B. L. Hempstead, S. O. Yoon*, and K. M. Giehl* *corresponding authors

MATERIALS AND METHODS

Animals and surgery.

Experimental procedures and maintenance of animals were approved by the local Animal Care Committee according to the German law regulating the experimental use of animals. Male Sprague-Dawley rats weighing 190-330 g, and p75 (6) and NGF mutant mice of both sexes at 5-9 weeks of age were used. P75 null mutants were back-crossed from a C57Bl/6 to a 129Sv genetic background for several generations until homozygous null mutants could again be obtained. NGF null mutant mice were generated by replacement of exon IV of the NGF gene by a lacZ cDNA (K. M. and M. M., unpublished). They were kept on a mixed C57Bl/6-129Sv background. The procedure and stereotaxic coordinates for internal capsule lesion, Fast Blue labeling of CSN, intracortical delivery of solutions in rats have been described (7), as well as the determination of the lesion and the cell-death areas (8).

Tissue processing.

At the times indicated, animals were sacrificed by an overdose of sodium-pentobarbital and chloral hydrate and transcardially perfused with PBS followed by 4% paraformaldehyde. The brains were processed as described (8). For biochemical
analyses, sensory motor cortices were quickly removed, frozen on dry ice and stored at -80°C until further processing. For preparation of tissue lysates, the tissues were thawed in the lysis buffer on ice, before being homogenized with Dounce homogenizer.

**Application of the antibodies to lesioned CSN.**

Alzet 2001 osmotic mini-pumps were used to deliver either 20 mM PBS, NGF-neutralizing mouse monoclonal NGF-antibody, Mab 27/21 (0.5 mg/ml in PBS) (9), rabbit polyclonal proNGF-neutralizing antiserum (anti-proNGF, 1:20 in PBS) (3), protein A column purified IgG fraction of anti proNGF (anti-proNGF-IgG, 2.4 mg/ml), rabbit serum (1:20 in PBS), protein A column purified IgG from rabbit serum (2.4 mg/ml), or mouse IgG (mouse IgG, 0.5 mg/ml in PBS) at a rate of 1 ml/h. All solutions contained 50 U/ml Penicillin/Streptomycin and were continuously infused over the indicated periods.

**Analysis of cell survival.**

The number of surviving CSN was assessed by blinded cell-counts of every 2nd section collected for cell-counts (i.e. every 4th section of the mice and every 10th section of the rat brains) (8). The criterion for a CSN was a Tracer-filled pyramidal-shaped profile larger than 4 µm (rats) or 3 mm (mice) in diameter (8). For the quantitative survival data, only the data from the "cell death areas" (8) were used. Within the cell death-area, percent survival is defined as "number of Fast Blue-labeled CSN on the lesion side / number of Fast Blue-labeled CSN in contralateral to the lesion side x 100%". The total number of cells counted was over 280,000 in rats and over 120,000 in mice. For
statistical analyses, one-way analysis of variance followed by a post hoc Newman-Keuls test was used.

**Analysis of CSF.**

Animals were anesthetized as described (8) and the dorsal aspect of the atlanto-occipital membrane was exposed to have access to the cerebello-medullar cisterna. A glass micropipette connected via teflon tubing to a 200 ml Hamilton syringe was used to puncture the cerebello-medullar cisterna, and the initial 100 ml of the aspirated CSF was collected for Western analyses. Ten ml of CSF was used for Western analyses, and 30-50 ml CSF was used for immunoprecipitation reactions. CSF-samples did not contain blood contamination.

**Immunoprecipitation/Western Analyses.**

Cortical tissues were homogenized according to Kim et al. (10). Briefly, tissues were homogenized in a lysis buffer containing 1% Nonidet P-40, 20mM Tirs, pH 8.0, 137mM NaCl, 0.5mM EDA, 10% glycerol, 10mM Na₂P₂O₇, 10mM NaF, 1mg/ml aprotinin, 10mg/ml leupeptin, 1mM vanadate, and 1mM phenylmethylsulfonyl fluoride. The procedures for immunodepletion and immunoprecipitation were as described (3). For NGF Western analyses, 27/21 mouse monoclonal (Chemicon), rabbit polyclonal H-20 and M-20 (Santa Cruz) were used.

**Oligodendrocyte culture and TUNEL assay.**

For quantification of apoptotic rat oligodendrocytes, cells were fixed after overnight
treatment with CSF from the lesioned or the control rats. The fixed cells were processed for TUNEL and MBP immunostained as described (3).

**Vascular smooth muscle culture and TUNEL assay.**

P75-expressing mouse vascular smooth muscle cells (11) were plated at 10,000/well in media containing 0.1% serum, and the temperature was shifted to 39.5°C to induce differentiation. Purified, recombinant cleavage-resistant proNGF (4), or diluent controls were added to cultures, in the presence or absence of protein A column purified IgG from rabbit polyclonal, anti-prodomain antibody, or comparably protein A column purified IgG from non-immune rabbit serum. Following 14h incubation, cells were fixed and processed for TUNEL reaction, and counterstained with DAPI. 5 fields were analyzed in each of duplicate wells, in a blinded manner, and experiments were performed twice.
RESULTS

P75 is induced by the lesion, and required for the death of lesioned CSN.

Differentiation and survival of corticospinal neurons (CSN) are largely governed by neurotrophins. During embryonic development, NT3 promotes differentiation of cortical precursors into BDNF-dependent neurons in culture (12, 13). Once fully differentiated, these neurons adopt the adult phenotype and lose these trophic requirements (5, 14). Following axotomy at internal capsule levels, the survival of adult CSN is again regulated by endogenous neurotrophins, with BDNF supporting survival, while NT3 promotes death of BDNF-dependent CSN (5, 14). NT3, however, appears to act via an indirect mechanism and not by activating its own death program. When NT3 action was neutralized, CSN no longer depended on BDNF for survival, suggesting that NT3 is required for CSN to become dependent on BDNF after the lesion.

In line with their responses to BDNF and NT3, CSN express TrkB and TrkC, but not TrkA (5, 7). Of the neurotrophin receptors, p75 is the only receptor whose mRNA expression is dramatically induced in lesioned CSN, while Trk receptor expression remains unchanged (5, 14). In agreement with previous in situ hybridization data (5), p75 protein was undetectable in the unlesioned cortex, but beginning 1 day after internal
capsule lesion, it was present in the lesioned cortex and reached highest levels by 3d post-lesion (Fig. 1A). This peak coincides with the peak of cell death of axotomized CSN (Fig. 1B). The p75 protein levels decreased by 14d post-lesion, the time when CSN no longer underwent cell death (Fig. 1A, B). In rats, infusion of p75-blocking antibody during the first week post-lesion prevented axotomy-induced death of CSN, suggesting that neurotrophin binding to p75 plays a role in inducing death among the lesioned CSN (5). We therefore investigated whether genetic depletion of p75 would similarly protect CSN from axotomy-induced death.

In p75 null mutant mice, death of CSN was completely circumvented, while the survival of CSN in the wild type controls remained at 68% (Fig. C, D). Together, these data demonstrate that CSN upregulate p75 expression after internal capsule lesion, and that p75 is a critical factor for the death of lesioned CSN.

**NGF gene product is required for the death of lesioned CSN.**

We next asked whether NGF contributes to the activation of p75 following axotomy, since the effects of BDNF and NT3 on CSN-survival are mediated primarily by TrkB/C (5). Continuous intracortical infusion of an NGF-neutralizing antibody, Mab 27/21, for the entire 7d post-lesion period resulted in 92% of adult rat CSN surviving after the lesion, while vehicle or mouse IgG infusion resulted in only 61-66% survival (Fig. 2A, B). This result suggests that an NGF gene product is involved in inducing death of CSN. As predicted from Mab 27/21 infusion data, 87% of CSN survived axotomy in
NGF heterozygous mice, while the survival remained at 60% in the wild type mice (Fig. 2C). These data together implicate an NGF gene product as a death-inducing ligand for the lesioned CSN.

**ProNGF is induced by CSN lesion and secreted in an active form**

It has been shown that the p75 level increased among oligodendrocytes after spinal cord injury (3). In the same study, it was suggested that activation of p75 among oligodendrocytes is likely to be mediated by proNGF in vivo: ProNGF expression was induced by the injury, and proNGF derived from the injured spinal cord lysates induced apoptosis of cultured oligodendrocytes in a p75-dependent manner. Although these results strongly suggest that proNGF was responsible (3), a question still remained as to whether the apoptotic action of proNGF in the lysates was due to proNGF that was secreted outside the cell, or proNGF that was synthesized and remained inside the cell. Activation of p75 has been predominantly mediated by soluble ligands that bind p75 on the cell surface. ProNGF was previously shown to be secreted only in vitro (15).

To assess whether proNGF is produced and secreted in vivo, we collected cerebrospinal fluid (CSF) after the lesion, since CSF is known to contain most molecules secreted from brain parenchyma (16). A 26-28 kDa product, immunoreactive for NGF, was detected from CSF at 3d post-lesion using two different NGF antibodies (Fig. 3A). The size of the NGF-reactive band indicates that the product is proNGF as we have observed after spinal cord injury (3). To test whether the immunoreactive band is indeed proNGF, we subjected the CSF samples to immunoprecipitation with the polyclonal, proNGF-specific antibody, and then the precipitated proteins were probed in Western
analyses using the monoclonal NGF antibody. A substantial amount of proNGF was detected at 3d post-lesion, but very little in the control CSF (Fig. 3B, left panel). The resulting supernatant from the immunoprecipitation reaction with proNGF antibody did not contain the 26-28 kDa band, indicating that the 26-28 kDa band on NGF Westerns is proNGF (Fig. 3B, right panel). When both CSF samples from unlesioned and lesioned animals were added to primary oligodendrocytes, only the 3d lesion CSF induced apoptosis, and not the non-lesion CSF (Fig. 3C). The apoptotic activity of the 3d lesion CSF was blocked by proNGF antibody, suggesting that the proNGF present in CSF is responsible for inducing the death of cultured oligodendrocytes. We therefore conclude that active, functional proNGF is induced and secreted into the CSF after cortical axotomy.

**ProNGF binds p75 in vivo, and inhibition of proNGF binding to p75 in vivo rescues CSN**

Since proNGF is secreted in a functional form, we next asked whether proNGF binds p75 in vivo. For this, cortical lysates were subjected to immunoprecipitation with p75 antibody, and the presence or absence of proNGF was assessed using the monoclonal NGF antibody in Western analyses. ProNGF was present in p75 immune complexes from the lysates of lesioned cortices, but not in control immunoprecipitates or in non-lesion samples (Fig. 4A). These data suggest that proNGF binds p75 in vivo after cortical axotomy.

We then investigated whether disrupting the binding between p75 and proNGF could result in a rescue of the lesioned CSN. To disrupt the binding between p75 and
proNGF, we chose to infuse the proNGF antibody intracortically at the time of lesion. This antibody, derived from the pro-domain of NGF, was previously shown to be proNGF-specific, and not to deplete mature NGF (3). In addition, the proNGF antibody was able to attenuate in a dose-dependent manner the proNGF mediated apoptosis in p75+ vascular smooth muscle cells, while comparable concentration of control IgG was not (Fig. 4B). This bioassay was utilized because this cell line exhibits highly reproducible and dose-dependent responsivity to proNGF (4, 17).

To test whether the infused proNGF antibody disrupts the binding between p75 and proNGF, we subjected the cortical lysates from rats that had been infused with proNGF antibody or the control IgG in immunoprecipitation reactions. For immunoprecipitation, we used anti-p75 or control IgG, which was followed by Western with NGF antibody. Little proNGF was immunoprecipitated with the control IgG in the lesion lysates, (Fig. 4C, lanes 5 and 6), or with the IgG or p75 antibody in the non-lesion lysates (Fig. 4C, lanes 1-4). In the lysates from lesioned rats that had been infused with IgG, a significant amount of proNGF was found in complex with p75 after immunoprecipitation with p75 antibody at 3d post-lesion, (Fig. 4C, lane 7). In contrast, in the lysates from the lesioned rats that had been infused with proNGF antibody, the amount of proNGF that immunoprecipitated with p75 was greatly reduced (Fig. 4C, compare lanes 7 and 8). These data suggest that infusion of proNGF antibody was effective in attenuating the proNGF binding to p75 after the lesion.

We then asked whether infusion of the proNGF antibody could rescue CSN from axotomy-induced death. Infusion with vehicle, preimmune serum, or control IgG
resulted in 61-66% of CSN surviving, while proNGF serum (92% survival) or the purified IgG fraction of proNGF antibody (100% survival) prevented axotomy induced death of CSN (Fig. 4D). Infusion of proNGF antibody at the same dose resulted in significant attenuation of proNGF binding to p75 (Fig. 4C).

Therefore, these results together indicate that proNGF binding to p75 is responsible for initiating a death-inducing signal *in vivo.*
DISCUSSION

In this report, we present data establishing that proNGF is the physiological ligand for p75 under pathological conditions. ProNGF was induced after axotomy on a time course similar to those of p75 and the subsequent death of CSN. More importantly, endogenous proNGF was secreted in a functional form that is capable of binding and activating p75, thereby inducing neuronal death in vivo. Consistent with this conclusion, disruption of the binding between endogenous proNGF and p75 resulted in complete rescue of the lesioned CSN. These data establish proNGF as a bona fide ligand for p75.

We have previously shown that endogenous NT3 also promotes the death of BDNF-dependent CSN; blocking NT3 binding to TrkC with TC89 antibody rescued lesioned CSN (5). These data and the data presented in this report suggest that the death of lesioned CSN can result from two different mechanisms. One is to induce death by activating a pro-apoptotic receptor, p75; and the other is to create a dependency on a survival factor that is present in a limiting amount, BDNF. Indeed, lesioned CSN do not require endogenous BDNF for survival if NT3 is simultaneously neutralized (5), indicating that NT3 signaling via TrkC is required to maintain or to cause the initial dependency of CSN on BDNF for survival. The role of NT3 here resembles its role during development, where NT3 promotes neuronal differentiation of cortical progenitors.
and their dependency on BDNF as they mature in culture (12, 13).

We have also reported that blockade of p75 with Rex antibody rescued the survival of lesioned CSN (5). We concluded at the time that Rex antibody was most likely interfering with p75 binding to NT3 rather than NGF (5), since infusion of sheep anti-NGF-serum did not produce a dramatic rescue of lesioned CSN (unpublished data). The lack of dramatic effect using sheep anti-NGF serum might have been due to a selective interaction of this serum with mature NGF or its insufficient neutralization of endogenous proNGF in vivo. Here, we clearly demonstrated that a NGF gene product is the responsible ligand activating p75: lesion induced death of CSN was significantly attenuated not only in NGF heterozygotes, but also after infusion with Mab 27/21. Mab 27/21 is a better antibody for blocking NGF binding to p75 in vivo (18). We would also like to emphasize that our biochemical data compliment our in vivo functional blocking experiments: ProNGF is selectively induced by the lesion, and interference in proNGF binding to p75 resulted in rescue of the lesioned CSN.

We demonstrated that proNGF is the predominant form of NGF present in the CSF. To our surprise, proNGF remained in an active state for an extended period of time (our unpublished data). Since secreted proNGF can potentially target p75+ cells at a distance after CNS injury, the extent of cell death may increase over time, affecting regions of the brain that were not initially damaged. P75 is expressed in the adult among Purkinje neurons in the cerebellum, and among cholinergic neurons of the basal forebrain, a neuronal population damaged in Alzheimer’s disease. Since proNGF can induce apoptosis of cultured SCG neurons (4) that express TrkA, such as cholinergic
neurons, the secondary damage can potentially have a pervasive impact. Matrix metalloproteinase 3 and plasmin were identified as the proteinases that can cleave proNGF in vitro (4), but it is not known whether the same proteinases cleave proNGF in vivo. It should be noted that the proNGF level has been shown to increase in Alzheimer’s disease (19), while the plasmin level was reduced in Alzheimer’s brain (20). Identification of proteinases that can cleave proNGF in vivo could aid the development of a therapeutic strategy to inactivate the apoptotic proNGF.

In this study, intracortical infusion of proNGF-specific antibody resulted in complete rescue of the lesioned CSN, due to the antibody interfering with the binding of proNGF to p75. These data reveal a new potential target for pharmacological intervention in diseases in which neuronal death is a pathogenetic factor. Once the specific binding sequences are identified, drugs may be designed to specifically impair the interaction between p75 and proNGF in order to prevent or reduce the rate of cell death in such diseases. Alternatively, identification of critical downstream players that mediate p75/proNGF signals would provide additional routes for potential therapeutic intervention.
REFERENCES


Beattie, M. S., Harrington, A. W., Lee, R., Kim, J. Y., Boyce, S. L., Longo, F. M.


Giehl, K. M., Rohrig, S., Bonatz, H., Gutjahr, M., Leiner, B., Bartke, I., Yan, Q.,
Reichardt, L. F., Backus, C., Welcher, A. A., Dethleffsen, K., Mestres, P. &

Lee, K. F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V. & Jaenisch, R.


Fig. 3.1  P75 is induced by cortical axotomy and is required for axotomy-induced death of corticospinal neurons.

(A) p75 is induced in the sensory motor cortex following axotomy. Thirty µg of cortical lysates were analyzed for each time point on Western analyses with anti-p75 antibody. (B) CSN undergo cell death after internal capsule lesion, in a temporally coordinated manner to p75 induction. Part of the time points was already published(7). (C) and (D) Axotomy-induced death of CSN is prevented in p75−/− animals. (C) Fast Blue labeled CSN in sections of representative animals. Size bar = 0.5 mm. (D) Quantification of CSN survival. Mean ± s.e.m.
Fig. 3.2 A NGF gene product is required for the induction of cell death of axotomized CSN. (a) and (b) Treatment with the 27/21 monoclonal NGF antibody prevents axotomy-induced death of CSN. (a) Fast Blue labeled CSN in representative sections of a control animal (PBS) and an antibody-treated animal. Size bar = 1 mm. (b) Quantification of survival after Mab 27/21 and control infusions. Mean ± s.e.m. (c) Death of axotomized CSN in NGF heterozygous mice is ameliorated. Mean ± s.e.m.
Fig. 3.3 ProNGF is induced after internal capsule lesion, and released as a biologically active form into CSF.

ProNGF is secreted into CSF. Left panel: Each lane in Western represents 1/10 of the total 100 µl of CSF collected from individual animals that were either unlesioned (cntr.) or received an internal capsule lesion 3 days prior to the analysis (3d). Note that two polyclonal NGF antibodies, SC H-20 and M-20, do not detect a 26-28kDa band in unlesioned CSF samples. The band marked by * is a non-specific band. (b) The proNGF antibody immunoprecipitates the 26-28kDa band from the lesioned CSF sample (undep). The resulting supernatant (dep) lacks the band, suggesting that the 26-28kDa band is proNGF. (c) ProNGF secreted to CSF is active in inducing death in culture. 0.2 µl of CSF was added to oligodendrocyte cultures for 16-18 hrs, and processed for TUNEL and MBP double staining. For quantification, 450-550 cells were counted in duplicates for a total of 900-1,100 cells counted. 0.2 µl of CSF from lesioned animals contains approximately 10 ng of proNGF, which yields 0.35 nM in estimated concentration in 1 ml media. Note that the control CSF does not induce apoptosis of oligodendrocytes, suggesting that the NGF immunoreactive band detected in direct NGF Western is not a functional proNGF.
Fig. 3.4 ProNGF binds p75 in vivo and its binding to p75 is responsible for axotomy-induced death of CSN in vivo.

(a) ProNGF binds p75 in vivo. Cortical lysates (500 µg) from unlesioned (cntr.) or 3d post-lesioned animals were subjected to immunoprecipitation using anti-p75 antibody or the control anti-IgG, followed by Western analyses with mNGF antibody. Actin Western is also shown as a loading control. (b) ProNGF antibody rescues the death of vascular smooth muscle cells in a dose-dependent manner. Purified, recombinant proNGF, or diluent controls were added at 2 or 10 ng/ml to cultures, in the presence or absence of IgG purified from rabbit anti-prodomain antibody, or comparably purified non-immune rabbit IgG. The amount antibodies were 40 or 100 µg/ml. Following 14h incubation, cells were fixed and processed for TUNEL reaction, and counterstained with DAPI. Five independent fields were analyzed in each of duplicate wells, in a blinded manner, and experiments were performed twice. (c) The proNGF antibody disrupts the binding between proNGF and p75. Cortical lysates (500 µg) from proNGF antibody treated or the control antibody treated animals were subjected to immunoprecipitation with the control IgG or p75 antibodies. The resulting immunoprecipitates were analyzed in Western analyses with mNGF antibody. Note that there is a significant reduction in the amount of proNGF immunoprecipitated with p75 after proNGF antibody infusion (compare lanes 7 and 8). (d) Infusion of proNGF antibody at the same concentration rescues axotomized CSN. Upper panel: Photographs of Fast Blue labeled CSN from representative animals of the indicated experimental groups. Size bar = 1 mm. Lower panel: Quantification of CSN survival. Mean ± s.e.m.
CHAPTER 4

INTRODUCTION

CNS axons do not regenerate, partly due to the actions of myelin inhibitory molecules, such as Nogo (Chen et al., 2000), MAG (Mukhopadhyay et al., 1994), and OMP (Wang et al., 2002b). When the Nogo receptor (NgR) was cloned using the Nogo-66 domain, which is exposed on the surface of oligodendrocytes (GrandPre et al., 2000), it was evident that NgR would require a coreceptor to signal, since NgR is tethered to the membrane via GPI-linkage (Fournier et al., 2001). P75 soon emerged as the co-receptor for NgR (Wang et al., 2002a; Wong et al., 2002), after it was shown that MAG-mediated RhoA activation was lost in p75−/− cerebellar granule neurons (CGN (Yamashita et al., 2002)). Since then, NgR was shown to bind MAG (Domeniconi et al., 2002) and OMP (Wang et al., 2002a), which all activate RhoA in a p75-dependent manner.

RhoA activation by p75-NgR was shown to involve a RhoGDI (Yamashita and Tohyama, 2003). RhoA is recruited to p75 via the RhoGDI that binds p75 directly, and the recruited RhoA is then released and activated by unknown means. Since p75 does not contain a GEF domain, the mechanism by which the recruited RhoA becomes activated has remained undetermined. Here, we present data indicating that Kalirin9, a dual Rho
GEF, is one of the key components of the signaling unit for RhoA activation by myelin inhibitory molecules both in vitro and in vivo.

Kalirins are mammalian orthologs of Trio in Drosophila and Unc 73, which were shown to play a critical role in axon guidance in Drosophila and C. elegans (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000). In the mammalian nervous system, several Kalirin isoforms are known to date, including Kalirin5, 7, 8, 9 and 12 (Hansel et al., 2001). These isoforms all contain Rac GEF, but only Kalirin9 and 12 additionally contain Rho GEF, like Trio. Kalirin12 contains a kinase-like domain in addition to Rac and RhoA GEF domains, but its kinase activity has not yet been characterized. During development, Kalirin9 promotes axon outgrowth (Penzes et al., 2001), and is the predominant form expressed (Hansel et al., 2001). As its role in axon outgrowth suggests, Kalirin9 is localized to growth cones and neurites in neuronal cultures (Penzes et al., 2001).

Although Trio dual Rho GEF’s is shown to play critical roles in axon outgrowth, the extracellular factors or receptors that regulate Kalirins’ actions are not known. Recently, it was reported that EphrinB and its receptor, EphB2, regulates Kalirin7-dependent Rac activation at synaptic sites (Penzes et al., 2003), but Kalirin7 is localized to post-synaptic compartments, unlike the other members of the family. Here, we present evidence that myelin inhibitory molecules serve as the ligands that induce Kalirin9 to activate RhoA. Inhibition of RhoA activation by endogenous Kalirin9 allows neurons to send out long processes on myelin inhibitory substrates. In addition, the activated NgR regulates binding of Kalirin9 to p75.
MATERIALS AND METHODS

Recombinant retro virus and adenovirus Constructs

*pLIA-GEF1\(\Delta\)-Kalirin9*: First, the EcoR I site in pLIA was changed to Asc I-Sfi I-Pme I sites, the Nco I site in pEAK- myc-GEF1\(\Delta\)-Kalirin9 was changed to Nco I-Asc I-Sac II-Nco I, and the Not I site in pEAK-myc-GEF1\(\Delta\)-Kalirin9 was changed to Pme I by inserting the respective linkers at each site. pEAK-myc-GEF1\(\Delta\)-Kalirin9 is identical to pEAK-mycKalirin9 (May et al., 2002), except that it lacks the entire GEF1, the putative Rac GEF domain. The myc-GEF1\(\Delta\)-Kalirin9 fragment was prepared by digesting with Asc I/Pme I, and inserted into Asc I/Pme I sites in pLIA.

*pLIA-GEF2\(\Delta\)-Kalirin9*: The GEF2, the putative RhoA GEF domain in Kalirin9, was deleted using a hybrid PCR strategy (Zhong and Bajaj, 1993). The forward primer used for deletion was 5’

GCCAAGGCTCTCTAAAGAGGCAGGATGTGTGTGATTGAGCTGGATGCTGGCATG

and the reverse primer was 5’

CATGCCAGCATCCAGCTCAATCACAACCATCCTGCCCTCTTAGAGCCTTGGC.

The PCR product was cloned into the pCR II vector, and sequenced for its entire length for any errors. The resulting product reads GRMF:viel at the deletion junction, where the capital letter denotes the amino acid residues before the GEF2 and the small capital letter denotes the residues after the GEF2 domain. The pCR II-GEF2\(\Delta\) was digested with Pac I
and Bsa BI and the resulting fragment was placed into pEAK-mycKalirin9 that was digested with the same enzyme to generate pEAK-myc-GEF2Δ-Kalirin9. To make pLIA-GEF2Δ-Kalirin9, the pEAK-myc-GEF2Δ-Kalirin9 was digested with AgeI and Bsa BI and the resulting fragment was placed into pLIA-GEF1Δ-Kalirin9 that was digested with the same enzyme.

**Generation of myc-GEF1Δ-Kalirin9 transgenic construct under a human p75 promoter**

*pMG2-p75*: In order to introduce 4Kb p75 promoter fragment within the exon 3 of the β-globin gene in pMG2, we exchanged the restriction sites Sac I to Xho I, Bam HI to Sac II, and Eco RI to Sal I-Pac I. Eco RI site is within the the exon 3 of the β-globin gene. The 4Kb p75 promoter fragment was isolated from pBRB511H3 (Huber and Chao, 1995) by Sal I and Sac II digestion. The digested fragment was ligated into pMG2 that was digested with Xho I and Sac II to remove the 1.9Kb MBP promoter fragment. The resulting construct, pMG2-p75, now contains the exon 2, intron 2, and exon 3 of the human β-globin gene (Gow et al., 1992).

*pMG2-p75- myc-GEF1Δ-Kalirin9-IRES-EGFP*: We first placed the myc-GEF1Δ-Kalirin9 into the pIRES-EGFP2 (Clonetech). The myc-GEF1Δ-Kalirin9 with restriction sites Asc I-Sac II-Nco I at the 5’ end and Pme I at the 3’ end was digested with Sac II and Pme I and placed into pIRES-EGFP2 vector that was digested with Sac II and Sma I. Prior to inserting myc-GEF1Δ-Kalirin9, the Not I site at the pIRES-EGFP2 vector was mutated to Pac I. The entire myc-GEF1Δ-Kalirin9-IRES-EGFP fragment was then isolated by Sal I and Pac I digestion, and placed into the Sal I-Pac I site at the pMG2-p75.
The order of the fragments in the final construct is 4Kb human p75 promoter-exon 2-intron 2, and the myc-GEF1Δ-Kalirin9-IRES-EGFP within the exon 3 of the β-globin gene. The final product was digested with Not I and used to generate transgenic mice at the Keck Genetics Research Facility at the Center for Molecular Neurobiology at the Ohio State University, using previously described methods (Hogan et al., 1994).

**Primary neuronal cultures**

For cerebellar granule neuron (CGN) cultures, cerebella were dissected from P5-6 neonatal rat pups, and plated in 10% FBS in DMEM as mixed cultures on laminin coated dishes. Next day, the cultures were infected with the myc-GEF1Δ-Kalirin9, myc-GEF2Δ-Kalirin9, or the control retrovirus in the presence of polybrene for 2hrs, and maintained in Neurobasal A media supplemented with B27.

**GST pulldown assays**

GST and GST-p75 were produced from bacteria expressing pGEX vectors. Cell lysates from 293T cells transfected with Kalirin9 or Kalirin9 that was synthesized in the presence of 35S-methionine using in vitro transcription/translation system (Promega) were incubated with glutathione sepharose beads bound with either GST or GST-p75. The samples were rotated overnight at 4 °C. The beads were then washed three times using 0.5 ml of lysis buffer. Pulled down proteins were detected by western blot or after fluorography.

**Immunohistochemistry**

Sagittal frozen rat brain sections (20 μm) were stained for pan-Kalirin and p75 antibody (Covance), and the immunoreactive signals were visualized using anti-rabbit-Cy3 (Sigma) for p75, and streptavidin-Alexa 488 (Molecular Probes) as described (Kim et al.,
2003). For spinal cord samples, the mice were perfused at 2-3 dpi, the 10mm spinal cord bloc was removed, cut in horizontal plane at 20 µm thickness, and processed for immunohistochemistry with GFP (Molecular Probes), p75, and/or myc antibody.

**Measurement of Rho-GTP:**

To measure Rho-GTP, the CGN cultures infected with the control or the DN-kalirin9 retrovirus were treated for 15min with 0.3 µg/ml of conjugated MAG-Fc (Sigma), 8 µg/ml conjugated AP-Nogo-66, 10 µg/ml soluble myelin, 1 µg/ml His-OMgP, or 30 µg/ml LPA (Sigma) for 3min. The cells were lysed in a buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 1 mM MgCl$_2$, 0.5 mM EDTA, 10 mM Na pyrophosphate, 1% NP40, 10mM Na$_2$P$_2$O$_7$, 10mM NaF, 1µg/ml aprotinin, 10µg/ml leupeptin, 1mM vanadate, 1mM phenylmethylfulfonyl fluoride, and 10% glycerol. After clearing by 13,000 g centrifugation, 90% of the cleared lysates was incubated for 45 minutes at 4°C with 30µl of Rhotekin coupled beads (Upstate Biotech). The beads were then washed three times in the same buffer. The remaining 10% of the cleared lysates was saved for protein loading controls. RhoA activity assay from 293T transfected cells was performed in an identical manner.

**Immunoprecipitation/Western Analyses**

The procedures for immunoprecipitation and Western analyses were identical to what was described (Yoon et al., 1997). The antibodies used in these assays include p75 (Covance), Nogo receptor (Alpha Diagnostics International), myc (Santa Cruz), pan-Kalirin antibody (Penzes et al., 2001), flag (Sigma), Rho, myc (Santa Cruz), RhoGDI
(Sigma), Alkaline phosphatase (Zymed), GFP (Molecular Probes), phospho-p38 (Cell Signaling).

**Lipid raft fractionation**

Lipid rafts were isolated using Optiprep (Accurate Chemical and Scientific). The spinal cord tissues were homogenized using Dounce B pestle in 1X HB buffer, which contains 250mM sucrose, 20mM Tricine-NaOH, pH 7.8, 1mM EDTA, and 2mM MgCl₂ (Wu et al., 2001). The nuclei were removed by 10min centrifugation at 2000rpm, and the supernatant was adjusted to 40% Optiprep in 1X HB buffer and loaded into a 5ml discontinuous Optiprep gradient of 10%, 30% and 40%. The sample was centrifuged for 16hrs at 150,000g and the fractions were collected. 10% of each fraction was analyzed in Western analyses with the indicated antibodies.

**Spinal cord injury and processing of the cord**

Mice were anesthetized with ketamin/xylazine and subjected to dorsal hemisection at the thoracic 9-10 position using mini-Vannas scissors. The control laminectomized mice underwent the exact procedure, except for the hemisection. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee and followed the NIH guidelines for the proper use and care of laboratory animals. At the indicated time, six mm the spinal cord tissue block was quickly dissected and frozen in liquid N₂. The spinal cords were homogenized in the lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 10 mM Na pyrophosphate, 1% NP40, 10mM Na₂P₂O₇, 10mM NaF, 1μg/ml aprotinin, 10μg/ml leupeptin, 1mM vanadate, 1mM phenylmethylfulfonyl fluoride, and 10% glycerol. The resulting lysates were used for Rho-GTP assays as well as Western analyses as described above.
RESULTS

Direct binding and coexpression of Kalirin9 with p75

P75 has been shown to regulate Rac and RhoA activity (Harrington et al., 2002; Yamashita et al., 1999), but whether p75 activates or inhibits these GTPases depends on which receptor p75 has associated with. In an effort to decipher the complex mechanisms by which p75 regulates Rac and RhoA, we searched the literature for a putative guanidine exchange factor (GEF) that modulates both Rac and RhoA activity, and whose expression pattern coincides with that of p75. We found that the Kalirin family fits both criteria. There are several Kalirin isoforms present in the mammalian nervous system known to date, including Kalirin5, 7, 8, 9 and 12, ranging in size from 180kDa to 400kDa (Hansel et al., 2001). These isoforms all contain Rac GEF, but only Kalirin9 and 12 contain RhoA GEF in addition to Rac GEF. Although Kalirin12 needs further characterization, Kalirin9 is known to be the predominant form expressed during development, while Kalirin7 is the major Kalirin isoform present in the adult (Hansel et al., 2001). When overexpressed, Kalirin9 promotes axon outgrowth (Penzes et al., 2001). As its role in axon outgrowth suggests, Kalirin9 is in fact localized to growth cones and neurites in cortical neuron cultures (Penzes et al., 2001). We therefore asked whether Kalirin9 plays a role in p75 signaling.
Immunohistochemical analyses of postnatal day 5 rat cerebellum preparations with p75 and pan-Kalirin antibodies revealed that Kalirin is coexpressed with p75 mainly among developing Purkinje (Figure 1A), and some granule neurons (data not shown). Kalirin9 is the major isoform present at postnatal day 5 in the rat cerebellum, based on Western analyses (Fig. 1A, lower panel). We next tested whether Kalirin9 binds p75 by incubating GST or GST-p75 intracellular domain (GST-p75ICD) with the 293T lysates that contained the full-length, myc-tagged Kalirin 9 or the control vector in pulldown assays. The presence of Kalirin 9 in the pulled fractions was detected in Western analyses using myc antibody. Kalirin9 was pulled down with the GST-p75ICD but not with the GST (Figure 1B), suggesting that Kalirin9 binds the cytoplasmic domain of p75 in vitro. Using the full-length Kalirin9 synthesized in vitro, we also determined that Kalirin9 binding to the p75 cytoplasmic domain is direct, requiring no intermediate molecule (data not shown).

We next investigated whether the full-length p75 could associate with Kalirin 9 using 293T cells, which expressed p75 and Kalirin9 transiently. Immunoprecipitation with a p75 antibody brought down Kalirin9 from the immune complex (Fig. 1C), suggesting that Kalirin9 binds the full-length p75 in 293T cells. Kalirin9 was not detected in the p75 immune complexes from the control lysates expressing p75 or Kalirin9 alone. These data together suggest that Kalirin9 has the potential to mediate Rac and RhoA regulation by p75.
NgR regulates Kalirin9 binding to p75

Yamashita and Tohyama (Yamashita and Tohyama, 2003) have recently reported that RhoA GDP dissociation inhibitor (RhoGDI)-RhoA complex binds p75-NgR, and upon binding, RhoGDI releases Rho, which results in RhoA activation. Since p75 does not contain a GEF domain, the mechanism by which the released RhoA is activated remained undetermined. We hypothesized that Rho GEF-containing Kalirin9 may interact with RhoA after being released from RhoGDI, thereby activating RhoA.

Beginning our experiments, we tested whether Kalirin9 exists as a part of the p75-NgR complex in vivo. Since Kalirin9 is expressed among p75+ Purkinje and granule neurons (Fig. 1A), we subjected the cerebellar lysates from P1 and P5 rat pups to immunoprecipitation with the control IgG or MC192, an anti-p75 antibody. The resulting p75 immune complexes were separated and probed for both Kalirin and NgR by dividing the membrane into two parts, and then using one for Kalirin9 and the other for NgR in Western analyses. As a control, the blot was stripped and reprobed for p75. Kalirin9 was detected in the p75 immune complex, as was NgR (Fig. 2A), but not in the control IgG immune complex. These data suggest that Kalirin9 is a component of the functional complex with p75-NgR in vivo, and may play a role in p75-NgR signaling.

We next examined the binding characteristics of Kalirin9 to p75 in the presence of NgR using transient transfection of the relevant constructs in 293T cells. NgR was detected in the p75 immune complex when cells were transfected with p75 and NgR (Fig. 2B, lane 1), which is in agreement with previous reports (Wang et al., 2002a; Wong et al., 2002). Similarly, Kalirin9 interacted with p75 (Fig. 2B, lane 2) as in Fig. 1D. When NgR was cotransfected along with p75 and Kalirin9, however, Kalirin9 dissociated from p75.
(Fig. 2B, compare lanes 2 and 3), suggesting that the presence of NgR facilitates the dissociation of Kalirin9 from p75, a likely prerequisite for GEF involvement in RhoA activation after RhoA is released from RhoGDI.

We next investigated the binding behavior of Kalirin9 to p75 in the presence of RhoGDI. For these experiments, the lysates from the transfected 293T cells were divided equally to produce two identical immunoprecipitation reactions with a p75 antibody, then one was probed in Western for RhoGDI and the other for Rho. RhoGDI interacted with p75 (Fig. 2C, lane 2 in upper panel), and this interaction brought RhoA to p75 (Fig. 2C, lane 2 in lower panel). RhoA was also found in the p75 immune complex even without the introduction of RhoGDI (Fig. 2C, lane 1 in lower panel), presumably via endogenous RhoGDI in 293T cells as reported earlier (Yamashita and Tohyama, 2003). When Kalirin9 is introduced, on the other hand, the amount of RhoGDI bound to p75 was significantly reduced compared to that without Kalirin9 (Fig. 2C, compare lanes 2 and 4 in upper panel), which correlated with the loss of RhoA from p75 (Fig. 2C, lane 4 in lower panel). These results suggest that Kalirin9 and RhoGDI compete for p75 binding. Such mutually exclusive binding behavior can be interpreted as suggesting that Kalirin9 may participate in the release of RhoA from p75 after RhoA is recruited to p75 via RhoGDI.

If Kalirin9 were the functional GEF in RhoA activation by p75-NgR, one would expect that RhoA should bind Kalirin9 after it is released from p75 upon activation. To test this hypothesis, we first examined whether RhoA binds Kalirin9 once it is released from p75 in 293T cells that were transfected with p75, NgR, and RhoGDI, with or without Kalirin9. The 293T lysates were divided into two independent
immunoprecipitation reactions, one with a p75 antibody, and the other, with a RhoA antibody. The p75 immune complexes were probed for the presence of RhoA in RhoA Western, while RhoA immune complexes were probed for Kalirin9 or RhoGDI using one divided blot in myc Western. In the absence of Kalirin9, RhoA still remained bound to p75, while in the presence of Kalirin9 RhoA dissociated from p75 and interacted with kalirin9 (Fig. 3A). Similarly, the amount of RhoA that associated with RhoGDI also decreased in the presence of Kalirin9, suggesting a transfer of RhoA from p75/RhoGDI to Kalirin9 under a condition that mimics the active state of p75-NgR.

We next investigated whether RhoA indeed jumps from p75-RhoGDI to Kalirin9 upon ligand-dependent activation of the endogenous p75-NgR in cerebellar granule neuron (CGN) cultures. Neonatal CGN cultures are known to respond to all known NgR ligands that activate RhoA in a manner dependent on p75-NgR (Mi et al., 2004; Wang et al., 2002a; Wong et al., 2002; Yamashita et al., 2002). Also in our P5-6 neonatal rat CGN cultures, the addition MAG-Fc resulted in RhoA activation (Fig. 3B). We asked whether a greater amount of RhoA interacts with Kalirin9 under the same condition. Following 15min MAG treatment, the lysates were prepared, and subjected to immunoprecipitation reactions using RhoA antibody or the control IgG. The presence of Kalirin9 was detected in Kalirin Western. The amount of Kalirin9 present in the RhoA immune complex increased significantly with MAG treatment, and also compared to the control IgG immunoprecipitation (Fig. 3B). These results suggest that activation of RhoA by p75-NgR includes Kalirin9, whose action is to receive and activate RhoA after it is released from the activated p75-NgR complex.
Kalirin 9 is required for RhoA activation mediated by MAG

Our biochemical data suggest that Kalirin9 is involved in RhoA activation by p75-NgR. If Kalirin9 is the necessary component for RhoA activation by the p75-NgR complex, one would expect that Kalirin9 will allow RhoA activation in an ectopic system, such as 293T cells, since the introduction of p75 with NgR alone fails to activate RhoA in such systems (Mi et al., 2004). 293T cells were therefore transfected with p75/NgR, p75/NgR/Kalirin9 or p75/NgR/Ephexin. Ephexin is a dual Rac/RhoA GEF specific for Ephrin A receptors (Shamah et al., 2001). Following 15min treatments with MAG-Fc (Ma) and OMgp (O), the lysates were prepared and subjected to GST-Rhotekin pulldown assays. There was no ligand-dependent RhoA activation with p75/NgR, while MAG and OMgp induced RhoA activation when Kalirin9 was added (Fig. 4A). In p75/NgR/Ephexin cultures, the basal Rho-GTP level was elevated, but there was no further increase in the level with any ligand treatment. These results suggest that Kalirin9 constitutes a missing component in the RhoA activation machinery of the p75-NgR complex, specifically in response to myelin inhibitory molecules.

We next examined whether Kalirin9 action is necessary for RhoA activation by myelin inhibitory molecules in CGN cultures. In order to establish such a causal relationship, we sought to inhibit the action of endogenous Kalirin9 using a dominant-negative mutant that lacks either the putative Rac (GEF1Δ) or Rho GEF (GEF2Δ) domain. Although the sequences of the GEF1 and GEF2 resemble a typical Rac and Rho GEF domain, respectively, both GEF domains in isolation bind Rac in vitro to a similar extent (Penzes et al., 2001), not yet clarifying the existence of a functional distinction. In addition, while the GEF1 in Trio was effective in rescuing the original Trio mutation in
Drosophila, the GEF2 in Trio did not exhibit any noticeable phenotype (Newsome et al., 2000). Due to such functional ambiguity between the two domains, we generated two Kalirin9 mutants in retroviruses, one lacking the GEF1 (GEF1Δ-Kalirin9), and the other, lacking the GEF2 (GEF2Δ-Kalirin9).

The P5-P6 CGN cultures were infected with the control, the GEF1Δ-Kalirin9, or the GEF2Δ-Kalirin9 retrovirus a day after plating, and the next day, the infected cultures were treated for 15min with soluble myelin (My), Nogo-66 (N), OMgP (O), MAG-Fc (Ma), or 3 min with lipolysophosphatidic acid (LPA; L), a well-known RhoA activator that is not dependent on p75-NgR (Ren et al., 1999). The resulting lysates were subjected to RhoA activity assays. In the presence of the GEF2Δ-Kalirin9, RhoA activation by all the myelin inhibitory ligands was completely blocked, while with the GEF1Δ-Kalirin9, the effect ranged from partial to complete, depending on the type of inhibitory ligands (Fig. 4B, C). In contrast, LPA, which signals via Edg-2, a G-protein coupled receptor (GPCR) (Moolenaar, 1999; Van Leeuwen et al., 2003), activated RhoA in the presence of the mutant. These results together suggest that Kalirin9 is a specific GEF for RhoA activation by p75-NgR in culture. In addition, our data from the two Kalirin9 mutants indicate that both GEF domains participate in overall RhoA activation, although the GEF1 appears to exert a greater effect on Rho-GTP levels in response to MAG and myelin than to Nogo66 and OMgP.

**Blocking endogenous Kalirin 9 action curtails inhibitory action of MAG**

The lack of RhoA activation in the presence of the mutant Kalirin9 suggests that neurons should be able to send out processes even on inhibitory substrates, as long as
Kalirin9 function is blocked. We therefore infected CGN cultures with the GEF2Δ-Kalirin9 or the control retrovirus after isolation, and plated them onto poly-d-Lysine (PDL) substrates or myelin. Both the control and the GEF2Δ-Kalirin9 infected neurons sent out long neurites on PDL, indicating that the virus does not affect their normal process outgrowth on permissible substrates (Fig. 4D). Quantification of these data indicates that inhibition of the endogenous Kalirin9 in DRG cultures resulted in a significant increase in neurite lengths on all the inhibitory substrates, compared to that obtained with the control virus infected cultures (Fig 4E). These data together suggest that Kalirin9 is necessary for p75-NgR-dependent RhoA activation and inhibition of neurite outgrowth.

**Kalirin 9 is involved in RhoA activation after spinal cord injury**

Dubreuil et al. have reported a robust activation of RhoA following spinal cord injury (Dubreuil et al., 2003). Since our results indicate that Kalirin9 is necessary for RhoA activation by p75-NgR in culture, we next asked whether Kalirin9 is also involved in activating RhoA after spinal cord injury. For our spinal cord injury model, we subjected the mice to dorsal hemisection at thoracic level 9-10, as we have reported (Beattie et al., 2002). At varying times after the injury, lysates were prepared from the 6mm tissue block around the injury epicenter, and processed for RhoA activity assays. Following spinal cord injury, Rho-GTP levels increased 1-3d post-injury (dpi), and remained elevated for 7-14 dpi (Fig. 5A). Kalirin9 and NgR levels also increased after injury, with its peak at 3 dpi, which coincided with p75 induction (Fig. 5A).
Next, we asked whether Kalirin9 is expressed among the same cells that express p75 and NgR after spinal cord injury. For these experiments, spinal cords were sectioned at 20µm in a horizontal plane, and adjacent sections were processed for immunohistochemistry with p75, NgR, or Kalirin antibody. In the control laminectomized samples, there was little p75 expression detected, while a few cells expressed NgR and Kalirin in the gray matter (Fig. 5B, a to c). At 3dpi, however, p75 was detected widely including the neurons in the gray matter (Fig. 5B, d). The neurons in the gray matter also expressed increased levels of NgR and Kalirin (Fig. 5B, e and f), suggesting that Kalirin is localized in the same cells that upregulate p75 and NgR expression. In addition to the neurons in the gray matter, p75 was also detected near the lesion site in fibrous processes (Fig. 5B, g and h). The endings of the p75+ processes appeared like “endballs”, a typical feature reported among denervated, dystrophic nerve endings (Silver and Miller, 2004). Some of these p75+ processes also stained for neurofilament, suggesting that p75 expression is upregulated not only the neuronal cell bodies but also in the damaged nerve fibers after spinal cord injury.

Colocalization of Kalirin9 among NgR+/p75+ cells after injury indicates the formation of a functional complex in vivo, where Kalirin9 acts as a functional GEF for p75 and NgR. We tested whether this is the case, by asking whether Kalirin9 forms a complex with RhoA after spinal cord injury. This is similar to data in Fig. 3B, where we documented that an increased amount of Kalirin9 interacts with RhoA after MAG activation in CGN cultures. For these experiments, we subjected the 3dpi spinal cord lysates to immunoprecipitation with RhoA antibody, and probed for the presence of Kalirin9 in Kalirin Western. As with MAG-dependent activation of CGN cultures, the
amount of Kalirin9 that associated with RhoA increased significantly at 3dpi, while there was no Kalirin9 detected in the control laminectomized lysates or IgG immunoprecipitation (Fig. 5C). These results suggest that Kalirin9 is likely to play a role in p75-NgR-dependent RhoA activation after spinal cord injury. We further interpret these results as suggesting that Kalirin9 may be the relevant GEF participating in RhoA activation after spinal cord injury.

In order to test whether Kalirin9 is indeed necessary for activating RhoA after spinal cord injury, we generated a transgenic mouse line where the expression of the GEF1Δ-Kalirin9 was directed by a 4Kb human p75 promoter fragment (Fig. 6A). The 4Kb p75 promoter was chosen, since the fragment was shown to be sufficient to allow expression of a transgene after a lesion (Huber and Chao, 1995). P75 is not normally expressed in the adult spinal cord (Beattie et al., 2002), and the use of an inducible promoter would allow us to inhibit RhoA activation only among the cells that upregulate p75 after injury. To aid the identification of cells that express the mutant Kalirin9, we also included the cDNA for GFP under IRES. As shown in Fig. 6B and C, the 4Kb p75 promoter fragment was sufficient to allow expression of the transgene after spinal cord injury, as it did after sciatic nerve lesion (Huber and Chao, 1995); both GFP and myc were detected only after injury, and its expression coincided with that of endogenous p75, whose expression is also induced by the injury.

We next assessed the extent of RhoA activation by subjecting the transgenic and the control mice to spinal cord injury. To account for the potential variability in the extent of injury, we also measured the active state of p38 as an internal control (Fig. 6D). The p38 pathway is activated mainly among microglial populations and not among
neurons or oligodendrocytes after spinal cord injury (our unpublished data). In the non-transgenic controls, Rho-GTP level increased beginning 1dpi, which remained elevated up to 5dpi, ultimately reaching a 3 fold increase over the control value (Fig. 6E). In the transgenic mice, however, the Rho-GTP level was significantly reduced compared to that in the control, although the extent of injury was comparable based on phospho-p38 Western (Fig. 6D, E). The phospho-p38 data also suggests that the GEF1Δ-Kalirin9 inhibited the RhoA pathway selectively without affecting the unrelated p38 pathway in the transgenic mice. These results therefore suggest that Kalirin9 plays a critical role in RhoA activation after spinal cord injury. Combined with our culture data, we conclude that Kalirin9 is a necessary component in p75-NgR signaling.
DISCUSSION

In this report, we present data that support the functional role for the Trio family of a dual Rac and Rho GEF, Kalirin9, in p75-NgR signaling. Our data not only demonstrate that binding of Kalirin9 to p75 is regulated by the active NgR, but also suggest that a transfer of RhoA to Kalirin9 from the p75-NgR-RhoA GDI complex is likely to be a prerequisite for the nucleotide exchange reaction that yields Rho-GTP in response to myelin inhibitory molecules. We also document that Kalirin9 is functionally involved in RhoA activation by p75-NgR: (1) Introduction of Kalirin9 in ectopic 293T cells is sufficient to restore p75-NgR-mediated RhoA activation, (2) Rac and RhoA GEF domains of Kalirin9 are necessary for RhoA activation in CGN cultures as well as after spinal cord injury, and (3) Kalirin9 function is required for the inhibitory action by all the known NgR ligands in culture. Together, these data help establish Kalirin9 as a GEF that plays a necessary role in the activation of RhoA by the p75-NgR complex both in vitro and in vivo. It should also be pointed out that our study identifies myelin inhibitory molecules as the first known ligands for activating Kalirin9, a mammalian homolog of Trio that is known to play critical roles in axon guidance (Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000; Steven et al., 1998).

Earlier studies have implicated p75 in regulating neurite outgrowth in response to neurotrophins (Brann et al., 1999; Dostaler et al., 1996; Wright et al., 1992), and analyses
of p75−/− mice supported such an interpretation (Kohn et al., 1999; Lee et al., 1994). Since p75 augments Trk function when they are expressed together, it was still not clear whether p75 was solely responsible for the effect on neurite outgrowth. It was only after the discovery that p75 inhibits RhoA activity in response to neurotrophins in the absence of Trk receptors (Yamashita et al., 1999), that the independent role of p75 in regulating axon outgrowth became far less debatable. With the recent findings that p75 forms a functional unit with NgR in response to myelin inhibitory factors (Wang et al., 2002a; Wong et al., 2002), the potential role of p75 in regulating axon outgrowth has become more established, at least in vitro when it complexes with NgR. Identification of Kalirin9 as a RhoA GEF that facilitates p75-mediated RhoA activation provides additional evidence that p75 modulates RhoA activity and its regulation is critical for neurite outgrowth in culture.

The finding that p75 is the signaling partner for NgR in response to myelin inhibitory proteins suggests that p75 may regulate the extent of re/degeneration after CNS injury. When p75−/− mice were subjected to spinal cord injury, however, both the ascending sensory fibers and descending corticospinal fibers failed to regenerate (Song et al., 2004). It is noteworthy in this context that RhoA activity was inhibited at 24 hr after spinal cord injury in p75−/− mice, but full activity returned by 3 dpi (Dubreuil et al., 2003). These data may be interpreted as suggesting that RhoA activation after spinal cord injury comprises at least two phases: an early phase that is regulated by p75 and a late phase that is regulated by a receptor other than p75. Recently, chondroitin sulfate proteoglycans, major components of the late forming glial scar (Silver and Miller, 2004), were shown to activate RhoA in a PKC-dependent manner (Sivasankaran et al., 2004).
These results suggest that it is plausible that the early RhoA activation phase is mediated by p75-NgR in response to myelin inhibitory molecules, while the late phase is initiated by distinct RhoA activation machinery that responds to signals from glial scars. Alternatively, p75 action after injury is multifaceted in that p75 may also downregulate RhoA activity in response to neurotrophins after injury. If this is the case, the balance between neurotrophins and myelin inhibitory molecules will be critical in determining the outcome of p75 activation.

To our surprise, we discovered that p75 protein that is induced after spinal cord injury resides outside the lipid rafts, where p75 is normally reported to be present in PC12 cells (Bilderback et al., 1997; Wu et al., 2001). In primary oligodendrocyte cultures, p75 is also localized exclusively within the lipid rafts (data not shown). Perhaps, p75 is modified differently when its expression occurs in the cell where p75 is not normally expressed, resulting in different targeting of the receptor. Although the reason for different localization is not clear at present, these data may provide an explanation for multiple, distinct partners that p75 is known to have, such as Trk receptors, NgR, sortilin, and Lingo (Chao, 2003). By changing its locale, p75 can now associate with different partners residing in that particular compartment, such as NgR. Differential localization of p75 would also allow p75 to recruit different signaling molecules, such as Kalirin9 that is outside of lipid rafts, thus signaling to activate RhoA.

In our study, deletion of either of the GEF domains in Kalirin9 was capable of inhibiting p75-NgR-dependent RhoA activation. This may be regarded as somewhat unexpected, since the GEF1 and GEF2 domains elicit opposite effects in regard to the length of neuritic processes, when they are individually expressed as an isolated domain.
in cortical neurons (Penzes et al., 2001). For instance, overexpression of the GEF1
induced shortening in process lengths, while the GEF2 caused process-elongation with
abundant filopodia (Penzes et al., 2001). Although the data may be unexpected,
investigating a function of a domain within the context of a whole molecule will be more
likely to have physiological relevance than assessing its role in isolation. Indeed, deletion
of the GEF1 in the context of Kalirin9 resulted in complete inhibition of fiber outgrowth
in SCG cultures (May et al., 2002). Our data indicate that the two domains cooperate in
activating the RhoA pathway, either by sharing a common downstream effector or
forming a hierarchical signaling unit. It should also be pointed out that, unlike the other
overexpression studies, we investigated here the role of each domain in ligand-dependent
regulation of RhoA within the context of the full-length Kalirin9.

While the GEF∆2-Kalirin9 was effective in blocking RhoA activation by all the
known NgR ligands, the effect of the GEF∆1-Kalirin9 depended on the types of ligands
used. For instance, the GEF∆1-Kalirin9 blocked RhoA activation by MAG completely,
but its effect was partial with Nogo-66 and OMgp. It has been reported that Nogo-66
differs from MAG in regard to its ability to activate RhoA (Niederost et al., 2002). For
instance, PI-PLC treatment that removes the GPI-linkage abrogated Nogo-66-mediated
RhoA activation, while it did not have any effect on RhoA activation by MAG-Fc
(Niederost et al., 2002). The authors of that study concluded that there may be another
NgR for MAG or NiG-Δ20, representing another active site in Nogo-A (Oertle et al.,
2003). Clarification will undoubtedly require more careful analyses, but it should be
pointed out that Kalirin9 was sufficient to facilitate RhoA activation in 293T cells in
response to both MAG and OMgp. If there were another NgR receptor, our data would suggest that Kalirin9 may also be involved in its signaling.

Recently, another GPI-linked member of the p75-NgR complex, Lingo-1, was identified (Mi et al., 2004). Lingo-1 does not bind myelin inhibitory molecules, but associates with p75 and NgR, at least in Cos cells (Mi et al., 2004). In addition, introduction of Lingo-1 in Cos cells results in RhoA activation with p75 and NgR, which was interpreted as suggesting that Lingo-1 was the missing component in Nogo signaling. Here, we demonstrated that the introduction of Kalirin9 also restores the ability of p75-NgR to activate RhoA in 293T cells. These data suggest that interaction of the p75-NgR complex with Lingo-1 somehow obviates the need for Kalirin9. Whether Lingo-1 affects Kalirin9 binding to p75-NgR is not yet known.

In conclusion, we document that Kalirin9 is a necessary, functional component for p75-NgR signaling, facilitating RhoA activation both in vivo and in vitro. We also identify myelin inhibitory molecules as the first known group of ligands that regulate Kalirin9 function in process outgrowth.


receptor mediates the effects of nerve growth factor on outgrowth of cultured hippocampal neurons. J Neurosci 19, 8199-8206.


Wright, E. M., Vogel, K. S., and Davies, A. M. (1992). Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron 9, 139-150.


Fig. 4.1 Kalirin9 binds p75 and is coexpressed with p75 in vivo.

(A) Kalirin is expressed among p75+ Purkinje neurons in the P5 cerebellum. P5 rat sagittal sections were stained with p75 (Covance) and pan-Kalirin antibodies (Penzes et al., 2000). The arrow points to doubly stained Purkinje neurons (Upper panel). Kalirin9 is the major form present in the developing cerebellum at P5 (Lower panel). The lysates from the cerebellum and the cortex were analyzed in Western analyses for the presence of Kalirin proteins. Note that Kalirin7 level is high in the neonate cortex, while Kalirin9 is the major form present in the cerebellum. Scale bar: 12.5 μm. (B) Kalirin9 binds the cytoplasmic domain of p75 in vitro. GST or GST-p75ICD was incubated with the lysates from 293T cells that were transfected with the vector or the myc-tagged wild type Kalirin9. The bound Kalirin9 was detected in myc Western. (C) Kalirin9 binds the full-length p75. 293T cells were transfected with p75, Kalirin9, or p75 plus Kalirin9. The lysates were subjected to immunoprecipitation with p75 antibody (Covance), and the precipitated Kalirin9 was detected in myc Western. The presence of p75 and Kalirin9 is also shown as control Western.
Fig. 4.2 NgR regulates Kalirin9 binding to p75.

(A) p75 associates with Kalirin9 and NgR in the developing cerebellum. Cerebellar lysates from P1, P3, and P5 rats were subjected to immunoprecipitation with anti-p75 antibody (192) and the bound proteins were simultaneously probed with pan-Kalirin and NgR antibodies using a divided membrane. +: positive controls for Kalirin9 and NgR. (B) Kalirin9 dissociates from p75 when NgR forms a complex with p75. The lysates from transfected 293T cells were subjected to immunoprecipitation with p75 antibody (Covance), and the immune complexes were probed with myc antibody to detect Kalirin9 using the upper part of the transferred membrane, and flag antibody to detect NgR using the lower part of the membrane. The control Westerns are also shown. (C) Kalirin9 competes with RhoGDI for p75 binding (Upper panel), which coincides with the loss of RhoA from p75 (Lower panel). The lysates from transfected 293T cells were divided into two equal parts, and each being subjected to immunoprecipitation with p75 antibody. One immunoprecipitate was probed for the presence of RhoGDI in myc Western (Upper panel), while the other immunoprecipitates were probed for RhoA in RhoA Western (Lower panel). The control Westerns are also shown.
Fig. 4.3 Activated NgR induces RhoA to bind Kalirin9.

(A) When Kalirin9 is present, the dissociated RhoA from p75 is found in complex with Kalirin9, instead of RhoGDI. The lysates from transfected 293T cells were equally divided, and one was subjected to immunoprecipitation with p75 antibody and probed for the presence of RhoA with RhoA antibody (Upper panels), the other, immunoprecipitated with RhoA antibody and probed for Kalirin9 with myc antibody (Lower panels). The control RhoA and myc Westerns are shown. (B) Activation of the p75-NgR complex with MAG-Fc induces RhoA to associated with Kalirin9, rather than RhoGDI in CGN cultures. The CGN cultures were treated with 0.3µg/ml conjugated MAG-Fc for 15min. The lysates were equally divided and one was subjected to immunoprecipitation with RhoA antibody and probed with pan-Kalirin antibody (Upper panel), and the other was also immunoprecipitated with RhoA antibody, probed with RhoGDI antibody (Lower panel). In parallel, MAG-Fc treated CGN lysates were subjected to RhoA activity assays using Rhotekin binding proteins in pulldown assays.
Fig. 4.4 Kalirin9 is functionally involved in p75-NgR signaling.

(A) Introduction of Kalirin9 in 293T cells reconstitutes a ligand-dependent RhoA activation by p75-NgR. The transfected 293T cells were untreated or treated with 0.3µg/ml conjugated MAG-Fc (Ma) and 1µg/ml conjugated OMgp (O) for 15min, and the resulting lysates were subjected to RhoA activity assays using Rhotekin binding protein pulldown assays. (B) The mutant Kalirin9 lacking either the Rac GEF domain (GEF1Δ-Kalirin9) or the RhoA GEF domain (GEF2Δ-Kalirin9) inhibits ligand-dependent RhoA activation in CGN cultures. CGN cultures were infected with either the control pLIA, pLIA-GEF1Δ-Kalirin9, or GEF2Δ-Kalirin9 retrovirus, and the next day, the cultures were treated with 10µg/ml soluble myelin (My), 8µg/ml conjugated Nogo-66 (N), 1µg/ml conjugated OMgp (O), 0.3µg/ml conjugated MAG-Fc (Ma) for 15min, or 30µg/ml LPA for 3min. The resulting lysates were subjected to RhoA activity assays using Rhotekin binding protein pulldown assays. The extent of infection is shown in Western with alkaline phosphatase antibody (AP), and the presence of the mutant virus, as myc Western. (C) Quantification of RhoA activity data with the control (n=6-13), the GEF1Δ-Kalirin9 (n=3-5), and the GEF2Δ-Kalirin9 (n=3-7) are shown in Fig. 4B. (D) Kalirin9 is necessary for inhibition of neurite outgrowth on myelin. A representative pictures of CGN cultures infected with the control GFP or the GEF2Δ-Kalirin9 retrovirus. (E) Quantification of neurite outgrowth in infected DRG neurons.
Fig. 4.5 Kalirin9 is involved in RhoA activation after spinal cord injury.

(A) Time course of RhoA activation after hemisection in mice after spinal cord injury. The spinal cord lysates from 3dpi were subjected to immunoprecipitation with the control IgG or RhoA antibody. The immune complexes were probed in Western assays with pan-Kalirin antibody. C: laminectomy control, T: 3d post-injury. (B) Kalirin9 binds RhoA after spinal cord injury. The spinal cord lysates from control laminectomized and injured mice were loaded into a discontinuous Optiprep gradient. Fractions 6 and 7 represent lipid-rafts, while fractions 12-14 are outside of the rafts, close to the bottom of the tubes. Note a weak p75 expression within the rafts, which represents p75 expressed among innervating fibers within the spinal cord. Injury-induced p75 is predominantly localized outside of the lipid rafts, suggesting that p75 signals differently depending on where it is localized.
Fig. 4.6 RhoA activation after spinal cord injury is attenuated in the mutant Kalirin9 transgenic mice.

(A) A diagram of the GEF1Δ-Kalirin9 transgenic construct driven by 4Kb human p75 promoter. The angled arrow indicates the start of transcription. Translation starts at the beginning of the transgene. Note that the construct allows the expression of GFP under IRES. (B) Induced expression of the GEF1Δ-Kalirin9 and GFP after spinal cord injury. At 2-3 dpi, 10mm spinal cord blocs were removed, cut in the horizontal plane at a 20 µm thickness, and processed for immunohistochemistry with GFP, p75, and/or myc antibody. Note the expression of GFP coincides with the endogenous p75 expression in the transgenic mice, but not in the non-transgenic control. Scale bar: 20 µm. (C) Detection of the GEF1Δ-Kalirin9. The lysates were subjected to immunoprecipitation with myc antibody and Western with Kalirin antibody. Note that a myc-reactive band is present only in injured transgenic mice, while the endogenous Kalirin9 is detected in all the samples. (D) Attenuation of RhoA activity in the transgenic mice after spinal cord injury. Mice were subjected to T8-9 hemisection, and the spinal cord lysates were prepared at the indicated time after the injury. 80% of the total lysate was used to measure Rho-GTP level in pulldown assays, while 20% of the lysates was used to measure the total RhoA protein level as well as the extent of injury using phospho-p38 antibody. (E) Quantification of RhoA activity data (n=7). The Rho-GTP levels are expressed as normalized values, taking the total RhoA protein levels into account.
CONCLUSION

Rac activation kinetics determines cellular outcome

It is our hypothesis that long term Rac activation is necessary for apoptosis, while short term Rac activation is involved in cytoskeletal changes in neuronal cells. There is substantial evidence to link an increase in Rac-GTP levels to neurite outgrowth and branching both in vitro (Yasui et al., 2001; Chen et al., 1999) and in vivo (Ozdinler et al., 2001; Li et al., 2000). Dominant negative mutants of Rac can block outgrowth neurite outgrowth neuronal cell lines. In addition to its role in cytoskeletal rearrangement, activation of Rac has also been shown to be necessary for JNK activation by EGF (Coso et al., 1995) and TNFa (Coso et al., 1995; Kim et al., 1999). In several cell types including neurons, JNK activation initiates apoptotic signaling. Therefore, how does activation of Rac bring about these two very different cellular events? Our results suggest the kinetics of Rac activation may be responsible.

Our results show that Rac activation by p75 is prolonged for four hours, while Rac activation by p75 and Trk receptors was occurred only at ten minutes. How can p75 activate Rac with two different kinetics. Two mechanisms of Rac activation may explain...
the data. The first possible mechanism involves Trk receptors activating a pathway that leads to suppression of p75-mediated Rac activation. Trk receptors could recruit or lead to the activation of a GTPase activating protein (GAP) to prevent Rac activation.

The second mechanism involves Trk receptors initiating a signal that leads to Rac activation, but with a different time course from that of p75. While our work was the first to demonstrate p75 mediated activation of Rac, it was known for some time that neurotrophin treatment of p75 and Trk expressing cells could lead to Rac activation. These effects were attributed to Trk mediated activation of the PI-3 kinase pathway (Yuan et al., 2002; Weiner et al., 2002). Certain phosphoinositides, products of PI-3 kinase, have been shown to activate the Rac GEF Vav leading to Rac activation (Han et al., 1998). The consequences of Rac activation by Trk and PI-3 kinase involve processes that alter the cytoskeleton including axon pathfinding, chemoattraction (Yuan et al., 2002) and migration (Altun-Gultekin et al., 1996). Interestingly, the time course of Rac activation observed in these systems is remarkably similar to what we observed in oligodendrocytes after NT3 treatment; a rapid increase in Rac-GTP that persists only minutes (Yuan et al., 2002; Yamaguchi et al., 2001). Further analysis of Rac activation by NT3 in the presence of Trk and PI-3 kinase inhibitors may lend support to this hypothesis. It would be interesting to examine the morphological effects of NT3 on oligodendrocytes to further support the hypothesis that short term Rac activation is initiated by Trk receptors to promote cytoskeletal changes, while p75 signals for long term Rac activation to promote JNK activation and apoptosis.

How does different Rac activation kinetics translate into separate signaling outcomes? Separate Rac effectors could mediate the different outcomes. In our system a
prolonged Rac activation led to apoptosis. Many downstream effectors and binding partners for Rac have been identified and characterized, some linking Rac to the JNK kinase cascade. A type of JNKKK known as Mlk3 was identified as one particular kinase which may link activated small G proteins to MAPK pathways. Activated Rac and Cdc42 were found to interact with Mlk3 and the presence of Mlk3 with these constitutively active Rac and Cdc42 mutants greatly enhanced their ability to activate JNK (Teramoto et al., 1996). In an NGF withdrawal paradigm, MLK3 was found to be required for the death of SCG sympathetic neurons (Mota et al., 2001). In this system however, Cdc42 activation, not Rac, was responsible for activation of MLK3, JNK and subsequent apoptosis. Other Rac activated kinases have been linked to cytoskeletal regulation. MRCKalpha is one such kinase found to link Cdc42 and Rac activation to myosin light-chain phosphorylation (Chen et al., 1999). Still, several other effectors of Rac cannot be classified into these two groups. PAK1, possibly the most well characterized Rac effector, has been shown to play a role in gene transcription, cell morphology, motility, and apoptosis (Jaffer et al., 2002).

Recent evidence suggests localization is crucial for active Rac to have the appropriate effect. Lack of dynamin was shown to alter Rac localization (Schlunck et al., 2004). Even though Rac was still activated in these cells, lamellipodia formation by fibronectin was inhibited in the absence of dynamin suggesting Rac-GTP must be in the right location for proper outcome. Rac, like other small GTPases, are prenylated to aid in their association with the plasma membrane. Consequently this places them in good position for rapid activation by receptors and changes in cytoskeletal dynamics. Rac is also known to contain a nuclear localization sequence which is responsible for
translocating Rac to the nucleus (Lanning et al., 2004). Although it has been
demonstrated that activation enhances its accumulation in the nucleus (Lanning et al.,
2003), it is not known what signaling Rac may activate in this context. We did not
investigate whether the localization of active Rac changes dependent on time in
oligodendrocytes. However, it is tempting to consider a system where Rac-GTP intended
for cytoskeletal changes is confined to the cell periphery, while longer activation times
cause a shift in Rac localization into the cytoplasm or nucleus where it is able to interact
with kinase cascades that initiate apoptotic signaling.

**Cell death by ProNGF activation of injury induced p75.**

Our data documented that p75 is a critical player for oligodendrocyte death both
in vivo and in vitro. In culture, apoptosis of oligodendrocytes was completely inhibited
in the absence of p75. In vivo, however, the absence of p75 resulted in a partial rescue.
The difference between in vivo and in vitro data suggests that there may be additional
signals activated in oligodendrocytes in the intact spinal cords that induce p75-
independent apoptosis. It is believed the mechanisms of secondary spinal cord injury are
complex and require a variety of contributing factors. Extraction of protein from injured
spinal cord likely contains recently secreted ProNGF as well as ProNGF still present
inside the cell producing it. It’s possible our preparation doesn’t include other signals
which exist in vivo. Some studies suggest activation of microglia following injury
increases cytotoxic cytokine production (Beattie et al., 2002). Tumor necrosis factor
alpha might play an apoptotic role by potentiating excitotoxic neuronal cell death by glutamate receptors (Hermann et al., 2001). The production of reactive oxygen species (ROS) is another proposed mechanism of cell death following spinal cord injury. ROS were shown to be increased in motor neurons after injury and there is evidence this could contribute to apoptosis (Xu et al., 2004). The environment of the cultured oligodendrocytes is very different compared to physiological conditions. They are isolated from other cell types and are bathed in different media conditions. Therefore, it is possible components of the culture media, such as insulin may have protecting effects against signals which would lead to apoptosis in vivo. Insulin-like growth factor (IGF) was demonstrated to protect spinal motoneurons from death after injury in rats (Haninec et al., 2003).

**Regulation of ProNGF mediated apoptosis**

The identification of ProNGF, as opposed to mature NGF, as the primary pathological ligand to p75 after injury has profound consequences. While the cellular response to mature NGF depends on the Trk receptor composition of the cell, ProNGF will activate apoptotic signaling from p75, regardless of Trk expression. Regulation of the processing of ProNGF is a possible means controlling ProNGF-mediated apoptosis. ProNGF contains consensus cleavage sites for the proteases plasmin, MMP-3, and MMP-7, which cleaved ProNGF to mature NGF in vitro (Lee et al., 2001). The convertases furin, PC1, PC2, PACE4, PC5 and the PC5 isoform PC5/6-B were also found to cleave
ProNGF in vitro (Seidah et al., 1996). Although the expression pattern of these proteases is consistent a role in ProNGF cleavage, which of these proteases is responsible for processing ProNGF in vivo is not yet known. ProNGF mediated apoptosis may be a general mechanism of injury induced cell death. Reports analyzing expression of pro-neurotrophin versus mature neurotrophin in Alzheimers disease patients’ brains have indicated significantly higher amounts of ProNGF versus mature depending on disease severity (Fahnestock et al., 2001; Peng et al., 2004).

It has been discovered that ProNGF requires a receptor in addition to p75 to initiate signaling. The Neurotensin-3 receptor, sortilin, binds ProNGF and is necessary for its signaling through p75 (Nykjaer et al., 2004). The expression of sortilin in the nervous system during embryogenesis suggests a possible role for ProNGF in mediating programmed cell death during development. Although the necessity of sortilin for injury mediated cell death needs to be examined, current evidence suggests sortilin could provide a form of regulation over which cells are killed by ProNGF. In support of our results showing a role for ProNGF in injury mediated cell death, levels of sortilin were found to significantly increase in brains of mice after CSN axotomy (data not shown). Sortilin is also abundantly expressed in cultured oligodendrocytes (Nykjaer et al., 2004) in which we have shown ProNGF can induce apoptosis (Beattie et al., 2002). These observations lead us to the conclusion that induction of ProNGF, p75, and sortilin serve to kill oligodendrocytes after axotomy injuries in the brain and spinal cord.
Kalirin as exchange factor for p75/NgR-mechanism of Rho activation by NgR

It is well known that Rho activation is critical for growth cone collapse by NgR. (Niederost et al., 2002). Small molecule inhibitors of Rho and its downstream effector ROK can stimulate nerve regeneration (Dergham et al, 2002) (Fournier et al, 2003). However the specific guanidine nucleotide exchange factor responsible for Rho activation by p75 and NgR is unknown. Here, data is presented that shows Kalirin 9, a Kalirin isoform containing both Rac and Rho activation domains interacts with p75 in a manner that is regulated my NgR. Kalirin 9 is found associated with Rho in situations where Rho is activated and deletion mutants lacking either activation domain suppress or block Rho activation by the various NgR ligands.

Further experiments will are necessary to fully understand how Kalirin 9 activity is regulated by p75 and NgR. Our hypothesis is that binding of Kalirin 9 to p75 is crucial for regulation of Kalirin GEF activity. Additional data from our lab suggests the interaction of Kalirin 9 with p75 decreases after NGF treatment in transfected cells. At the same time, Rho activity increases in these cells, suggesting the dissociation of Kalirin 9 from p75 is responsible for Rho activation. Based on our data that the binding of Kalirin 9 competes with RhoGDI for binding to p75, it is also possible in the “off” state, Kalirin 9 acts to inhibit RhoGDI/p75 association and Rho release. Once the signal is activated that causes Kalirin 9 to dissociate from p75, RhoGDI is now able to bind and release Rho to be activated by Kalirin 9. The association with p75 may also serve to
localize Kalirin 9 close to the plasma membrane, in a position to determine neurite outgrowth and directional changes.

Although only the second DH/PH domain of Kalirin 9 is believed to bind and activate Rho, deletion mutants of both DH/PH domains were able to suppress Rho activation by NgR. This may suggest that activation of Rac, which is activated by the first DH/PH domain, is required for activation of Rho. However, this is unlikely as Rho and Rac activities are well documented to oppose one another. It is possible that the structure of Kalirin 9 protein plays a role in determining the function of each DH/PH domain. Kalirin 9 GEF deletions 1 and 2 have a very similar structure with the exception of the source of the DH/PH domain. Based on this hypothesis, both these mutants would lack Rho activation while retaining the ability to activate Rac. In this case, the location of the DH/PH domain within the context of the entire protein would determine its preference for Rac or Rho activation. To address this hypothesis, point mutations in a specific DH/PH domain could be used to knockout Rac or Rho activation without affecting the overall conformation of the protein. The specificity of dominant negative GEFs used in these experiments is also a concern. Recently, point mutations in the Rac activation domain of the Kalirin homologue Trio was found to only affect Rac activation by Trio, leaving Rho activation and Rac activation by means other than Trio unaffected (Debreceni et al., 2004). Taking this approach with Kalirin may lead us to develop better and more specific dominant negatives.

Another relevant question that emerges from the data is: why would the GEF responsible for NgR-mediated Rho activation have a domain for Rac activation as well? Constitutively active and dominant negative mutants of Rac and Rho are commonly used
to ask questions about the role of these proteins in certain cellular processes. However as new data demonstrates the importance of things such as localization and kinetics for certain signals, use of these mutants can only answer so much. Many reports suggest the balance between Rac and Rho is the critical issue (Allen et al., 2000; Leeuwen et al., 1997). Therefore, it is relevant that we have identified Kalirin 9 as a mediator of cytoskeletal changes by p75. P75 has been implicated in neurite extension through neurotrophin stimulation and retraction through NgR activation. Current studies in our lab are focused on identifying possible roles for Kalirin proteins in other facets of p75 signaling.

The modulation of the small G proteins Rac and Rho by p75 has emerged as a critical theme in cell death as well as in neurite growth and collapse. Because of the lack of any catalytic properties, p75 is thought to signal exclusively through interaction with downstream effectors. Indeed, many proteins have been found to associate with p75; however linking these interacting proteins with downstream signaling has proven difficult in some cases. The Rho family of small G proteins may be the critical link between p75 and these effects. Both Rac and Rho have effects on cytoskeleton arrangement, either resulting in neurite growth, neurite guidance (Dalpe et al., 2004), or neurite collapse and retraction. Data presented here suggests members of the Kalirin family of guanidine nucleotide exchange factors may be the proteins necessary for p75 action on Rac and Rho. Further experiments will indicate specifically how these GEFs are regulated and activated to provide greater understanding of p75 signaling.
REFERENCES


Dalpe G, Zhang LW, Zheng H, Culotti JG. Conversion of cell movement responses to Semaphorin-1 and Plexin-1 from attraction to repulsion by lowered levels of specific RAC GTPases in C. elegans. Development. 2004 May;131(9):2073-88


Domeniconi, M., Cao, Z., Spencer, T., Sivasankaran, R., Wang, K., Nikulina, E.,
interacts with the Nogo66 receptor to inhibit neurite outgrowth. Neuron 35, 283-290.

Frédéric Doussau, Stéphane Gasman, Yann Humeau, Francesco Vitiello, Michel
Popoff, Patrice Boquet, Marie-France Bader, and Bernard Poulain. A Rho-related
GTPase Is Involved in Ca2+-dependent Neurotransmitter Exocytosis. J Biol Chem,

G.R. Fanger, T.K. Schlesinger and G.L. Johnson, Control of MAPK signaling by
Ste20- and Ste11-like kinases. In: J.S. Gutkind, Editor, Signaling Networks and Cell
Cycle Control, Humana Press, Totowa, New Jersey (2000) 183–211.pp

Fahnestock M, Michalski B, Xu B, Coughlin MD. The precursor pro-nerve growth
factor is the predominant form of nerve growth factor in brain and is increased in

Nathalie Lamarche, Nicolas Tapon, Lisa Stowers, Peter D. Burbelo, Pontus
Aspenström., Tina Bridges, John Chant, and Alan Hall. Rac and Cdc42 Induce Actin
Polymerization and G1 Cell Cycle Progression Independently of p65PAK and the
JNK/SAPK MAP Kinase Cascade Cell 1996 87: 519-529


APPENDIX A

INTRODUCTION

Regulation of cell survival and death by NGF depends on the receptor composition in a given cell, since pro-survival signal from Trk receptors override the apoptotic signal mediated by p75 (Friedman, 2000; Harrington et al., 2002; Yoon, 1998). ProNGF, on the other hand, can initiate the apoptotic cascade by selectively activating p75 even in presence of TrkA (Lee et al., 2001). ProNGF was the predominant form of NGF induced by spinal cord hemisection, which suggests that proNGF-mediated activation of p75 may be critical for injury-mediated cell death in vivo (Beattie et al., 2002). Whether proNGF activates p75 during the developmental period of program cell death is not yet known. The classical experiments, where injection of blocking antibody against mature NGF in vivo induced death of developing neurons, suggest functional role of mature NGF in the developing nervous system (Levi-Montalcini and Booker, 1960). It is likely, therefore, that the receptor composition still plays a role in governing the death of excess neural populations during development.
Upon binding NGF, p75 induces ceramide production (Dobrowsky et al., 1994) and activates Rac GTPase (Harrington et al., 2002) and JNK (Casaccia-Bonnefil et al., 1996), as part of its apoptotic machinery. For p75 to induce apoptosis, activation of Rac GTPase and JNK was necessary (Harrington et al., 2002). Trk receptors promote cell survival by activating Ras-dependent pathways, such as extracellular regulated kinase (ERK) and phosphatidylinositol-3 kinase (PI-3 kinase) pathways (Kuruvilla et al., 2000; Segal and Greenberg, 1996). For optimal survival, Ras activation was both necessary and sufficient in neuronal cultures (Klesse and Parada, 1998; Mazzoni et al., 1999). When both Trk receptors and p75 were activated concurrently, Trk signaling led to selective inhibition of the JNK pathway, without affecting the NF-κB pathway, highlighting the critical role that the JNK pathway plays (Yoon, 1998). In regard to specific downstream effectors of Trk that are involved in JNK inhibition, PI-3 kinase has emerged as a potential key player, since inhibition of PI-3 kinase in the presence of NGF resulted in increase in phospho-c-jun level in NGF-withdrawal paradigm (Tsui-Pierchala et al., 2000). Whether activation of PI-3 kinase is both necessary and sufficient for NGF-dependent survival, however, still remains unresolved (Crowder and Freeman, 1998; Kuruvilla et al., 2000; Philpott et al., 1997; Tsui-Pierchala et al., 2000; Yao and Cooper, 1995).

We have previously reported that although JNK activation was necessary (Yoon, 1998) (Harrington et al., 2002), it was not sufficient for p75-mediated apoptosis, since co-activation of TrkC and p75 resulted in activation of JNK, but no cell death (Harrington et al., 2002). Addition of NGF in PC12 cells resulted in
similar activation of JNK (Minden et al., 1994). These results may be interpreted as that Trk receptors activate two distinct pathways, one of which may inhibit JNK, while the other may initiate a pro-survival pathway.

In this report, we examined the role of PI-3 kinase and ERK pathways that are activated by Trk receptors in counteracting p75 signaling machinery of Rac and JNK. We report that activation of the PI-3 kinase pathway by Trk receptors is required, but not sufficient, to inhibit the Rac-JNK pathway by p75. For optimal survival, activation of the ERK pathway was also required. Together, these results reaffirm that Trk-mediated survival requires inhibition of the pro-apoptotic pathway and simultaneous activation of the pro-survival pathway.
MATERIALS AND METHODS

Materials:
The phospho-Akt, Akt, phospho-Erk, Erk, JNK, and cleaved caspase 3 antibodies were purchased from Cell Signaling Technology (Beverly, MA). The JNK1 (C-17) antibody used for immunoprecipitation/kinase assays was from Santa Cruz Biotech (Santa Cruz, CA), and PY 4G10 and Rac antibodies, from Upstate Biotechnology (Lake Placid, NY). LY294002 was from BioMol, and PD98059, from Cell Signaling. The TUNEL kit and anti-MBP antibody were from Roche Molecular Biochemicals (Indianapolis, IN).

Primary Oligodendrocytes Cultures:
Primary rat oligodendrocytes were isolated from Sprague-Dawley rat pups as previously described (Yoon, 1998). JNK3+/+ and JNK3−/− mice were obtained from Dr. Richard Flavell. For mouse oligodendrocytes cultures, JNK3+/+ and JNK3−/− mice were obtained from heterozygote mating as littermates. The culture procedure has been described (Harrington et al., 2002). The genotype was determined by PCR analyses of tail DNA according to Yang et al. (Yang et al., 1997).
Generation of Recombinant Adenovirus:

The DN-JNK2 has been previously described (Harrington et al., 2002). The cDNA for the CA-p110 virus was isolated from pCG-CA-p110 (p110*; (Hu et al., 1995)) by digesting it with Xba I (partial) and Sca I, and sub-cloned into Track CMV shuttle vector {He, 1998 #120} that was digested with Xba I and EcoR V. The CA-p110 contained the SH2 domain of p85 in its N-terminus, thus it no longer requires binding to p85 for activation. Subsequently, Pme I site within the SH2 domain of p85 was deleted by creating a silent mutation in phenylalanine residue, in order to generate adenoviruses which required a unique Pme I site in the shuttle vector (AAG TTT AAA CGC to AAG TTc AAA CGC; c in small cap indicates a point mutation). The resulting mutant was sequenced for any errors. The cDNA for the DN-p110 virus was isolated from pCG-DN-p110, which contained a deletion in ATP binding motif (p110*Δkin; (Hu et al., 1995)). pCG-DN-p110 was digested with Xba I and Sca I, and the cDNA fragment of the DN-p110 was sub-cloned into Track CMV shuttle vector (He et al., 1998) that was digested with Xba I and EcoR V. This removes the SH2 domain of p85 that was fused in pCG-DN-p110*Δkin. The removal of SH2 domain of p85 in pCG-DN-p110*Δkin was shown to produce a more effective DN-p110 mutant (Bilderback et al., 2000). For RasV12 construct, we introduced the flag tag at its 5’ end by PCR using primers that contained the flag sequence. The resulting construct in pCR II was sequenced for any errors, and was subsequently digested with Sal I and Hind III and ligated into Track CMV. The recombinant adenovirus
constructs of all three mutants were generated in RecA+ bacteria according to He et al. (He et al., 1998). The virus was then produced in 293 cells by transfection and further purified using two rounds of CsCl centrifugation. The CsCl present in the virus preparation was removed by dialysis.

**Immunoprecipitation/Solid Phase Kinase Assays:**

JNK immunoprecipitation/kinase and solid phase JNK kinase assays were performed using a JNK1 polyclonal antibody (C-17, Santa Cruz Biotech), as described (Harrington et al., 2002).

**Measurement of Rac-GTP:**

The Rac activity was as described previously (Harrington et al., 2002).

**JNK1/2 Immunodepletion:**

Lysates of oligodendrocytes treated or untreated with NGF were subject to two rounds of immunodepletion with an anti-JNK1(G151) and anti-JNK2 antibodies. Before depletion, 35S-labeled JNK1 and JNK2 proteins were added to the lysates to serve as a tracer as described (Harrington et al., 2002). Depleted and undepleted lysates were then assayed for JNK activity by solid phase kinase assay. The extent of depletion of JNK1 and 2 was assessed following fluorography.
**Immunocytochemistry:**

The procedures for double staining of mouse oligodendrocytes for MBP and TUNEL were as described (Harrington et al., 2002). For double staining of MBP and cleaved Caspase 3, cells were fixed and stained simultaneously in the presence of 0.3% Triton X100. Quantification of TUNEL+ cells among GFP+ infected cells as well as TUNEL+ among MBP+ cells were described (Harrington et al., 2002).


RESULTS

JNK3 is necessary for p75 mediated apoptosis and caspase 3 activation

We have reported that p75 activates JNK3 in oligodendrocyte cultures {Harrington, 2002 #574}, and in vivo, p75 plays a critical role in apoptotic death of oligodendrocytes following spinal cord injury {Beattie, 2002 #624}. Since JNK3 is an injury-specific form of JNK {Yang, 1997 #372}, we examined whether JNK3 is necessary for p75-mediated apoptosis using cortical oligodendrocytes from JNK3–/– mouse pups and their wild-type littermates. Mouse oligodendrocytes express p75 in culture, but not TrkA (Harrington et al., 2002). In JNK3+/+ oligodendrocyte cultures, NGF treatment for 24 hrs caused a 4-5 fold increase in the proportion of TUNEL+ oligodendrocytes, while NGF addition had no effect on JNK3–/– oligodendrocytes (Fig. 7A). These data indicate that JNK3 is indeed essential for p75-mediated apoptosis.

Since p75 activates caspases and induces cytochrome C release, and their activation was shown to be necessary for NGF-dependent apoptosis {Gu, 1999 #423} {Wang, 2001 #638} {Troy, 2002 #637}, we asked whether JNK3 is required for caspase activation. Cortical oligodendrocytes were prepared from JNK3+/+ and JNK3–/– littermates, and NGF-dependent changes in the proportion of caspase 3+ cells were determined among MBP+ cells. In JNK3+/+ oligodendrocytes, NGF treatment for 24 hrs...
resulted in 4 fold increase in the proportion of oligodendrocytes that were positive for active caspase 3, while it produced no increase in JNK3+/− oligodendrocytes. These results therefore indicate that JNK3 activation is necessary for activation of the caspase pathway and apoptosis in p75-mediated apoptotic signaling.

**Ras activation is sufficient to suppress p75 mediated cell death**

We have previously reported that concurrent activation of Trk A can override the apoptotic action of p75 by inhibiting p75-mediated JNK activity (Yoon, 1998). When we activated endogenous TrkC, instead of introducing TrkA into oligodendrocytes, however, p75-mediated JNK activation remained unaffected, leading to the conclusion that JNK activation was necessary, but not sufficient (Harrington, 2002 #574). We therefore decided to analyze the downstream effectors that Trk receptors utilize to block p75-mediated apoptosis.

As a member of receptor tyrosine kinases, Trk receptors activate Ras upon binding neurotrophins. In sympathetic and sensory neurons, Ras activation was shown to be both necessary and sufficient for NGF-dependent survival (Klesse and Parada, 1998; Mazzoni et al., 1999). We therefore examined whether Ras activation

The data with the CA-p110 mutant suggest that there are additional signaling pathways that play roles in inhibiting p75-mediated JNK activation and apoptosis. A candidate is the Ras-ERK pathway. In sympathetic and sensory neurons, Ras activation was shown to be both necessary and sufficient for NGF-dependent survival (Klesse and Parada, 1998; Mazzoni et al., 1999). We therefore infected oligodendrocytes with
constitutively-active Ras, RasV12, or the control GFP virus and determined whether RasV12 can override the apoptotic action of p75. With RasV12, p75-mediated apoptosis was completely blocked even in the presence of NGF (Fig. 4A). These data indicate that Ras activation is also sufficient to inhibit p75-mediated apoptosis, in addition to NGF-withdrawal-mediated death.

RasV12 activated both the ERK and PI-3 kinase pathways in oligodendrocytes (Fig. 4B). Since the ERK pathway was shown to play a role in promoting survival (Klesse and Parada, 1998; Mazzoni et al., 1999), we treated oligodendrocytes with PD98059 to inhibit ERK kinase, MEK, and assess its effect on cell survival. Addition of 25 µM PD98059 prior to BDNF and NT3 addition resulted in attenuation NGF-dependent survival (Fig. 5A). 25 µM PD98059 was effective in these cultures to block ERK activation as shown by reduction in phospho-ERK Western (Fig. 5B). These data therefore indicate that the ERK pathway plays a role as one of the effectors that are activated by Trk receptors to counteract the apoptosis by p75.

**Ras activation inhibits NGF-dependent JNK activation, including JNK3**

Since RasV12 was sufficient to protect oligodendrocytes from apoptosis and activated both the PI-3 kinase and ERK pathways, we tested the effect of RasV12 on p75-mediated JNK activation. Expression of RasV12 in oligodendrocytes by itself led to an increase in the basal level of JNK activity (Fig. 6A), as has been reported (Minden et al., 1994). With NGF treatment, however, there was no increase in JNK activity in RasV12 infected cultures, while NGF induced JNK activation in cultures infected with the GFP
control virus. These results are in agreement with our earlier report that JNK activation is necessary, but not sufficient for p75-mediated apoptosis (Harrington et al., 2002).

The lack of sufficiency of JNK suggests that p75 may activate additional pathways to induce apoptosis. Alternatively, activation of specific isoform of JNK may be necessary. We have previously reported that NGF binding to p75 induces JNK3 activation (Harrington et al., 2002). Since JNK3 plays a role in injury-mediated apoptosis (Yang et al., 1997), and p75 is one of the critical components for injury-mediated apoptosis in vivo (Beattie et al., 2002), we next asked whether the high basal level of JNK activity that we observed with RasV12 represents the activity from JNK1 and 2, and not JNK3. For this, the lysates were subjected to immunodepletion using JNK1 and 2 antibodies, and the resulting depleted lysates were used in JNK kinase assays as a measure of JNK3 activity. The extent of depletion is shown in Fig. 6B (lower panel). The basal level of JNK3 was higher with RasV12 compared to GFP control (Fig. 6B, upper panel), suggesting that activation of Ras induces JNK3 activity as well. In GFP control cultures, NGF addition led to JNK3 activation as we have previously reported (Harrington et al., 2002). With RasV12, NGF addition caused a slight reduction in JNK3 level, suggesting that RasV12 may inhibit JNK3 activation by p75.
Co-activation of Trk’s and p75 leads to activation of the PI-3kinase pathway in oligodendrocytes

We have previously reported that concurrent activation of Trk receptors can override the apoptotic action of p75 (Yoon, 1998; Harrington et al., 2002). The downstream effectors that Trk receptors utilize to block p75-mediated apoptosis are not known. A likely candidate is PI-3 kinase, since the PI-3 Kinase pathway has been shown to play an essential role NGF-dependent neuronal survival (Crowder and Freeman, 1998; Kuruvilla et al., 2000). We first tested whether the PI-3 Kinase pathway is activated by co-activation of TrkA, B, or C in oligodendrocytes. Since oligodendrocytes do not express TrkA under our culture conditions, we introduced TrkA into these cells by infecting them at the progenitor stage with TrkA harboring retrovirus as described (Yoon, 1998). Activation of the PI-3 kinase pathway was first assessed by the extent of tyrosine phosphorylation of p85, the regulatory subunit of PI-3 kinase. Following treatment with purified NGF for 5 min at 100 ng/ml, p85 was tyrosine phosphorylated among TrkA infected cells, and not among the cells that were infected with a control virus (Fig. 1A, upper panel). NGF treatment of TrkA expressing cells also led to an increase in AKT phosphorylation (Fig. 1A, lower panel). Similarly, activation of the endogenous TrkB and TrkC with BDNF and NT3, respectively, resulted in an increase in AKT phosphorylation in oligodendrocytes (Fig. 1B). These results indicate that co-activation of p75 with TrkA, TrkB, or TrkC led to activation of the PI-3 kinase pathway in oligodendrocytes.
Activation of the PI-3 Kinase pathway is necessary to prevent p75-mediated JNK activation and apoptosis

We next asked whether activation of the PI-3 kinase is necessary for Trk receptors to prevent p75-mediated JNK activation and apoptosis. For this, the oligodendrocytes infected with TrkA retrovirus were treated with LY294002, a PI-3 Kinase inhibitor, at 25 µM, 50 µM, or with the vehicle alone, prior to NGF treatment for 4 hrs. JNK activity was measured in immunoprecipitation-kinase assays using GST-c-jun as a substrate. In vehicle treated cells, the addition of NGF did not activate JNK, although there was an increase in the basal level of JNK activity (Fig. 2A). In oligodendrocytes treated with LY294002, there was a NGF-dependent increase of JNK activity, suggesting that blocking the PI-3 kinase pathway restored NGF-dependent JNK activation even in the presence of TrkA. Treatment with LY294002 was effective in blocking the PI-3 kinase pathway as shown by significant reduction in phosphorylated AKT level (Fig. 2A, middle panel). These data suggest that PI-3 Kinase is necessary for TrkA to suppress p75-mediated JNK activation.

Since NGF-dependent JNK activity was restored with the inhibition of PI-3 kinase, we tested the effect of LY294002 on Trk receptor-mediated survival, via the endogenous TrkB and TrkC without introducing TrkA. We have previously demonstrated that oligodendrocytes express TrkB and TrkC (Harrington et al., 2002). Oligodendrocytes were treated with LY294002 at 25 µM or vehicle for 30 minutes, followed by NGF, BDNF, or NT3 for 4 hrs. The effect of LY294002 on cell survival was assessed by counting the number of TUNEL⁺ cells among myelin basic protein, MBP⁺, oligodendrocytes. In the vehicle treated control, the proportion of TUNEL⁺ cells
increased 10 fold following NGF treatment, but not with BDNF and NT3 (Fig. 2B). This is consistent with our previous report (Harrington et al., 2002). In cells treated with LY294002, however, BDNF and NT3 induced apoptosis to the same extent as NGF (Fig. 2B). This result suggests that PI-3 kinase activation by Trk receptors is required to prevent p75-induced apoptosis. Since LY294002 could potentially act on targets other than PI-3 kinase, we also utilized a dominant negative mutant of PI-3 kinase to inhibit the PI-3 kinase pathway directly. The dominant negative mutant of PI-3 kinase was modified from the original construct, p110*Δkin, (Hu et al., 1995), by deleting the SH2 domain of p85 as described (Bilderback et al., 2000). The effect of the DN-p110 is shown in Western analyses, where NT3-mediated increase in phospho-AKT was abrogated in the presence of the DN-p110 (Fig. 2C, lower panel). To assess the effect of the DN-p110 on cell survival, oligodendrocytes were then infected with the DN-p110 or the control GFP adenovirus. 24 hrs after infection, cells were treated with NT3 at 100 ng/ml or left untreated for 4 hrs, and TUNEL assays were performed. The number of TUNEL+ cells among virus infected GFP+ oligodendrocytes was then quantified. As shown in Fig. 2C, the DN-p110 blocked the survival effect of NT3. These data, together with the LY294002 data, indicate that PI-3 kinase is necessary for Trk receptors to counteract the apoptotic action of p75.

**PI-3 kinase is not sufficient to prevent JNK activation and apoptosis by p75**

Since PI-3 kinase is one of the downstream effectors of Trk receptors that are required for counteracting the action of p75, we tested whether independent activation of PI-3 kinase in the absence of Trk activation is sufficient to promote cell survival. For
this, we generated an adenovirus with a constitutively active form of PI-3 Kinase, CA-p110. The CA-p110 has been described (Hu et al., 1995). Oligodendrocytes were infected with the CA-p110, or the GFP control virus, and treated with NGF for 4 hrs or left untreated. Surprisingly, oligodendrocytes expressing the CA-p110 underwent apoptosis to a similar extent as the control virus infected cells following NGF treatment, (Fig. 3A). Based on phospho-AKT Western, the CA-p110 was effective in activating the PI-3 kinase pathway (Fig. 3A, lower panel). To confirm the effect of the CA-p110 on apoptosis, we analyzed its effect on activation of the caspase pathway, a different measure of apoptosis. Activation of caspase pathway was shown to be necessary for p75-mediated apoptosis in oligodendrocytes (Gu et al., 1999). Following NGF treatment, the extent of caspase activation was assessed by counting the number of cells that were stained for active, cleaved caspase 3. In cells infected with the control GFP virus, NGF induced an increase in the proportion of cells that were positive for cleaved caspase 3 (Fig. 3B). Similar increase in the proportion of cleaved caspase 3+ cells was observed in the presence of the CA-p110, while the NGF-dependent increase was abrogated with a dominant-negative JNK (Fig. 3B). The role of the JNK pathway as an upstream regulator of the caspase pathway has been shown (Tournier et al., 2000). These results therefore suggest that activation of PI-3 kinase is not sufficient to block p75-mediated apoptosis as well as activation of the caspase pathway in oligodendrocytes.

We have previously shown that Rac activation was required for p75-mediated JNK activation and subsequent apoptosis (Harrington et al., 2002). Co-activation of Trk and p75 resulted in either attenuation or complete inhibition of Rac activity, suggesting that Trk receptor signaling intercepts p75 signaling at or above the level of Rac GTPase
(Harrington et al., 2002). We therefore examined the effect of the CA-p110 on p75-mediated activation of Rac and JNK. NGF addition resulted in activation of Rac and JNK even in the presence of the CA-p110 (Fig. 3C, and D), which is in correlation with its failed effect to counteract the apoptotic signaling by p75. These data indicate that although activation of the PI-3 kinase pathway is necessary for Trk receptors to suppress apoptotic signaling by p75, activation of PI-3 kinase alone is not sufficient to inhibit p75-mediated JNK activity and apoptosis.
DISCUSSION

We have previously identified the Rac-JNK pathway as the component of apoptotic machinery in p75 signaling (Harrington et al., 2002). In this report, we present the data that JNK3 activity is required for NGF-dependent apoptosis and also for activation of the caspase pathway. These data therefore help establish the linear relationship from Rac to JNK3 and to the caspase pathway in p75 signaling. To counteract this apoptotic signal from p75, Trk receptors utilize Ras. On one hand, Ras blocks NGF-dependent increase in JNK activity by activating the PI-3 kinase pathway. At the same time, Ras induces, in parallel, activation of the anti-apoptotic, MEK-ERK pathway. In support, Ras activation was sufficient to completely block p75-mediated apoptosis and NGF-dependent JNK3 activity. These results together reaffirm that Trk-mediated survival signaling requires inhibition of the pro-apoptotic pathway and simultaneous activation of the pro-survival pathway.
PI-3 kinase is necessary but not sufficient to inhibit p75-mediated JNK activity and apoptosis

PI-3 kinase plays an important role for NGF-dependent cell survival. In PC12 cells, inhibition of PI-3 kinase induced cell death in the presence of NGF (Yao and Cooper, 1995), suggesting that PI-3 kinase activity is necessary for NGF-dependent cell survival. In sympathetic neurons, activation of PI-3 kinase was shown to promote cell survival in the absence of NGF (Mazzoni et al., 1999; Philpott et al., 1997), suggesting that PI-3 kinase activation is sufficient to promote cell survival. Whether PI-3 kinase is also necessary for NGF-dependent survival of sympathetic neurons, however, remains unresolved with opposite results reported. LY294002 inhibited NGF-mediated survival almost completely (Crowder and Freeman, 1998; Kuruvilla et al., 2000), while it caused a partial inhibition (Tsui-Pierchala et al., 2000), or completely failed to block NGF-dependent survival response (Philpott et al., 1997). The reason for the difference is not clear, but it may reflect multiple roles that PI-3 kinase plays in overall survival of these neurons. In compartmentalized culture paradigm, PI-3 kinase was shown to be critical in initiating a retrograde transport of NGF from the distal axons to the cell body (Kuruvilla et al., 2000). This result may suggest that PI-3 kinase regulates certain aspects of cytoskeletal reorganization, since retrograde transport would involve changes in cytoskeletal components. This function may be a prerequisite in vivo for PI-3 kinase to act as a pro-survival signal in the cell body. Rather than using NGF withdrawal as a paradigm to study the signaling mechanisms that TrkA and p75 utilize to regulate cell survival, here we activated p75 alone or p75 with Trk receptors in oligodendrocytes. Our results suggest that Trk-mediated survival response includes PI-3 kinase, but Trk needs to
activate additional pathways to completely override the apoptotic signal by p75. The MEK-ERK pathway is one such pathway, since inhibition of the MEK-ERK pathway resulted in partial block in Trk-mediated survival. This result is in agreement with the conclusion from Tsui-Pierchala et al. (Tsui-Pierchala et al., 2000) that additional pathways other than PI-3 kinase contribute to Trk-mediated survival.

In oligodendrocytes, inhibition of Trk-mediated PI-3 kinase action resulted in an increase in JNK activity, and coordinate increase in apoptosis (Fig. 2). This is in agreement with the data in sympathetic neurons, where inhibition of PI-3 kinase in the presence of NGF also resulted in increase in JNK activity, as measured by phospho-c-jun level (Tsui-Pierchala et al., 2000). These results together suggest a functional link between PI-3 kinase and JNK. The constitutively active mutant of PI-3 kinase, on the other hand, failed to inhibit p75-mediated JNK activation and apoptosis (Fig. 3), while RasV12 blocked NGF-dependent JNK activation (Fig. 6). These results suggest that p75 activates additional distinct pathways that could contribute to JNK activation. Alternatively, Ras inhibits a specific isoform of JNK, such as JNK3, that is required for p75-mediated apoptosis. Ras activation may inhibit all the pathways involved including the additional pathway, while PI-3 kinase may modulate only one of the JNK pathways. A candidate for such pathway may be ceramide-activated JNK pathway. Neurotrophin binding to p75 has been shown to induce an increase in intracellular ceramide (Dobrowsky et al., 1994), and it was recently shown that inhibition of neutral sphingomyelinase led to inhibition of JNK and subsequent protection of cells from NGF-mediated death (Brann et al., 2002). We have previously reported that p75 to Rac and JNK forms a linear pathway that is required NGF-dependent apoptosis (Harrington et al.,
2002). It remains to be determined whether ceramide production is directly linked to the Rac-JNK pathway, or whether it forms a divergent pathway that eventually converges on JNK.


Figure A1.1 Neurotrophins Activate PI-3 Kinase in Oligodendrocytes.

A, NGF activates PI-3 Kinase in oligodendrocytes ectopically expressing TrkA. Oligodendrocyte precursors were infected with a retrovirus encoding TrkA. After differentiation, NGF (100 ng/ml) was added to the cells for 10 minutes. The regulatory subunit of PI-3 Kinase, p85, was immunoprecipitated and subject to Western blot for phospho-tyrosine (PY). The blot was stripped and re-probed for p85. As a control, oligodendrocytes not expressing TrkA were also tested. Lysates from TrkA expressing oligodendrocytes were also probed for phosphor-Akt. The blot was stripped and reprobed for total Akt. 

B, BDNF and NT3 activated PI-3 Kinase in oligodendrocytes. Oligodendrocytes were treated with BDNF or NT3 at a concentration of 100 ng/ml for 10 minutes. Lysates were probed for phospho-Akt. The blots were stripped and reprobed for total Akt.
Figure A1.2 PI-3 Kinase is Necessary to Prevent P75-Mediated JNK Activation and Cell Death.

A, PI-3 Kinase is required to suppress JNK activation by p75. Oligodendrocytes infected with the TrkA retrovirus were pretreated with LY294002 at 25 and 50 µM concentrations, or DMSO, followed by NGF (100 ng/ml) for four hours. Lysates were prepared and JNK immunoprecipitation/kinase assay was performed. Lysates were also subject to Western blot to analyze phospho-Akt and total Akt levels. B, PI-3 Kinase is required to prevent p75-mediated cell death. Oligodendrocytes were pretreated with LY294002 at 25 µM or DMSO, followed by NGF, BDNF, or NT3 at 100 ng/ml for four hours. Samples were fixed stained for TUNEL and MBP. The number of TUNEL+ cells among MBP+ cells was counted. C, NT3 induces apoptosis in cells expressing a dominant negative PI-3 Kinase. Oligodendrocytes were infected with adenoviruses expressing DN-p110 or GFP as a control. Twenty-four hours later, cells were treated with NT3 at 100 ng/ml for four hours. The TUNEL assay was performed and apoptosis was quantified by counting TUNEL+ cells among cells expressing GFP and displaying oligodendrocyte morphology.
**Figure A1.3  PI-3 Kinase is Not Sufficient to Prevent P75-Mediated JNK Activation or Cell Death.**

A. Oligodendrocytes were infected with an adenovirus expressing CA-p110 or a GFP control. Cells were treated with NGF at 100 ng/ml for four hours. Apoptosis was quantified as in Figure 2C. B. JNK and Rac are activated by p75 in CA-p110 expressing oligodendrocytes. Oligodendrocytes infected with CA-p110 or GFP adenoviruses were treated with NGF for four hours. Cells were harvested and a solid-phase JNK kinase assay was performed. As an internal control, a western blot for JNK was performed. A Rac activity assay was done on lysates from oligodendrocytes infected with either GFP or CA-p110 adenoviruses and treated with NGF for a timecourse.
Figure A1.4 Ras is Sufficient to Prevent P75-Mediated Apoptosis.

A, RasV12 prevents p75-induced cell death. Oligodendrocytes were infected with GFP or RasV12 expressing adenoviruses. Cells were then treated with NGF at 100 ng/ml for four hours and apoptosis was quantified as before. B, RasV12 activates Erk and Akt. Oligodendrocytes were infected with GFP, CA-p110, or RasV12 adenoviruses. Lysates from infected cells were analyzed by western blot for phospho-Akt, and phospho-Erk. Blots were stripped and reprobed for total Akt and Erk.
Figure A1.5 Erk plays a role in suppressing p75-mediated apoptosis.

A. BDNF and NT3 can induce apoptosis in oligodendrocytes after Erk inhibition. Oligodendrocytes were pretreated with PD98059 at 25 µM or DMSO followed by NGF, BDNF, or NT3 at 100 ng/ml for four hours. Cells were fixed and stained for TUNEL and MBP. The number of TUNEL* among MBP* was counted. B. PD98059 effectively inhibits Erk. Lysates from oligodendrocytes treated with DMSO or PD98059 were probed for Erk and phospho-Erk.
Figure A1.6 RasV12 Prevents NGF-Dependent Activation of JNK, Yet Increases Basal JNK Activity.

A. JNK activity was measured by solid phase kinase assay from oligodendrocytes infected with RasV12 or GFP adenovirus and treated with or without NGF for four hours. B. RasV12 increases basal JNK3 activity. A JNK solid phase kinase assay was performed on oligodendrocytes lysates depleted of JNK1 and 2. Radiolabeled JNK1 and 2 protein was added to the lysates before immunodepletion to serve as a tracer. Lysates before and after depletion were run on SDS-PAGE. Labeled JNK1 and 2 proteins were detected by enhanced autoradiography.
Cdk5 and its regulators p35 and p39 are essential for neuronal migration and for proper layering of the cortex. It was found that Rac and its effector PAK1 were present in complexes of Cdk5 and p35, associating directly with p35 (Nikolic et al., 1998). We asked whether Cdk5 played a role in Rac activation by p75. Cdk5 kinase activity was assayed from oligodendrocytes treated with NGF or NT3 for various times. NGF treatment of oligodendrocytes led to a significant increase in Cdk5 activity at 1 hour and 4 hour timepoints based on its ability to phosphorylate H1 histone. NT3 treatment, on the other hand, did not activate Cdk5 (Figure 1A).

We next asked whether this activation of Cdk5 was necessary for p75 to induce apoptosis. Treating NGF to cultured oligodendrocytes causes p75 dependent apoptosis starting around 4 hours. Inhibiting Cdk5 with the drug roscovitine, suppressed this apoptosis slightly at higher concentrations, although did not completely rescue cells (Figure 1B). This leads us to conclude that while Cdk5 activation is not necessary, its activation may contribute to p75 dependent apoptosis.
MATERIALS AND METHODS

CDK5 kinase measurement

Oligodendrocyte treated with NGF (100 ng/ml) or NT3 (100 ng/ml) for various times were lysed in a buffer containing 25mM HEPES (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1% NP-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 25 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 2 mM PMSF. The lysates were immunoprecipitated using an antibody to Cdk5 (Santa Cruz Biotech). Immunoprecipitates were incubated with 3 ug histone H1 and 2 uCi ³²P-ATP for 20 minutes at 30 degrees Celcius. Samples were run on SDS-PAGE and the gel processed for autoradiography.

Quantification of apoptotic oligodendrocytes

Cultured oligodendrocytes were pretreated with roscovitine or DMSO for 30 minutes prior to the addition of NGF (100 ng/ml). After 4 hours, the cells were fixed and processed for counting as described previously (Harrington et al., 2002).
A.

Figure A2.1  Cdk5 activation by p75 contributes to apoptosis.

Oligodendrocytes treated with NGF or NT3 for various timepoints were assayed for Cdk5 activity. B. Oligodendrocytes treated with various concentrations of roscovitine were analyzed for NGF dependent apoptosis. Increasing roscovitine concentrations decreased the amount of cell death.
APPENDIX C

RESULTS

We previously observed that Rac GTPase is activated by p75 and this activation is critical for p75 to illicit apoptosis in oligodendrocytes (Harrington et al., 2002). P75 can also affect the activity of Rho GTPase as reported previously (Yamashita et al., 1998). We asked whether activation of the small G proteins Rac and Rho were altered in brains of p75 null mice. To test this, pull down assays using GST fusion proteins of PAK1 and Rhotekin were used to quantify activated Rac and Rho, respectively. Interestingly, levels of Rac activity were similar between wild type and p75 null in the cortex, but in the cerebellum Rac activity was lower in p75 null mice (Figure 1). This result was opposite for Rho, where in the cerebellums of p75 null mice the activity was increased compared to wild type (Figure 1). No active Rho was detected in the cortexes of these animals. These results suggest in vivo regulation of Rac and Rho activities by p75.
MATERIALS AND METHODS

Rac/Rho Activity Assay

Mouse brains were dissected from wild type or p75-/- mice around 3 months of age. Cerabella and cortices were separated and homogenized in a buffer containing 25mM HEPES (pH 7.5), 150 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA, 10% glycerol, 1% NP-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 25 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 2 mM PMSF. Lysates were rotated for 5 minutes at 4°C, and clarified by centrifugation at 14,000 rpm for 5 minutes at 4°C. 10% of the supernatant of each sample was saved for Western analysis as a control for the total Rac protein using anti-Rac 1 antibody (Upstate Biotechnology, Lake Placid, NY), while the remainder was incubated with approximately 30 µg of GST-PBD bound to glutathione Sepharose beads. Lysates were rotated with beads for 1 hour at 4°C and washed 3 times with lysis buffer. Bound Rac-GTP protein was detected by Western analysis using anti-Rac 1 antibody (Upstate Biotechnology, Lake Placid, NY). Assay for active Rho was done identically, with the exception of using GST-Rhotekin for the pull down assay, and an incubation time of 45 minutes as opposed to 1 hour.
Figure A3.1  Altered Rac and Rho activity in the cerebellum of p75 null mice.

Brains were dissected from wild type or p75 knockout mice and cerebella were divided from cortexes. Tissues were homogenized, and lysate used for pull down assays. Activated Rac and Rho were detected by Western blot. Total Rac and Rho levels and Actin Western were performed as controls.
REFERENCES


Bentley CA, Lee KF (2000) p75 is important for axon growth and schwann cell migration during development [In Process Citation]. J Neurosci 20: 7706-7715.


Widenfalk, J., Lundstromer, K., Jubran, M., Brene, S., and Olson, L. (2001). Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. Journal of Neuroscience 21, 3457-3475.


Dalpe G, Zhang LW, Zheng H, Culotti JG. Conversion of cell movement responses to Semaphorin-1 and Plexin-1 from attraction to repulsion by lowered levels of specific RAC GTPases in C. elegans. Development. 2004 May;131(9):2073-88


