UNIVERSITY OF CALIFORNIA, SAN DIEGO

The regulation of cAMP by the p75 Neurotrophin Receptor – a novel mechanism for the inhibition of scar resolution after injury

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Benjamin David Sachs

Committee in charge:

Professor Katerina Akassoglou, Chair
Professor Joan Heller-Brown
Professor Laurence L. Brunton
Professor Sylvia M. Evans
Professor Paul A. Insel
Professor Jason Xiao-Jian Yuan

2008

The Dissertation of Benjamin David Sachs is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2008

DEDICATION

This thesis is dedicated to my wife, Lindsey, my mom, my dad, my sister, and to my best friend, Jedd.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

aa	amino acid
AD	Alzheimer’s Disease
AEC	3-amino-9-ethylcarbazole
AKAR	A kinase activity reporter
ANOVA	analysis of variance
ARMS	ankyrin-rich membrane spanning protein
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
C-terminus	carboxy terminus
CGN	cerebellar granule neuron
CNS	central nervous system
COPD	Chronic Obstructive Pulmonary Disease
CRD	cysteine rich domain
Cy3	cyanine 3
DAPI	4',6-Diamidine-2'-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol

EAE	experimental autoimmune encephalomyelitis
ECD	extracellular domain
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
Epac	exchange protein activated by cAMP
ERK	extracellular regulated kinase
FAP-1	Fas associated phosphatase – 1
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FL	full length
g	force of gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
HA	hemagluttinin
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
Hepes	*N*-(2-Hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid)

HSC	hepatic stellate cell
IBMX	isobutylmethylxanthine
ICD	intracellular domain
Ig	immunoglobulin
IKK-β	IκB kinase β
IL	interleukin
IP	immunoprecipitation
i.p.	intraperitoneal
IRAK	interleukin-1 receptor associated kinase
JNK	Jun kinase
K_D	dissociation constant
kDa	kilodalton
LINGO-1	LRR and Ig domain containing, Nogo receptor interacting protein
LMP	low melting point
LPS	lipopolysaccharide
LR	linker region
LRR	leucine rich repeat
LTP	long term potentiation
M	molar
MAG	myelin associated glycoprotein
MAGE	melanoma antigen
MS	multiple sclerosis

NaCl	sodium chloride
NADE	$p75^{NTR}$ associated cell death executor
NF-κB	nuclear factor-κB
NGF	nerve growth factor
NGFR	nerve growth factor receptor
NgR	nogo receptor
NT	neurotrophin
N-terminus	amino terminus
NRAGE	neurotrophin receptor interacting MAGE homolog
NRIF	neurotrophin receptor interacting factor
OCT	optimal cutting temperature
OMgP	oligodendrocyte myelin glycoprotein
$p75^{NTR}$	p75 neurotrophin receptor
PAI-1	plasminogen activator inhibitor 1
PA	plasminogen activator
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PFA	paraformaldehyde
PI3K	phosphatidyl inositol 3 kinase
PKA	protein kinase A
PKC	protein kinase C
PNS	peripheral nervous system

PrP	prion protein
PTX	pertussis toxin
RACK-1	receptor for activated C- kinase -1
RT	reverse transcription
RIP	regulated intramembrane proteolysis
Rho-GDI	Rho guanine nucleotide dissociation inhibitor
RNA	ribonucleic acid
RVG	rabies virus glycoprotein
SC	Schwann cell
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
TBS	tris buffered saline
TGF-β	transforming growth factor - β
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
tPA	tissue plasminogen activator
Trk	tropomycin related kinase
Traf6	tumor necrosis factor receptor associated factor 6
UCR	upstream conserved region
uPA	urokinase plasminogen activator
wt	wild-type

LIST OF FIGURES

ACKNOWLEDGMENTS

During the course of my Ph.D. work, I have benefited immensely from the assistance of numerous people both within and outside of the UCSD community. First, I would like to thank my thesis advisor, Katerina Akassoglou, who has provided me with an unparalleled environment in which to pursue and ultimately acquire this degree. She has given me great projects and has been a constant source of scientific guidance and encouragement. Her dedication to her work and to her lab is unmatched, and she has set a great example for how I might make my own lab successful one day.

I am also extremely grateful to have been able to work with all the other members of the Akassoglou lab. I would like to thank all of the original lab members, Ryan Adams, Melissa Passino, and Shoana Sikorski for welcoming me into the lab, showing me the ropes, and always being willing to lend me a helping hand. Shoana, in particular, has provided amazing technical support throughout my entire graduate career and was always willing to go the extra mile for me. I have also received a great deal of support from the second generation of Akassoglou lab members who have repeatedly shared their expertise: especially Christian Schachtrup for help with primary neuronal culture, Dmitrios Davalos with microscopy, and Jerry Lu and Bernat Baeza Raja for a variety experimental assistance and helpful discussions. I would also like to thank all of the undergraduate assistants who tolerated my last minute ordering and autoclaving requests with a smile, starting with Adrienne Andres, who later became a technician in the lab, Priscila Kim, and Jasmine Lee.

My dissertation research was also greatly advanced by the hard work and dedication of a number of graduate students that I have had the pleasure of supervising, especially Julianne McCall, whose incredible work ethic and excitement for science helped make the summer of 2005 one of the most productive periods of my graduate career. I would also like to thank Diane Bushman, my last rotation student, some of whose work also appears in this dissertation.

I am indebted to David Loskutoff, Moses Chao, Miles Houslay, Marco Conti, and Jing Zhang and the members of their laboratories for providing reagents and assistance with experiments. I would also like to thank all the members of my thesis committee, Joan Heller Brown, Lawrence Brunton, Sylvia Evans, Paul Insel, and Jason Yuan for their feedback, advice, and support. I would like to extend additional thanks to Joan Heller Brown and Paul Insel, along with Alexandra Newton, Morton Printz, Palmer Taylor, and Jing Yang and all of their laboratories for allowing me access to equipment. I am grateful to Kenneth Humphries of Susan Taylor's lab for assistance with the PKA assays and to Lisa Gallegos and Maya Kunkel in Alexandra Newton's lab for assistance with FRET experiments. I thank Binhai Zheng for advice on CGN culture.

I would like to thank all my family and friends, starting with my parents, who instilled in me their strong work ethic and have always been a constant source of support. I would like to thank my big sister, Rebecca, for being both a great role model and a great friend through the years. Finally, I thank my wife, Lindsey, whose continued love and support is invaluable for everything I do.

Chapter 3, in part, has been published and appears in The Journal of Cell Biology 2007. Sachs, Benjamin; Baillie, George; McCall, Julianne; Passino, Melissa; Schachtrup, Christian; Wallace, Derek; Dunlop, Allan; MacKenzie, Kirsty; Klussmann, Enno; Lynch, Martin; Sikorski, Shoana; Nuriel, Tal; Tsigelny, Igor; Zhang, Jin; Houslay, Miles; Chao, Moses; Akassoglou, Katerina, The Rockefeller University Press 2007. The dissertation author was the primary investigator and author of this paper.

Chapter 5, in part, has been published and appears in Biochemical Society Transactions 2007. Sachs, Benjamin; Akassoglou, Katerina, Portland Press 2007. The dissertation author was the primary investigator and author of this paper.

VITA

EDUCATION:

2003-2008: *University of California, San Diego*
Ph.D., Biomedical Sciences Graduate Program

1999-2003: *Boston University*
B.A. Biochemistry and Molecular Biology, summa cum laude, with honors
B.A. Psychology, summa cum laude, with honors, with distinction

AWARDS:

2007 **Ruth Kirschstein – NRSA Predoctoral Fellowship,**
"Exploring the role of phosphodiesterase 4A in axonal regeneration"
National Institute of Neurological Disorders and Stroke
2006 **Ray Thomas Edwards Foundation Travel Award,** Society for Neuroscience 36th Annual Meeting
2005 **Pharmacology Training Grant Fellow**, University of California – San Diego
2004 **NIH Scholar,** University of California - San Diego
2003 **Book Award,** Boston University Department of Biochemistry and Molecular Biology
Psychology Excellence in Research Award, Boston University Department of Psychology
Awarded College Honors
Golden Key National Honor Society, lifetime member
Phi Beta Kappa, lifetime member
National Society for Collegiate Scholars, lifetime member
2001 **Undergraduate Research Opportunities Program Award Recipient,** Boston University Department of Psychology
2000 **Dean's List,** Boston University College of Arts and Sciences
1999 **National Merit Scholar**

PUBLICATONS:

1. **B.D. Sachs**, K. Akassoglou. Regulation of cAMP by the p75 neurotrophin receptor: insight into drug design of selective phosphodiesterase inhibitors. 2007. **Biochem Soc Trans**, 35:1273-1277.

2. C. Schachtrup, P. Lu, L.L. Jones, J.K Lee, J. Lu, **B.D. Sachs**, B. Zheng, K. Akassoglou. 2007. Fibrinogen inhibits neurite outgrowth via beta3 integrin-mediated phosphorylation of the EGF receptor. **Proc Natl Acad Sci USA**, 104:11814-11819.
3. **B.D. Sachs,** G. S. Baillie, J. R. McCall, M. A. Passino, C. Schachtrup, D. A. Wallace, A. J. Dunlop, K. F. MacKenzie, E. Klussmann, M. J. Lynch, S. L. Sikorski, T. Nuriel, I. Tsigelny, J. Zhang, M. D. Houslay, M. V. Chao, and K. Akassoglou. 2007. p75 neurotrophin receptor regulates tissue fibrosis through inhibition of plasminogen activation via a PDE4/cAMP/PKA pathway. **J Cell Biol**, 177:1119–1132. (Data from Chapter 3.)

   Editor's Choice: CELL BIOLOGY: N. R. Gough, Scar Formation. ***Sci. STKE*** 2007, tw226 (2007).
4. R.A. Adams, M. Passino, **B.D. Sachs,** T. Nuriel, K. Akassoglou. 2004. Fibrin mechanisms and functions in nervous system pathology. **Molecular Interventions**, 4:163-76.
5. B.E. Thompson, **B.D. Sachs,** Kathleen M. Kantak, J.A. Cherry. The Type IV Phosphodiesterase Inhibitor Rolipram Interferes With Drug-Induced Conditioned Place Preference but not Immediate Early Gene Induction in Mice. **Eur J Neurosci**, , 19:2561-8.
6. D.A. Gold, S.H. Baek, N.J. Schork, D.W. Rose, D.D. Larsen, **B.D. Sachs,** M.G. Rosenfeld, B.A. Hamilton, 2003. RORalpha coordinates reciprocal signaling in cerebellar development through sonic hedgehog and calcium-dependent pathways. **Neuron**. 40:1119-1131.

MANUSCRIPTS IN PREPARATION

1. **Sachs B.D.,** Passino MA, Akassoglou K. The biological functions of $p75^{NTR}$: Intracellular signaling pathways as novel targets for drug discovery. *Invited review,* ***Drug Discov Today***
2. M. Xu, C. V. Remillard, **B. D. Sachs**, A. Makino, O. Platoshyn, W. H. Dillmann, K. Akassoglou, and J. X.-J. Yuan. p75 Neurotrophin Receptor Regulates Agonist-induced Pulmonary Vasoconstriction. Submitted to **Am J Physiol Heart Circ Physiol.**

PRESENTATIONS:

1. **B.D. Sachs,** G. S. Baillie, J. R. McCall, C. Schachtrup, A. J. Dunlop, K. F. MacKenzie, E. Klussmann, M. D. Houslay, M. V. Chao, and K. Akassoglou. Functional interactions between the p75 neurotrophin receptor and phosphodiesterases. **Society for Neuroscience Meeting**, San Diego, California, 2007. Oral Presentation.
2. **B.D. Sachs,** G.S. Baillie, J.R. McCall, C. Schachtrup, A. J. Dunlop, K. F. MacKenzie, E. Klussmann, M. D. Houslay, M. V. Chao, and K. Akassoglou.

Functional interactions between the p75 neurotrophin receptor and phosphodiesterases. **Glial Cells in Health and Disease VIIIth Annual European Meeting**, London, UK, 2007. Poster Presentation.

3. **B.D. Sachs**, M. Passino, S. Sikorski, M.V. Chao, K. Akassoglou. The p75 neurotrophin receptor regulates plasminogen activation and fibrinolysis via a PDE4/cAMP/PKA pathway. **Neurons and Disease**, Atlanta, Georgia, 2006 and **Society for Neuroscience Meeting**, Atlanta, Georgia, 2006. Poster Presentation.
4. **B.D. Sachs**, S. Sikorski, J. Yuan, K. Akassoglou. The p75 neurotrophin receptor regulates pulmonary fibrosis via upregulation of plasminogen activator inhibitor – 1. **Gordon Research Conference on Plasminogen Activation and Extracellular Proteolysis**, Ventura, California, 2006. Poster Presentation.
5. B.D. Sachs, M. Passino, S. Sikorski, M.V. Chao, K. Akassoglou. p75 neurotrophin receptor regulates plasminogen activation and fibrinolysis via a cAMP/PKA pathway. **18th International Society for Fibrinolysis and Proteolysis Meeting,** San Diego, California. 2006. Poster Presentation
6. K. Akassoglou, **B.D. Sachs**, M. Passino, S. Sikorski, T. Nuriel, M.V. Chao. The p75 neurotrophin receptor regulates extracellular proteolysis. **Moving Toward Axon Protection and Remyelination Therapies in Man**, a Satellite Meeting of the Society for Neuroscience Meeting, Washington, D.C., 2005. Poster presentation.
7. B.E. Thompson, **B.D. Sachs,** J.A. Cherry. The PDE4 inhibitor rolipram prevents conditioned place preference induced in mice by cocaine and morphine, but not food. **Society for Neuroscience Meeting**, Orlando, Florida 2002. Poster presentation.

ABSTRACT OF THE DISSERTATION

The regulation of cAMP by the p75 Neurotrophin Receptor – a novel mechanism for the inhibition of scar resolution after injury

by

Benjamin David Sachs

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2008

Professor Katerina Akassoglou, Chair

The p75 neurotrophin receptor ($p75^{NTR}$), a tumor necrosis factor (TNF) receptor superfamily member upregulated after tissue injury, has been extensively studied as a modulator of cell death and survival within the nervous system. However, $p75^{NTR}$ exhibits a widespread pattern of expression during development, after injury and during a number of pathologic conditions within and outside of the nervous system. In many cases, the biological consequences of $p75^{NTR}$ re-expression after injury have yet to be elucidated. My dissertation research has revealed a novel function for $p75^{NTR}$ in the regulation of fibrinolysis after injury. Clearance of fibrin through proteolytic degradation is a critical step in extracellular matrix remodeling that contributes to tissue repair in a variety of pathological conditions, such as sciatic nerve injury, stroke, atherosclerosis, and pulmonary disease. After sciatic nerve crush injury and lipopolysaccharide (LPS) induced lung injury, we demonstrate that $p75^{NTR}$ blocks fibrinolysis by downregulating the serine protease, tissue Plasminogen Activator (tPA), and upregulating Plasminogen Activator Inhibitor-1 (PAI-1).

Furthermore, we have identified a novel signaling pathway downstream of $p75^{NTR}$ in which $p75^{NTR}$ interacts with a specific phosphodiesterase (PDE) isoform, PDE4A5, to regulate cyclic adenosine monophosphate (cAMP) degradation and inhibit plasminogen activation. This mechanism is supported *in vivo* by $p75^{NTR}$ deficient mice, which show increased proteolysis and decreased fibrin deposition after sciatic nerve injury and lung fibrosis. *$p75^{NTR}$-/-* mice also exhibit increased tPA activity within the cerebellum *in vivo,* suggesting that the regulation of cAMP by $p75^{NTR}$ is a general mechanism occurring within the CNS as well. In the CNS, we have demonstrated that the regulation of cAMP by $p75^{NTR}$/PDE4A5 plays a role in myelin induced inhibition of neurite outgrowth in addition to its role in plasminogen activation. Our results reveal a novel pathogenic mechanism by which $p75^{NTR}$ promotes degradation of cAMP, inhibits fibrin clearance, and regulates tissue repair after injury.

Chapter 1: Introduction

This dissertation focuses on the signaling pathways and biological functions regulated by $p75^{NTR}$ after injury. We report a novel role for $p75^{NTR}$ as an inhibitor of fibrinolysis and identify cAMP/PKA signaling as a novel pathway downstream of $p75^{NTR}$. We also provide a mechanism for the regulation of cAMP by $p75^{NTR}$ via the recruitment of phospodiesterase 4A5 (PDE4A5). This pathway becomes activated in response to $p75^{NTR}$ upregulation within the central and peripheral nervous systems (CNS and PNS) as well as outside of the nervous system. We demonstrate that this signaling mechanism regulates scar resolution after sciatic nerve and lung injury, and also regulates myelin inhibition of neurite outgrowth via crosstalk with RhoA signaling. This introductory chapter provides background information about $p75^{NTR}$ signaling and provides the rationale behind my dissertation research.

1. $p75^{NTR}$

1.1. Discovery of $p75^{NTR}$

In the first half of the twentieth century, relatively little was known concerning the regulation of neuronal growth and differentiation. One of the initial clues that neuronal growth is a regulated process came from the observation that mouse sarcoma tumors implanted within the body wall of a three day old embryo promoted the growth of spinal and sympathetic ganglia [1, 2]. At the time, it was not clear whether direct contact with the sarcoma was required to promote growth or whether growth resulted from a diffusible factor released by the sarcoma. This question was answered by the discovery that mouse sarcomas could promote neuronal growth even when implanted

outside the embryo on the allantoic membrane (i.e. in the absence of physical contact), thus suggesting the activity of a diffusible agent. Over the course of the following decade, work by Rita Levi-Montalcini {reviewed in [3]} and others led to the purification of this nerve growth promoting diffusible agent, which was named nerve growth factor (NGF). After NGF was purified and identified, the search began to find the receptor, or receptors, through which NGF mediates its effects. Thirty years later, Dan Johnson, while working in the laboratory of Moses Chao, cloned the nerve growth factor receptor (NGFR), the first identified receptor for NGF [4].

Shortly after NGFR was identified and cloned, a second neurotrophic factor, brain derived growth factor (BDNF), was purified [5] and subsequently cloned [6]. The following year, a third family member, neurotrophic factor 3 (NT-3), was discovered and shown to have high homology to both NGF and BDNF [7]. Soon thereafter, a fourth member of the neurotrophin family, neurotrophin-4 (NT-4) was identified [8]. Subsequent radioligand binding studies revealed that NGFR binds not only to NGF but to all four identified neurotrophins with similar affinity ($K_D = 10^{-9}$ M) [9, 10]. Given that NGFR binds to all four neurotrophins and due to its 75 kDa size, NGFR has been renamed the p75 neurotrophin receptor ($p75^{NTR}$).

1.2 $p75^{NTR}$ Signaling

1.2.1. $p75^{NTR}$ structure

$p75^{NTR}$ is a type one single transmembrane-spanning protein with an amino-terminal extracellular domain (ECD) and carboxy-terminal (C-terminal) intracellular domain (ICD) and was the first member of the tumor necrosis factor receptor (TNFR)

superfamily to be identified. The ECD of $p75^{NTR}$ contains 4 cysteine-rich domains (CRDs) required for ligand binding, N-linked and O-linked glycosylation sites, as well as a stalk domain that plays a role in receptor sorting. A crystal structure for the ECD has been solved [11], however, there is no published crystal structure for the intracellular domain of $p75^{NTR}$ ($p75^{NTR}$-ICD). $p75^{NTR}$-ICD lacks catalytic activity, but does contain a palmitoylation site as well as a juxtamembrane region capable of mediating protein-protein interactions, a death domain near the C-terminus involved in apoptotic signaling, and an extreme C-terminal PDZ binding domain {see Fig. 1, reviewed in [9, 12]}.

1.2.2. Intracellular mediators of $p75^{NTR}$ signaling

Despite its lack of intrinsic enzymatic activity, $p75^{NTR}$ can induce a number of intracellular signaling cascades via the differential engagement of multiple intracellular interactors {see Fig. 2, reviewed in [13]}. Through these different pathways, $p75^{NTR}$ is able to induce a variety of cellular effects. For example, $p75^{NTR}$ interacts with tumor necrosis factor receptor associated factor – 6 (Traf6) [14], $p75^{NTR}$ associated cell death executor (NADE) [15], neurotrophin receptor interacting factor (NRIF) [16], or neurotrophin receptor interacting MAGE protein (NRAGE) to influence cell survival and apoptosis [17]. In addition, $p75^{NTR}$ can regulate cellular proliferation via its interaction with SC-1 [18] which represses cyclin E and inhibits cell cycle progression [19]. RhoA activation through $p75^{NTR}$ is mediated via interactions between $p75^{NTR}$ and RhoA and Rho-GDI [20, 21] and regulates neurite outgrowth and cellular differentiation [22]. $p75^{NTR}$ also regulates NF-κB activation

via its association with Fas associated phosphatase – 1 (FAP-1), a phosphatase originally reported to interact with the TNFR superfamily member, Fas [23]. These intracellular $p75^{NTR}$ interacting proteins play important roles in the transduction of signals from extracellular ligands as well as during ligand independent $p75^{NTR}$ signaling.

1.2.3. Ligand dependent signaling through $p75^{NTR}$

1.2.3.1. Neurotrophin signaling through $p75^{NTR}$

Neurotrophins can play a major role in signal transduction via $p75^{NTR}$ by modulating the interactions between $p75^{NTR}$ and its aforementioned intracellular signaling mediators. In general, neurotrophin binding to $p75^{NTR}$ is associated with the activation of signaling pathways that promote cell survival. For example, neurotrophin binding to $p75^{NTR}$ promotes the association between the $p75^{NTR}$-ICD and TNF receptor associated factor 6 (Traf6). In response to Traf6 binding $p75^{NTR}$, interleukin-1 receptor associated kinase (IRAK) and an atypical protein kinase C (aPKC) get recruited to the complex and subsequently recruit and activate IκB kinase β (IKK-β). Active IKK-β then phosphorylates IκB, which dissociates IκB from NF-κB, ultimately leading to NF-κB activation and neuronal survival [24, 25].

As stated previously, signaling through $p75^{NTR}$ does not always result in cell survival, but can also induce cell death. Like other members of the TNF receptor superfamily, such as TNFR1 and 2, Fas, and CD40, $p75^{NTR}$ has a death domain and induces apoptosis via the activation of caspases [26-28]. In contrast to the other TNF receptor superfamily members, the induction of apoptosis by $p75^{NTR}$ occurs via a non-

traditional, caspase-8 independent manner [26]. The promotion of apoptosis by $p75^{NTR}$ depends upon the activation of the Jun kinase (JNK) signaling cascade [29]. Several important components of this signaling pathway have been identified, including neurotrophin receptor interacting factor (NRIF) [16], and Traf6 [14]. Genetic loss of either NRIF or Traf6 prevents $p75^{NTR}$ induced JNK activation and apoptosis. It has been proposed that NRIF, Traf6, and $p75^{NTR}$ form a complex in which Traf6, a ubiquitin ligase, ubiquitinates NRIF and allows it to enter the nucleus and induce apoptosis [30]. As Traf6 can also promote the survival of neurons via the recruitment of IRAK, it is possible that the relative levels of IRAK and NRIF recruited to $p75^{NTR}$ determines whether signaling results in cell survival or cell death.

Another potential explanation for $p75^{NTR}$'s intriguing ability to promote either cell survival or cell death comes from the fact that $p75^{NTR}$ undergoes regulated intramembrane proteolysis (RIP) upon cleavage by gamma secretase [31]. The nuclear translocation of NRIF has been shown to depend upon RIP of $p75^{NTR}$ [30]. It is possible that in the absence of proteolytic cleavage, $p75^{NTR}$ activates the NF-κB pathway and that cleavage induces the switch to the pro-apoptotic JNK activation. These results fit in well with data suggesting that the pro-apoptotic ligands of $p75^{NTR}$ promote RIP, while the ligands associated with cell survival, such as the neurotrophins, do not [30]. In addition to NRIF translocation and JNK activation, $p75^{NTR}$ RIP has also been shown to regulate interactions between $p75^{NTR}$ and its co-receptor TrkA [32], and to play a role in the inhibition of neurite outgrowth [33], but the precise mechanisms underlying these effects remain unclear. It is known that RIP of $p75^{NTR}$ generates a soluble intracellular fragment, $p75^{NTR}$-ICD, that has been

reported to translocate to the nucleus [34]. However, documenting $p75^{NTR}$-ICD translocation to the nucleus has proven quite difficult, and its precise role upon nuclear entry remains to be determined. It is likely that the cleaved ICD of $p75^{NTR}$ has additional, unidentified signaling capabilities {reviewed in [10]}. Future research will determine the contribution of RIP of $p75^{NTR}$ to physiological and pathophysiological processes.

In some cases, $p75^{NTR}$ binding to neurotrophins activates sphingomyelinase and generates the second messenger, ceramide [35]. Ceramide generation by $p75^{NTR}$ activation has been reported to promote either apoptosis [29] or cell survival [36] depending on the specific cell type. These differing effects of $p75^{NTR}$ induced ceramide generation likely stem from ceramide's own ability to modulate multiple signaling pathways {reviewed in [37]}. For example, ceramide can influence NF- κB (pro-survival), JNK (pro-apoptotic), and extracellular regulated kinase (ERK) signaling. The specific contribution of $p75^{NTR}$ induced ceramide generation to each of these signaling pathways has yet to be completely determined.

1.2.3.2. Nontraditional ligands of $p75^{NTR}$

In addition to the well established role of $p75^{NTR}$ as a neurotrophin receptor, more recent work has revealed that $p75^{NTR}$ also binds several additional ligands to induce signaling. For example, it was reported by Barbara Hempstead's group in 2001 that the pro-neurotrophins, the uncleaved precursors of neurotrophins, can be secreted and bind to $p75^{NTR}$ to regulate cell survival [38]. This group proposed that the pro-forms of the neurotrophins act as high affinity ligands for $p75^{NTR}$ and induce cell

death, while the mature forms of the neurotrophins preferentially activate a different class of neurotrophin receptors to promote survival, the tropomycin related kinase (Trk) receptors.

The role of $p75^{NTR}$ as a receptor extends beyond the neurotrophins and their precursors. Recent work has revealed that several unrelated proteins can also engage in ligand-receptor interactions with $p75^{NTR}$. For example, work from the Gilchrest group has revealed that beta amyloid binds to $p75^{NTR}$ with nanomolar affinity to induce cell death, revealing a potential role for $p75^{NTR}$ in Alzheimer's disease (AD) [39]. $p75^{NTR}$ has also been implicated in the pathogenesis of prion diseases through work demonstrating that a prion protein specific peptide (PrP106-126) binds to $p75^{NTR}$ and induces apoptosis [40]. In addition, $p75^{NTR}$ also serves as a receptor for rabies virus glycoprotein (RVG) and is hypothesized to allow rabies virus to infect neurons [41]. Future research will establish the specific contribution of RVG, beta amyloid, and PrP binding to $p75^{NTR}$ during disease pathology *in vivo*. Despite the lack of *in vivo* data, these studies reveal that $p75^{NTR}$ is a promiscuous receptor, capable of interacting with numerous ligands to induce cellular signals (Fig. 3).

1.2.4. $p75^{NTR}$ as a co-receptor

To further complicate the story of $p75^{NTR}$ signaling, $p75^{NTR}$ also activates signaling cascades in conjunction with its co-receptors, which include the Trk family of neurotrophin receptors, the Nogo receptor (NgR), and sortilin (reviewed in [13]). The Trk family of receptors consists of three receptors, TrkA, TrkB, and TrkC. Although highly homologous, these receptors differ in their specificity for

neurotrophins. TrkA preferentially binds to NGF, BDNF and NT-4 bind to TrkB, while TrkC binds NT-3 {reviewed in [42]}. Neurotrophins can induce signaling through either the Trk receptors or $p75^{NTR}$ alone. However, there is also evidence that $p75^{NTR}$ and the Trk receptors can engage in crosstalk, either by forming a co-receptor complex, or via more downstream interactions. Transiently transfecting both $p75^{NTR}$ and TrkA into heterologous cell types results in higher affinity NGF binding than expression of either $p75^{NTR}$ or TrkA alone [43-45]. $p75^{NTR}$ potentiates NGF-TrkA signaling both in PC12 cells [46] and MAH cells [47] and is required for NGF-induced TrkA phosphorylation and survival of hippocampal neurons [48]. In contrast, $p75^{NTR}$ reduces TrkB autophosphorylation in response to both BDNF and NT-4 in PC12 cells [49]. Thus, expression of $p75^{NTR}$ can either positively or negatively modulate Trk signaling. Similarly, expression of Trk receptors has been suggested to modulate $p75^{NTR}$ function. In the absence of Trk receptors, neurotrophin binding to $p75^{NTR}$ promotes apoptosis. In contrast, when both $p75^{NTR}$ and Trk receptors are present, neurotrophins promote cell survival {reviewed in [9]}. Determining the precise mechanisms underlying the crosstalk between $p75^{NTR}$ and Trk receptors is currently an active area of research and the subject of much debate. Recent crystallographic studies have shed new light on this topic and have cast doubt upon one prominent model of $p75^{NTR}$ and Trk interactions.

The crystal structure of $p75^{NTR}$ in complex with NGF reveals that each NGF homodimer binds to a single molecule of $p75^{NTR}$ [11]. This result raised the possibility that TrkA might bind to the opposite face of the same NGF homodimer to form a trimolecular signaling complex [11]. The trimolecular model gained popularity

as it could potentially explain how $p75^{NTR}$ and Trk influence each other's signaling and also could provide a molecular basis for the existence of the poorly understood high affinity binding sites for NGF [44, 50]. This model is supported by the fact that both the ECD and ICD of $p75^{NTR}$ co-immunoprecipitate with Trk receptors, indicating that $p75^{NTR}$ and TrkA might indeed form a complex [51, 52]. Whether NGF was also present in this complex remained unknown.

More recent crystallographic studies indicate that $p75^{NTR}$ and TrkA likely do not directly interact and form a trimolecular complex with a single dimer of NGF [53]. The crystal structure of TrkA bound to NGF reveals that for TrkA and $p75^{NTR}$ to bind to the same NGF dimer, the ECDs of both receptors would have to lie parallel to the membrane and sandwich NGF between them [53]. In this highly unlikely, contorted conformation, $p75^{NTR}$ and TrkA would not come into direct contact with each other [53], thus calling the existence of a trimolecular complex into question. β-galactosidase complementation experiments designed to determine if $p75^{NTR}$ and TrkA come into close physical contact revealed no evidence of $p75^{NTR}$ interacting directly with TrkA [53], casting further doubt on the trimolecular model. Although this data suggests that a direct interaction between one NGF molecule binding to both $p75^{NTR}$ and TrkA simultaneously is quite unlikely, it does not disprove the existence of a larger complex containing both receptors. Indeed, both $p75^{NTR}$ and TrkA have been shown to be part of a complex with an ankyrin-rich membrane spanning protein (ARMS) [54] and both receptors also interact with caveolin [55]. It is possible that the NGF-binding complex including $p75^{NTR}$ and TrkA requires additional scaffolding

proteins, such as ARMS or caveolin, and that the structural definition of the entire complex would require further crystallographic investigation.

In contrast to its pro-survival role as a co-receptor with the Trk receptors, $p75^{NTR}$ can also promote cell death via its role as a co-receptor with sortilin [56]. Sortilin is a single transmembrane receptor also known as the neurotensin receptor 3 [57] and has been shown to regulate intracellular trafficking of proteins, such as BDNF [58]. Pro-NGF binds to the extracellular domain of sortilin either in the presence or the absence of $p75^{NTR}$ [56]. Although $p75^{NTR}$ is not required for pro-NGF binding to sortilin, both $p75^{NTR}$ and sortilin are required for pro-neurotrophins to induce apoptosis *in vitro* [56, 59]. However, the *in vivo* relevance of pro-neurotrophin binding $p75^{NTR}$ remains unclear. In fact, in contrast to initial reports that pro-neurotrophins can be secreted and cleaved extracellularly [38], recent work has demonstrated that neurotrophins are cleaved almost exclusively intracellularly [60], prior to their release into the extracellular space. These results call into question whether pro-neurotrophins actually do interact with $p75^{NTR}$-ECD *in vivo*. Future research will determine whether pro-neurotrophins bind the $p75^{NTR}$-sortilin complex *in vivo*, and the functional relevance of this interaction in disease models.

In addition to its role as a co-receptor for the Trks and sortilin, $p75^{NTR}$ has also been shown to serve as a co-receptor for the Nogo receptor [61]. NgR binds to a variety of ligands, including myelin ligands such as myelin associated glycoprotein (MAG) [62], Nogo [63], and oligodendrocyte myelin glycoprotein (OMgP) [64]. However, the Nogo receptor is a GPI linked receptor and lacks a transmembrane and intracellular domain [63]. Thus, in order to transmit intracellular signals, NgR

requires a transmembrane co-receptor. $p75^{NTR}$ has been shown to interact with NgR and to serve as the signal transducing component of this co-receptor complex [20]. In this system, $p75^{NTR}$ modulates RhoA activity by acting as the displacement factor to release RhoA from its inhibitor, Rho GDI [21]. Myelin ligands, such as MAG, binding to the Nogo receptor complex strengthens the association between $p75^{NTR}$ and Rho-GDI and sequesters Rho-GDI away from RhoA, leading to RhoA activation [21]. Neurotrophins, in contrast to MAG, decrease the interaction between $p75^{NTR}$ and Rho-GDI and inhibit the $p75^{NTR}$ induced RhoA activation in HEK293 cells [21]. The activation of RhoA by $p75^{NTR}$/NgR has been shown to inhibit neurite outgrowth *in vitro*, and may contribute to nerve regeneration *in vivo*. More recently, a third component of this receptor complex has been identified, LRR and Ig domain containing, Nogo receptor interacting protein (LINGO-1) [65]. This transmembrane protein co-immunoprecipitates with Nogo receptor and is also required for RhoA activation and neurite outgrowth inhibition in response to myelin ligands. *In vivo* studies examining the role of the Nogo receptor complex in spinal cord injury models have revealed that genetic depletion of either $p75^{NTR}$, Nogo receptor, or Nogo receptor ligands is not sufficient to promote regeneration [66, 67]. Future studies will determine the extent to which interactions between $p75^{NTR}$, Nogo receptor, and LINGO contribute to axonal regeneration *in vivo*, and will likely identify additional functions for this receptor complex.

1.2.5. Neurotrophin independent signaling

Although $p75^{NTR}$ has been extensively characterized as a neurotrophin receptor, it is noteworthy that $p75^{NTR}$ can also activate signaling pathways in the absence of ligands. Three major signaling pathways downstream of $p75^{NTR}$ have been shown to be induced in a neurotrophin independent manner: the JNK, PI3K, and RhoA pathways. In the absence of exogenous neurotrophins, transfecting low levels of $p75^{NTR}$ into PC12nnr5 cells, which do not express $p75^{NTR}$ endogenously, activates PI3K-Akt signaling and suppresses apoptosis [68]. In this system, inhibition of Trk receptors has no effect on $p75^{NTR}$ expression induced Akt activation, but does prevent neurotrophin induced activation of Akt via the Trk receptors [68], demonstrating that $p75^{NTR}$ expression induced Akt activation is independent of Trk receptors. At high levels of expression, $p75^{NTR}$ activates JNK and promotes apoptosis in PC12nnr5 cells [68]. As further proof that ligands are not involved in the $p75^{NTR}$-induced activation of JNK and PI3K, $p75^{NTR}$-ICD alone, like the full length receptor, can activate either pathway, depending on the level of expression [68]. These findings provide a mechanistic explanation for the earlier observations that expression of $p75^{NTR}$ in neurons can be sufficient to induce apoptosis in the absence of ligand [69]. Overall, these results suggest that $p75^{NTR}$ can modulate cell survival and apoptosis by activating either JNK or PI3K even in the absence of neurotrophins.

$p75^{NTR}$ induced RhoA activity is most commonly associated with the role of $p75^{NTR}$ as the signal transducing component of the Nogo receptor complex (described above). However, in addition to modulating RhoA activity by sequestering Rho-GDI, $p75^{NTR}$ can also directly activate the RhoA pathway by interacting with RhoA itself,

even in the absence of MAG [70]. Expression of $p75^{NTR}$-ICD, similar to full length $p75^{NTR}$, induces robust RhoA activation in HEK293 cells, thus demonstrating that the extracellular domain (and therefore extracellular ligands) is not required for this effect [70]. Taken together, these data indicate that $p75^{NTR}$ regulates RhoA activation by two distinct mechanisms: via direct interactions with RhoA itself and via the sequestration of its inhibitor, Rho-GDI. Overall, it appears that expression of $p75^{NTR}$ serves as the trigger to induce RhoA activation and that ligands (either the neurotrophins or MAG) play modulatory roles, regulating the level of activation of RhoA.

1.3. Genetic models to study $p75^{NTR}$

Given the multiplicity of signaling pathways and functions regulated by $p75^{NTR}$ *in vitro*, there has been a great deal of interest in determining the precise physiological roles of $p75^{NTR}$ *in vivo*. Over the past fifteen years, a number of genetic mouse models to evaluate the role of $p75^{NTR}$ *in vivo* have been developed. The $p75^{NTR}$ knockout mouse ($p75^{NTR}$-/-), published in 1992 by Lee et al., uses a targeting strategy against Exon III, which encodes the second, third, and fourth CRDs. This mouse exhibits deficits in peripheral nerve development, including a decrease in Schwann cell number and axon diameter, and a dramatic loss of sensory innervation and insensitivity to heat [71]. A targeting strategy directed against Exon IV [72], which encodes the transmembrane domain [73], results in a mouse with a similar, but slightly more severe phenotype than the Exon III knockout. The Exon IV mouse exhibits a greater reduction in Schwann cell number and sciatic nerve diameter than the Exon III

mouse and also has defects in vascular system development [72]. Although the Exon IV mouse lacks full length $p75^{NTR}$, it has been shown to express a truncated $p75^{NTR}$ protein product consisting of the extracellular stalk domain of $p75^{NTR}$ coupled to the entire ICD [74]. This fragment has been shown to induce apoptosis through the activation of $p75^{NTR}$ signaling cascades [74]. In fact, a transgenic mouse overexpressing a similar $p75^{NTR}$-ICD fragment has been made by Phil Barker's group, and has been shown to exhibit neuronal apoptosis, loss of sympathetic and peripheral sensory neurons, and decreased numbers of neurons in the neocortex [75]. Thus, it is likely that some of the phenotype exhibited by the Exon IV mouse stems from a gain of function, rather than a loss of function mutation (discussed in [74]). The work presented in this dissertation examines the Exon III $p75^{NTR}$-/- mouse.

In addition to mice genetically deficient for $p75^{NTR}$ and those overexpressing $p75^{NTR}$-ICD, a transgenic mouse expressing the entire human $p75^{NTR}$ along with its promoter has also been generated [76]. This mouse, the TG81 line, expresses $p75^{NTR}$ in basal forebrain and cerebellum neurons, the developing sensory and sympathetic ganglia of the spinal cord, and in Schwann cells of the peripheral nerve. The presence of 8 kb upstream and 7.5 kb downstream of flanking regulatory elements of $p75^{NTR}$ is sufficient to confer appropriate regulation of $p75^{NTR}$ expression as sciatic nerve transection leads to a similar increase in $p75^{NTR}$ levels in TG81 mice as observed in wt mice after transection [76]. The expression of human $p75^{NTR}$ in these animals did not lead to any developmental or behavioral abnormalities; however, detailed analyses of the TG81 mouse line in disease models have not been reported.

1.4. p75^{NTR} expression and functions *in vivo*

Early work examining the cellular and tissue distribution of p75^{NTR} demonstrated that expression of this receptor is not restricted to the nervous system, but occurs in numerous tissues throughout the body [77]. Due to the fact that p75^{NTR} was originally identified as a receptor that promotes neuronal growth, most subsequent work has focused on nervous system functions for p75^{NTR}. However, this receptor also plays a number of important roles outside of the nervous system as well.

1.4.1. p75^{NTR} expression and functions in the nervous system disease *in vivo*

In the nervous system, expression of p75^{NTR} is increased during a wide range of pathological conditions. Initial examinations of the phenotypes of *p75^{NTR}-/-* animals and transgenic animals overexpressing p75^{NTR}-ICD in mouse models of CNS disease has revealed that p75^{NTR} plays multiple, sometimes opposing roles in nervous system repair. For example, analysis of *p75^{NTR}-/-* mice after spinal cord injury reveals less apoptosis of oligodendrocytes when compared to wildtype controls [78]. This decreased apoptosis in the absence of p75^{NTR} corresponds well with *in vitro* evidence that p75^{NTR} induces oligodendrocyte apoptosis upon binding to pro-NGF, its high affinity ligand [78]. After spinal cord injury, *p75^{NTR}-/-* mice also exhibit more intraspinal sprouting when treated with either NGF or NT-3 compared to wildtype controls [79], indicating that p75^{NTR} can inhibit sprouting *in vivo*. However, studies examining functional recovery after spinal cord injury have found no significant improvement in *p75^{NTR}-/-* animals [66]. These results were somewhat unexpected given the role of p75^{NTR} in inhibition of sprouting *in vivo* and the wealth of *in vitro*

evidence implicating $p75^{NTR}$ in the myelin inhibition of neurite outgrowth {reviewed in [80]}. However, given the fact that $p75^{NTR}$ regulates numerous functions in many cell types, it is likely that its deletion is beneficial for some aspects of the regeneration process and deleterious for others. For example, $p75^{NTR}$ has been shown to be a positive regulator of myelination [81], a step in regeneration that follows axonal re-extension [82]. The antagonistic nature of $p75^{NTR}$ functions after spinal cord injury may partially explain the minimal overall differences in regeneration observed in the *$p75^{NTR}$-/-* mouse. Another potential explanation for the minimal effect of $p75^{NTR}$ deletion is compensation. TROY, another tumor necrosis receptor family member, has been identified by two independent groups as a functional homolog of $p75^{NTR}$ in the inhibition of neurite outgrowth *in vitro* [83, 84]. It is possible that in *$p75^{NTR}$-/-* mice, expression of TROY can substitute for the absence of $p75^{NTR}$, thus masking the effects of $p75^{NTR}$ deletion. An investigation of nerve regeneration in mice genetically depleted of both TROY and $p75^{NTR}$ would shed light on the contribution of the $p75^{NTR}$/TROY/NgR/LINGO receptor complex to axonal regeneration *in vivo*.

In addition to its roles in acute CNS injury models, $p75^{NTR}$ can also affect the progression of CNS disease. Analysis of human Alzheimer's disease (AD) brains has revealed that the intracellular domain of $p75^{NTR}$ accumulates in nuclei of neurons in the entorhinal cortex [85], an integral part of the brain's memory system that is damaged during the early stages of AD. Although the biological consequences of $p75^{NTR}$-ICD accumulation in AD were not determined in this study, separate studies in transgenic mice overexpressing $p75^{NTR}$-ICD have shown that $p75^{NTR}$-ICD is sufficient to induce neuronal apoptosis *in vivo* [75]. The accumulation of $p75^{NTR}$-ICD observed

in AD brains may contribute directly to cell death, even in the absence of ligand. Neurotrophin-induced cell death may also play a role in AD as pro-NGF, the high affinity ligand for $p75^{NTR}$, is upregulated in AD brains and could potentially induce apoptosis through $p75^{NTR}$ [86]. Whether the pro-NGF is secreted and actually participates in neuronal apoptosis remains to be determined. Nonetheless, it is possible that $p75^{NTR}$ may contribute to neuronal death in AD using both ligand-dependent and independent pathways.

In an animal model of MS, there are conflicting reports about the function of $p75^{NTR}$. Copray *et al.* report that $p75^{NTR}$*-/-* mice show exacerbated symptoms of experimental autoimmune encephalomyelitis (EAE, an experimental model of MS), suggesting that $p75^{NTR}$ plays a protective role [87]. Detailed analysis of the cellular infiltrates invading the spinal cord during EAE in wildtype and $p75^{NTR}$*-/-* mice reveals that $p75^{NTR}$ exerts beneficial effects by regulating the infiltration of T-cells and other immune system cells during inflammatory demyelination [88]. In contrast, Soilu-Hanninen *et al.* show that mice treated with $p75^{NTR}$ antisense oligonucleotides to knock down $p75^{NTR}$ expression fare better than control-treated mice, suggesting $p75^{NTR}$ expression can be harmful in inflammatory demyelination [89]. The differences observed between the knockout and the knockdown experiments likely reflect the fact that at low levels of $p75^{NTR}$ expression, such as that observed after treatment with antisense oligonucleotides, $p75^{NTR}$ activates PI3K signaling and promotes cell survival [68]. At higher levels of expression, similar to those observed in disease states, $p75^{NTR}$ activates JNK signaling and promotes apoptosis [68]. Thus, siRNA treated animals may retain some of the beneficial effects of low level $p75^{NTR}$

expression, while being spared the deleterious consequences of $p75^{NTR}$ upregulation during disease.

In addition to its functions in the CNS, $p75^{NTR}$ also plays a role in tissue repair in the PNS. After peripheral nerve injury, $p75^{NTR}$ exhibits both beneficial and potentially damaging effects on the regeneration process. $p75^{NTR}$ may play beneficial roles by promoting sympathetic sprouting of dorsal root ganglia after axotomy [90] and by serving as a positive regulator of myelination [81, 91, 92]. Indeed, transplantation of $p75^{NTR}$ deficient Schwann cells into axotomized nude mice results in deficient myelination, motoneuron growth, and impaired functional recovery when compared to transplantation of wildtype Schwann cells [92]. However, $p75^{NTR}$ can also induce Schwann cell apoptosis in the injured sciatic nerve [93], which may prevent optimal nerve regeneration. Thus, after peripheral nerve injury, $p75^{NTR}$ regulates opposing functions during the repair process. Overall, it is unclear whether $p75^{NTR}$ expression is beneficial or detrimental to functional recovery after peripheral nerve injury. Studies to determine the overall contribution of $p75^{NTR}$ using $p75^{NTR}$-/- mice are complicated by developmental deficits observed in $p75^{NTR}$-/- peripheral nerves, which exhibit Schwann cell loss and reduced sciatic nerve diameter that result in $p75^{NTR}$-/- mice exhibiting gait abnormalities even in the absence of injury [71, 72]. Studies in inducible knockout mice that express $p75^{NTR}$ during development, but could be depleted of $p75^{NTR}$ prior to nerve injury would greatly enhance efforts to determine the overall contribution of $p75^{NTR}$ to the process of peripheral nerve injury.

1.4.2. p75^{NTR} expression and function in non-nervous system disease *in vivo*

Early work examining expression patterns of p75^{NTR} RNA revealed that p75^{NTR} has an extremely wide pattern of expression both within and outside of the nervous system during development and in the adult. Outside of the nervous system, p75^{NTR} is expressed in a wide variety of tissues, including the liver [94, 95], kidney [96], pancreas [97], skeletal muscle [98], and cells of the immune system [99, 100]. Immunohistochemical analysis has identified specific cell types that express p75^{NTR} outside of the nervous system, including stellate cells in the liver [94, 95] and pancreas [97], dendritic and mast cells [99, 100], epithelial cells in the testes [101], and vasculature smooth muscle cells [102]. Much of the *in vivo* evidence for biological functions of p75^{NTR} outside of the nervous system focuses on the role of p75^{NTR} as a regulator of apoptosis. For example, *p75^{NTR}-/-* mice exhibit reduced apoptosis of vascular smooth muscle cells after carotid artery injury, suggesting that p75^{NTR} promotes smooth muscle cell apoptosis *in vivo*. p75^{NTR} has also been implicated in the regulation of keratinocyte apoptosis as *p75^{NTR}-/-* mice exhibit greater hair growth and decreased apoptosis of hair follicle keratinocytes [103]. In the lung, NGF/p75^{NTR} signaling enhances local neurogenic inflammation [104] and exacerbates allergic bronchial asthma [105]. In the case of bronchial asthma, loss of p75^{NTR} reduces hyperreactivity of nerve fibers within the lung, and can thus be considered a nervous system effect [104].

p75^{NTR} has also been implicated in the pathogenesis of cancer. In humans, p75^{NTR} is expressed in a number of malignancies including: prostate cancer [106], squamous cell carcinoma of the esophagus [107], medulloblastoma [108], olfactory

neuroblastoma [109], epithelioid and spindled melanomas [110], breast carcinomas [111], and acute leukemias [112]. Recently, $p75^{NTR}$ has been reported to have a functional role in cancer as putative tumor suppressor. Evidence that $p75^{NTR}$ may be a tumor suppressor was first shown when SCID mice, injected subcutaneously with prostate cancer cell lines stably transfected with $p75^{NTR}$ in a dose dependent fashion, demonstrated decreased tumor volume and metastases with increased $p75^{NTR}$ expression [113]. $p75^{NTR}$ may also be a tumor suppressor in other organ systems such as the bladder and stomach [114, 115]. Although most evidence points toward $p75^{NTR}$ being a tumor suppressor, there are also reports that $p75^{NTR}$ may promote the survival of melanoma cells and also enhance migration and invasion in gliomas [116]. It is possible that $p75^{NTR}$ may play opposing roles in different cancer types, likely due to the differences in expression of ligands, co-receptors, and intracellular signaling molecules.

In a genetic model of liver disease (the plasminogen knockout mouse, *plg-/-*) genetic loss of $p75^{NTR}$ exacerbates liver pathology and inhibits hepatocyte proliferation [22]. In this model, pathology develops as a result of widespread fibrin accumulation, including massive fibrin deposits in the liver [117]. *$p75^{NTR}$-/-plg-/-* mice exhibit extreme wasting and shortening of lifespan when compared to *plg-/-* controls, but do not exhibit increased fibrin deposition within the liver [22]. Rather, the exacerbated phenotype results from the failure of *$p75^{NTR}$-/-* hepatic stellate cells to differentiate and identify $p75^{NTR}$ as a regulator of cell differentiation outside of the nervous system [22]. Differentiation of HSCs promotes hepatocyte proliferation, which is an integral component of liver repair and regeneration after both acute injury

and chronic disease [118]. The failure of *p75^{NTR}-/-* HSCs to differentiate has been shown to result from the loss of p75^{NTR} dependent, neurotrophin independent RhoA activation [22]. These results indicate that p75^{NTR} plays a major role in liver pathology caused by fibrin deposition. Taken together the data from the lung, liver, and cancer models reveals that the role of p75^{NTR} in disease pathology outside of the nervous system extends far beyond the regulation of apoptosis.

2. p75^{NTR}, fibrin, and tissue scarring

2.1. p75^{NTR} in diseases characterized by fibrin deposition

Tissue scarring, characterized by cell activation, excessive deposition of extracellular matrix (ECM) and extravascular fibrin deposition, is considered a limiting factor for tissue repair in numerous injury and disease states, such as peripheral nerve injury [119], idiopathic pulmonary fibrosis [120], glomerulonephritis [121], atherosclerosis [122], breast cancer [123], stroke, MS [124, 125], liver fibrosis, and acute lung injury [126]. Each of these conditions is characterized by leaky vasculature, either as the result of trauma, inflammation, or breakdown of the BBB and results in the extravasation of blood proteins into the surrounding parenchyma {reviewed in [127]}. Interestingly, many of these pathological conditions characterized by tissue scarring also exhibit p75^{NTR} upregulation, indicating a potential role for p75^{NTR} in these disorders.

One major component of the provisional matrix formed during tissue scarring is the protein, fibrin {reviewed in [127]}. Indeed, previous work has demonstrated a correlation between fibrin deposition and expression of p75^{NTR} after nerve injury.

Similarly, upregulation of $p75^{NTR}$ is frequently observed in MS [128], stroke [129], spinal cord [78] and sciatic nerve injury [130], all of which are also associated with fibrin deposition. $p75^{NTR}$ is also upregulated in a variety of non-nervous system diseases associated with defects in fibrin degradation, such as atherosclerosis, pancreatitis [97], melanoma formation [131], lung inflammation [105], and liver disease [94]. $p75^{NTR}$ has been primarily characterized as a modulator of cell survival in non-neuronal tissues. However, the expression of $p75^{NTR}$ by cell types such as smooth muscle cells [132] and hepatic stellate cells [95], which actively participate in tissue repair by migration, and secretion of ECM and extracellular proteases, raises the possibility for additional functional roles of $p75^{NTR}$ in disease pathogenesis. Given the striking correlation of $p75^{NTR}$ upregulation and fibrin deposition and the fact that $p75^{NTR}$ is expressed by cells that regulate ECM composition, we hypothesized that $p75^{NTR}$ might regulate fibrin deposition.

2.2. Fibrin

Fibrin is generated from its precursor, fibrinogen, via the coagulation cascade and the proteolytic action of thrombin. Fibrinogen, a 340 kDa glycoprotein synthesized in the liver, is one of the most abundant proteins in the bloodstream, with a physiological concentration ranging from 2-4 mg/ml. Local procoagulant responses to tissue injury trigger the conversion of fibrinogen to the insoluble fibrin which forms a provisional matrix within injured or diseased tissues {reviewed in [127]}. Fibrin has been extensively studied as a clotting factor within the bloodstream. However, the role of fibrin in tissues during conditions of injury and disease is less well understood.

Recent work from our laboratory has demonstrated that fibrin can contribute to activation of microglia within the CNS thus promoting disease progression during inflammatory demyelination [124]. Fibrin has also been implicated in the inhibition of nerve regeneration both in the CNS [133] and the PNS [119]. Due to the inhibitory role of fibrin in nervous system disorders, enhanced clearance of fibrin may promote tissue repair.

2.3. Fibrinolysis

Clearance of fibrin occurs through the process of proteolytic degradation or fibrinolysis, a process catalyzed by the serine protease, plasmin [134]. Although plasmin can cleave a number of proteins, including matrix metalloproteinases [135], basic FGF [136], and several ECM glycoproteins [137], its major physiological substrate is fibrin [117]. Local generation of plasmin is regulated by two Plasminogen Activators (PAs): the serine proteases, tPA and urokinase type PA (uPA). PAs trigger plasmin activity by cleaving the inactive zymogen, plasminogen, and converting it into active plasmin [138]. Plasminogen activation promotes in fibrin degradation and ECM remodeling and is required for tissue repair. Thus, PAs and their inhibitors, such as plasminogen activator inhibitor -1 (PAI-1), are key modulators of scar resolution by spatially and temporally regulating the conversion of plasminogen to plasmin.

In the peripheral nervous system, previous work by Katerina Akassoglou and others showed that inhibition of fibrinolysis in mice deficient in plasminogen or tPA results in exacerbated axonal damage [139] and impaired functional recovery [140] after nerve injury. In accordance, mice deficient for fibrinogen [141] show increased

regenerative capacity [119]. Studies of fibrin deposition in human diseases, in combination with experiments from gene-targeted mice deficient in plasminogen and PAs, have provided information about a wide range of physiological and pathological conditions that are exacerbated by defective fibrin degradation, such as wound healing, metastasis, atherosclerosis, lung ischemia, rheumatoid arthritis, muscle regeneration and multiple sclerosis (MS) [127, 141]. However, the molecular mechanisms that regulate fibrinolytic activity after injury remain unclear.

3. Rationale, Aims and Significance

The dramatic upregulation of $p75^{NTR}$ expression during a tissue injury and disease both within and outside of the nervous system points to the possibility that $p75^{NTR}$ may play a functional role in pathogenesis or during tissue repair. The close association between $p75^{NTR}$ upregulation and sites of fibrin deposition after injury further highlights the possibility of functional interplay between these two proteins. The primary goal of my dissertation research is to investigate the functional roles of $p75^{NTR}$ in tissue injury models characterized by fibrin deposition.

The first section of my dissertation deals with the role of $p75^{NTR}$ in regulating fibrin clearance after sciatic nerve injury. Preliminary data from our lab indicated that *$p75^{NTR}$-/-* mice exhibited reduced fibrin deposition after sciatic nerve crush injury, when compared to wt controls. However, the molecular mechanisms underlying this decreased fibrin deposition were unknown. Thus, the primary question I set out to address was:

What is the role of $p75^{NTR}$ in regulating fibrin deposition?

We began this work focusing exclusively on sciatic nerve injury, but later expanded our investigation to determine if the role of $p75^{NTR}$ in regulating fibrin deposition was specific to sciatic nerve injury, or if it was a general mechanism occurring during other pathologic states as well. To address this question, we examined the role of $p75^{NTR}$ in regulating fibrin deposition in an LPS-induced model of lung injury. Finally, we set out to determine

What is the precise molecular mechanism whereby $p75^{NTR}$ mediates its effects?

Upon identifying a novel pathway downstream of $p75^{NTR}$, we investigated additional roles for this new $p75^{NTR}$ signaling mechanism within the central nervous system.

The increased $p75^{NTR}$ expression and deposition of fibrin in tissues after injury indicates that these proteins may exhibit specific functions during disease pathology. Through the identification of the signaling pathways employed during pathogenesis, it may be possible to target disease-specific functions of proteins, such as $p75^{NTR}$ and fibrin, while leaving their normal physiological functions largely intact. We hope that the research performed for this dissertation will aide in the development of new techniques to combat human diseases characterized by fibrin deposition and $p75^{NTR}$ upregulation.

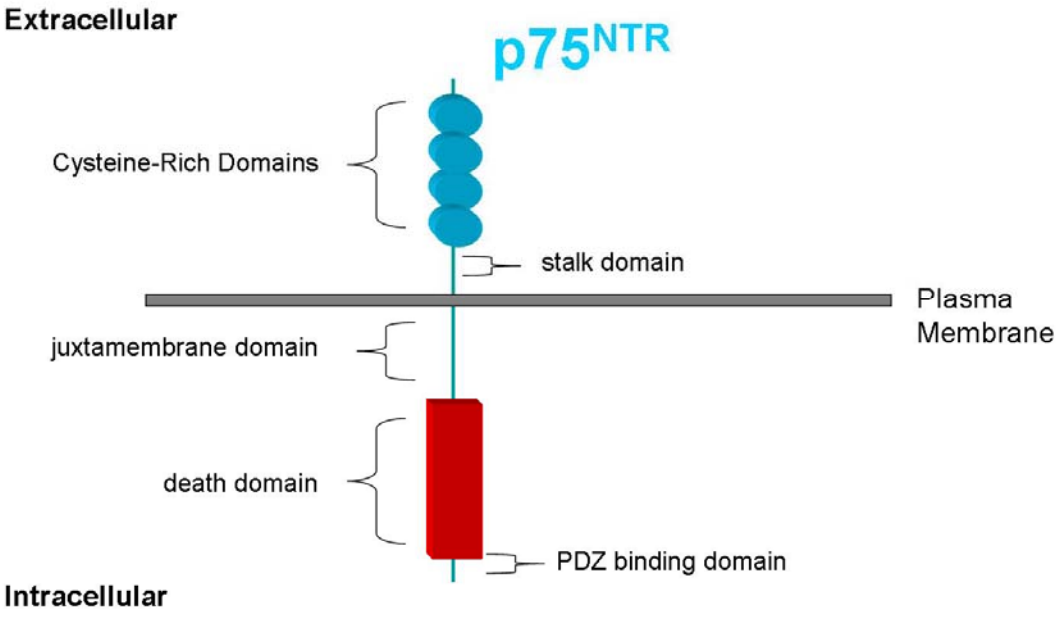

Figure 1. Schematic of p75^{NTR} structure.
p75^{NTR} is a single transmembrane-spanning protein with an amino-terminal extracellular domain and carboxy-terminal intracellular domain. The extracellular domain contains four cysteine-rich domains involved in binding neurotrophins, followed by a stalk domain that is involved in cellular sorting. The intracellular domain of p75^{NTR} contains a juxtamembrane domain required for interaction with intracellular signaling molecules, a death domain which is involved in apoptotic signaling, and an extreme C-terminal PDZ binding domain.

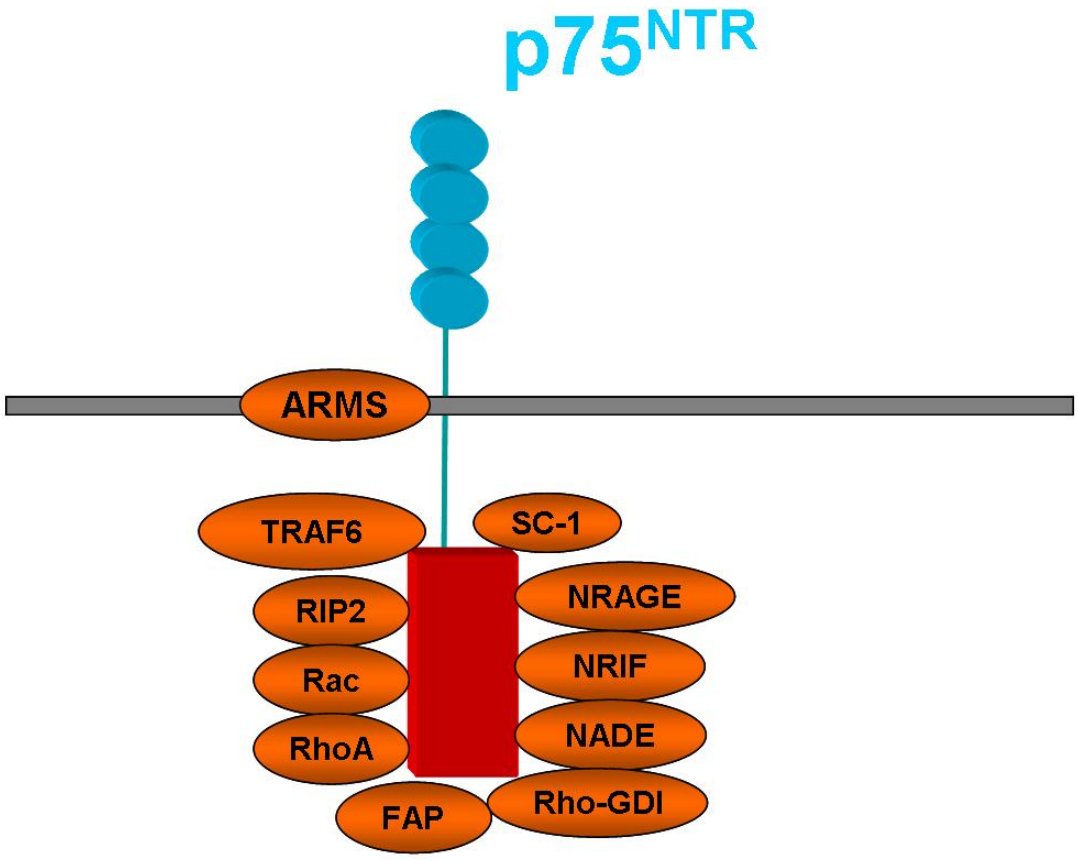

Figure 2. Mediators of p75^{NTR} signaling.
Signaling through p75^{NTR} involves the recruitment of a number of intracellular mediators. Recruitment of NADE, NRAGE, NRIF, Rac, and SC-1 are primarily associated with p75^{NTR} induced apoptosis and cell cycle arrest. TRAF6, RIP2, and FAP are involved in the activation of NF-kB. Interactions between p75^{NTR} and RhoA or Rho-GDI regulate RhoA activation and play a role in cellular differentiation and the inhibition of neurite outgrowth. ARMS is a transmembrane protein involved in mediating cross-talk between p75^{NTR} and a family of its co-receptors - the Trk receptors.

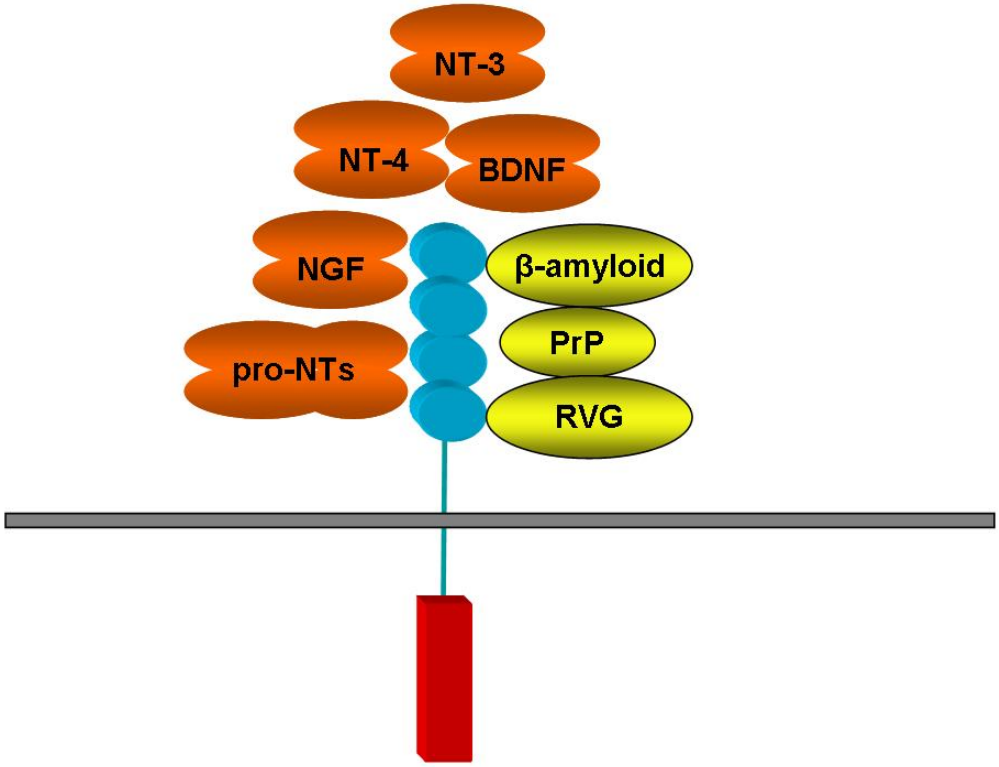

Figure 3. p75^{NTR} Ligands.
Schematic showing p75^{NTR} with its ligands. p75^{NTR} was originally identified as a receptor for the neurotrophins (NGF, BDNF, NT-3 and NT-4). However, it can also serve as the receptor for the pro-neurotrophins (pro-NTs, which include pro-NGF and pro-BDNF, shown in orange). In addition, p75^{NTR} can bind to a number of unrelated pathological ligands: β-amyloid, prion protein peptide (PrP), and rabies virus glycoprotein (RVG).

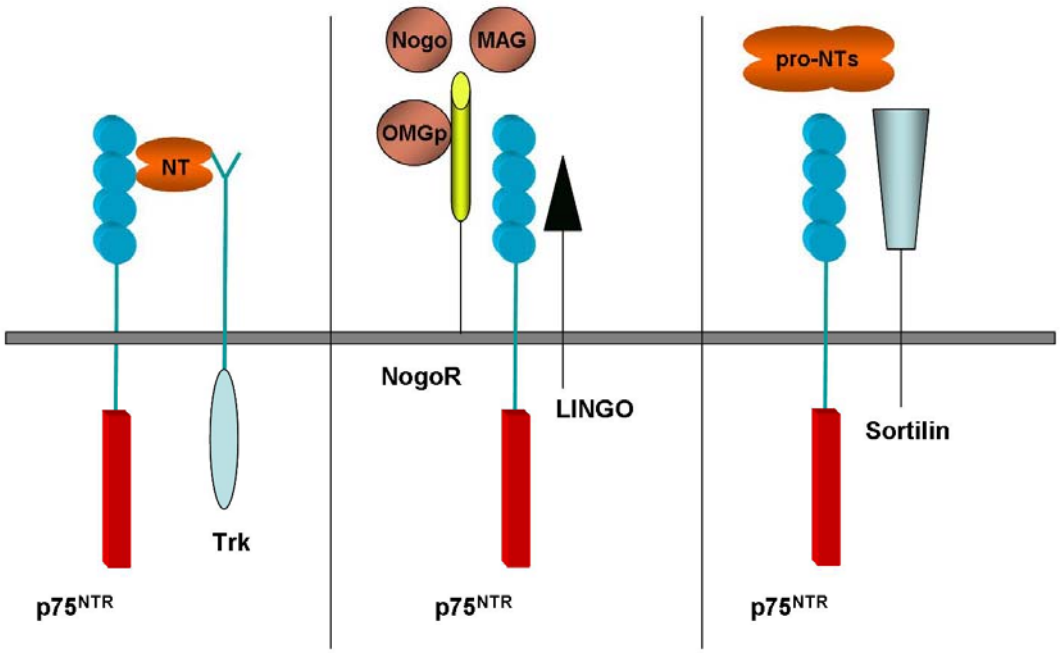

Figure 4. p75^{NTR} and its co-receptors.
p75^{NTR} interacts with Trk receptors (blue, far left) to promote neuronal survival and differentiation via binding to neurotrophins (NTs). p75^{NTR} and LINGO-1 (black) also serve as co-receptors for the Nogo receptor (NogoR) (yellow), forming a complex that binds the myelin-based proteins Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMGp) and transduces signals to RhoA. This receptor complex has been implicated in the inhibition of neurite outgrowth in neurons. p75^{NTR} can also interact with Sortilin (blue, far right) to form a complex that binds the proneurotrophins (pro-NTs) and promotes apoptotic signaling.

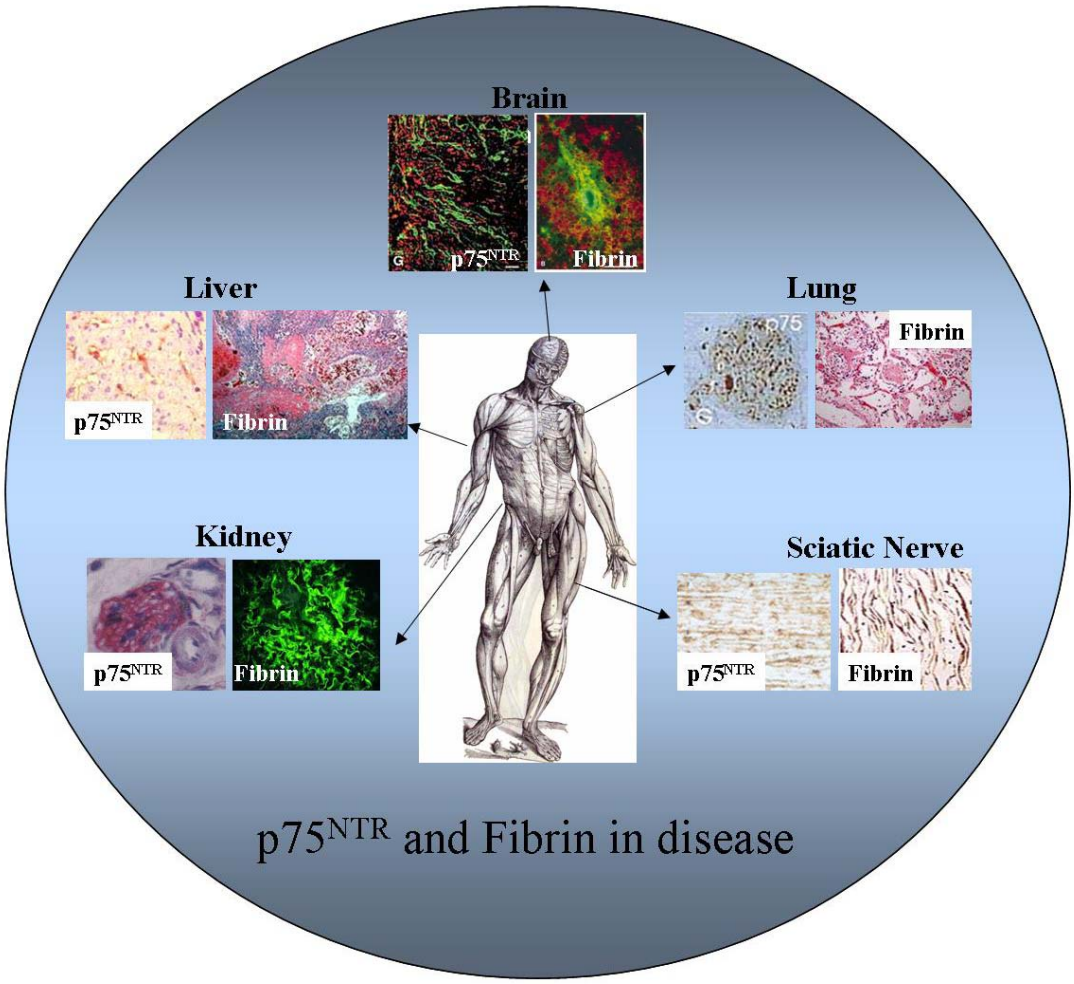

Figure 5. p75^{NTR} and fibrin during injury and disease.
Upregulation of p75^{NTR} expression is observed in a wide variety of disease states throughout the body, including Multiple Sclerosis in the brain [142], in lung tissue from human smokers [143], after sciatic nerve crush injury [119], in the kidney [144], and in the liver [95]. Fibrin immunostaining in brain [139].

Chapter 2. Materials and methods

1. $p75^{NTR}$ in fibrinolysis in sciatic nerve

1.1. Animals

$p75^{NTR}$-/- mice [71] and *tPA-/-* mice [145] were in a C57BL/6 background and purchased from Jackson Labs. Double *$p75^{NTR}$-/- tPA-/-* mice were generated by crossing *$p75^{NTR}$-/-* mice with *tPA-/-* mice. C57BL/6J mice were used as controls. Mice were kept on a twelve hour light dark cycle and had access to food and water *ad libitum.* Animal procedures were performed in accordance with the guidelines set by the University of California San Diego Institutional Animal Care and Use Committee and the National Institutes of Health.

1.2. Sciatic Nerve Crush

Sciatic nerve crush was performed as described [139] with slight modifications. Briefly, adult mice were anesthetized with an intraperitoneal injection of 2.5% avertin (18µl/g body weight, Aldrich Chemical Company). Sciatic nerves were exposed and crushed at the mid-thigh level two times (10 seconds each) with watchmaker's forceps, and rotating the forceps 90 degrees in between the two crushes. Forceps were sterilized in between crushes using a glass bead sterilizer and were allowed to cool prior to use. Skin incisions were closed using surgical staples. For rolipram treatment after sciatic nerve injury, mice were injected with rolipram (1 mg/kg) once daily for 8 days until tissue was harvested and processed for immunostaining.

1.3. Immunohistochemistry

Immunohistochemistry was performed as described [119]. Sciatic nerve sections were fixed in 4% paraformaldehyde for 30 minutes. After rinsing three times in PBS, sections were blocked for 30 minutes in 3% bovine serum albumin (BSA) diluted in PBS with 0.1% triton. Primary antibodies used were sheep anti-human fibrin(ogen) (1/200; US Biologicals), rabbit anti–human tPA (1/300; Molecular Innovations, Southfield, MA), rabbit anti-$p75^{NTR}$ clone 9651, (1/1,000), goat anti-$p75^{NTR}$ (1/200; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), mouse anti-S100 (1/200; Neomarkers, Fremont, CA) and rabbit anti-cAMP (1/1000, Chemicon Temecula, CA).. Primary antibody was diluted in 1% BSA in PBS with 0.1% triton and incubated overnight at 4 degrees. After three rinses with PBS, sections were incubated with appropriate secondary antibody (1/200, Jackson Immunochemicals, West Grove, PA) diluted in 3% BSA in PBS with 0.1% triton for 30 minutes, followed by three final rinses with PBS. Bound antibodies were visualized either using the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) with 3-amino-9-ethylcarbazole (AEC, Sigma) as a chromogen or with immunofluorescence. For immunofluorescence, sections were mounted with SlowFade Gold Antifade Reagent (Invitrogen) + 4',6-Diamidine-2'-phenylindole (DAPI) (1 μg/mL) and coverslipped. Images were acquired with an Axioplan II epifluorescence microscope (Carl Zeiss MicroImaging, Inc.) using dry Plan-Neofluar lenses using 10×0.3 NA, 20× 0.5 NA, or 40× 0.75 NA objectives equipped with Axiocam HRc digital camera and the Axiovision image analysis system.

1.4. Isolation of plasma

Plasma isolation was performed as described [124]. Briefly, whole blood was collected into one tenth volume of 4% sodium citrate and samples were spun down at 2500g for 10 minutes at room temperature. The supernatants were taken and analyzed by western blotting.

1.5. *In situ* zymography

For *in situ* zymographies [146], 10-μm unfixed, cryostat sciatic nerve sections were prepared. Sections were overlaid with a mixture of 2.5% milk, 1% low melting point (LMP) agarose and 25mg/ml plasminogen (American Diagnostica) and coverslipped. Control experiments were performed with overlay mixtures lacking plasminogen. To determine if the proteolytic activity was due to tPA, control experiments were performed in the presence of tPA-STOP (2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one dihydrochloride salt; American Diagnostica) at a concentration of 50 mg/ml. Sections were incubated at 37°C in a humidified chamber. Conversion of plasminogen into plasmin resulted in lysis of casein (from milk) and the appearance of dark lytic zones when photographed under dark field illumination. Quantification of *in situ* zymographies was performed by measuring the area of the lytic zone surrounding each nerve, and dividing that value by the area of the nerve. Images were collected after 8 hours of incubation for the sciatic nerve and 4 hours of incubation for the lung. For cell zymographies, cultures were washed four times with 1% BSA in PBS and overlaid with 200 μl of DMEM containing 1% LMP agarose, 2.5% boiled nonfat milk, and 25 μg/ml human plasminogen. The overlay was

allowed to harden, and plates were incubated in a cell culture incubator at 37ºC. Pictures of lytic zones were taken using an inverted microscope under dark field (Zeiss).

1.6. Immunoblots and quantification of $p75^{NTR}$ and fibrin

Immunoblot was performed as described [119]. Antibodies used were rabbit anti-$p75^{NTR}$ clones 9992 and 9651 (1/5000), mouse anti-fibrin (1/500; Accurate Chemical & Scientific Corp., NY), and rabbit anti-GAPDH (1/5000; Abcam, Cambridge, MA). Quantification was performed on the Scion NIH Imaging Software. Fibrin precipitation and quantification from lung tissues was performed exactly as described [147]. Briefly, the tissue was homogenized in 10 mM sodium phosphate buffer, (pH 7.5) 0.1 M ε - aminocaproic acid, 5 mM EDTA, with protease inhibitors added (Calbiochem). The homogenate was then agitated overnight at 4°C, centrifuged at 10,000 X *g* for 10 min, resuspended in extraction buffer, centrifuged again, and the pellet was resuspended in 3 M urea. This suspension was agitated for 2 h at 37 ° C, vortexed, and centrifuged at 14,000 *g* for 15 min. The supernatant was aspirated, and the pellet was dissolved at 65 ° C in reducing SDS sample buffer (60 mM TrisHCl (pH 6.8), 10% glycerol, 2% SDS, and 0.1 M β-mercaptoethanol). The samples were then subjected to SDS-PAGE, and transferred to a PVDF membrane (Immobilon-P; Millipore Corp., Bedford, MA). Fibrin was detected using a mouse anti-fibrin antibody (1/500; Accurate Chemical & Scientific Corp., NY) followed by anti-mouse HRP (1/2000, Santa Cruz).

2. Mechanisms of p75^{NTR} induced inhibition of fibrinolysis

2.1. Co-immunoprecipitation

Co-IP was performed as described [148]. Cell lysates were prepared in 1% NP-40, 200 mM NaCl, 1mM EDTA, 20 mM Tris-HCl pH 8.0. IP was performed with an anti- p75^{NTR} antibody (9992) and immunoblot with anti-PDE4A, PDE4B, PDE4C and PDE4D (Fabgennix). For mapping experiments, PDE4A5 cDNA was co-transfected with HA-tagged p75^{NTR} deletion constructs into HEK293 cells. IP was performed with an anti-HA antibody (Cell Signaling). Cell lysates were probed with an anti-PDE4A or an anti-p75^{NTR} antibody (9651). For co-ip experiments using recombinant proteins, equimolar amounts (2μM) of purified MBP-PDE4 constructs and GST-p75^{NTR}-ICD were mixed in binding buffer (50mM Tris-HCl pH 7.5, 100mM NaCl, 2mM MgCl2, 1mM DTT, 0.5% Triton-X100 and 0.5% BSA) and incubated for 1 hour at 4°C. Washed glutathione Sepharose beads were added according to the manufacturer's instructions for an additional hour. Beads were sedimented by centrifugation (10000g for 1 min) and washed 3 times. Proteins associated with the beads were eluted by boiling in loading buffer and separated by SDS-PAGE.

2.2. RT-PCR and Real-Time PCR

RT-PCR was performed as described [22]. Primers for *tPA, uPA, GAPDH, actin,* and *PAI-1* genes were used as described [149]. Primer sequences were:

tPA: 5' - CTGAGGTCACAGTCCAAGCAATGT

3'- GCTCACGAAGATGATGGTGTAAAGA

uPA:	5’ - GCTCCTATAATCCTGGAGAGATGAA
	3’ - ACCTGTCTTTTCAGCTTCTTCCCTCC
PAI-1:	5’ – TCAGAGCAACAAGTTCAACTACACTGAG
	3’ – CCCACTGTCAAGGCTCCATCACTTGCCCCA
GAPDH:	5' - CAAGGCCGAGAATGGGAAG
	3’ – TGTAGTTTACCCCACTCCGG
Actin:	5’ – GTCCCTGTATGCCTCTGGTC
	3’ – TCGTACTCCTGCTTGCTGAT

Real time PCR was performed using the Opticon DNA Engine 2 (MJ Research, Waltham, MA) and the Quantitect SYBR Green PCR kit (Qiagen). Results were analyzed with the Opticon 2 Software using the comparative Ct method as described [150]. Data were expressed as ΔΔCt normalized against *GAPDH* or *actin*.

2.3. Quantification of tPA and uPA activity

Quantification of tPA and uPA activity in SC and fibroblast in lysates and supernatants was performed according to the directions of the activity assay kits from American Diagnostica and Chemicon respectively. Briefly, equal amounts of cell lysates or supernatants were incubated in a solution containing 0.1 M Tris pH 8.0, 0.3 mM chromogenic Spectrozyme tPA substrate, and 0.42μM plasminogen at 37 degrees. Reactions were monitored by observing a color change from clear to yellow. Once a color change became visible, samples were analyzed for absorbance at 405 nm using a

plate reader and compared against a standard curve. To elevate cAMP cells were treated either with 2 mM dibutyryl-cAMP (db-cAMP, Sigma) or with 10 μM forskolin (Sigma) for 16 hours. To block PKA activity, cells were treated with 200 nM KT5720 (Calbiochem). Induction with neurotrophins was performed using 100 ng/ml NGF (Peprotech), 50 ng/ml BDNF (Peprotech) for 16 hours prior to tPA assay.

2.4. Fibrin degradation assay

Coating with fibrin was prepared as described [151]. Briefly, fibrinogen was diluted to 2mg/ml in DMEM and added to each well of a twelve well plate. Thrombin was added to stimulate conversion to fibrin. After two hours, fibrin gels had solidified, and cells were plated on top of the fibrin gel. After 48 hours, the supernatant was aspirated and the remaining gel was weighed using an analytical balance to quantitate fibrin degradation. The weight of remaining gel in each well was subtracted from the weight of a gel incubated with DMEM with no cells. The difference in the weight between the remaining fibrin gel with cells and without cells corresponded to the amount of fibrin degraded.

2.5. Cell culture and transfections

Murine SCs were isolated as described [152]. Briefly, 10-15 mice or 5-6 rat pups, aged 0-4 days, were decapitated and sciatic nerves were excised and placed into Hanks Balanced Salt Solution (HBSS). Nerves were pooled, minced, and digested in 0.03% collagenase and 0.1% Trypsin in HBSS for 30 minutes at 37 degrees. The digestion was stopped by the addition of pre-warmed DMEM with 10% FBS. SCs

were then plated onto tissue culture dishes. After three days, cells were lifted with 0.05% Trypsin –EDTA and were replated onto a plate coated with 50 ug/ml Thy1.2 antibody. After 30 minutes, nonadherent Schwann cells were separated from the adherent contaminating fibroblasts and transferred to a new tissue culture plate.

CGNs were isolated from P10 animals as described [64]. Briefly, 7 to 10 day old mice were sacrificed by decapitation and brains were removed into ice cold HHGN buffer (1X Hanks Balanced Salt Solution, 2.5mM Hepes, 35mM Glucose, 45 mM $NaHCO_3$). Meninges were removed and the cerebellum was dissected away from the rest of the brain. Two cerebella were pooled into one 15 mL Falcon tube and were washed 3 times in ice cold HHGN. Cerebella were then digested in 5 mL of TDN solution (Trypsin Dissociation media, 200 units/mL DNase 1 and 10 mg/mL in HHGN) for 15 minutes at 37 degrees followed by three addition washes in HHGN. DNB solution (Dissociation media, 200 units/mL DNase 1 in Basal Medium Eagle) was then added (5mL/cerebellum) and cerebella were triturated 25 times with a 10 mL pipette. Cells were allowed to settle for 5 minutes, after which time the supernatant was collected and transferred to a new 15 mL Falcon tube. The trituration process was repeated once and the two supernatants were pooled. CGNs were then centrifuged at 200*g for 5 minutes and then either resuspended in CBC media (DMEM/F12 supplemented with N2 supplement, pen/strep, 2.5% horse serum and 1.25% fetal bovine serum) for plating or lysed immediately for co-IP, without plating.

siRNA directed against $p75^{NTR}$ (Dharmacon, Chicago, IL) was transfected into SCs and NIH3T3$p75^{NTR}$ cells using Dharmafect (Dharmacon). NIH3T3 or HEK293 cells were co-transfected either with $p75^{NTR}$ FL, ICD or deletion constructs and

PDE4A5 cDNAs using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.6. cAMP and PKA assays

One million fibroblasts (either HEK293 or NIH3T3 cells) or 500,000 primary SCs were lysed in 0.1N HCl solution and cAMP was measured using a competitive binding ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. For screenings of pharmacologic inhibitors, cells were pre-treated with 100 ng/ml PTX, 500 μM Isobutyl methylxanthine (IBMX) (Calbiochem), 18.7 μM 8-methoxymethyl-3-isobutyl-1-methylxanthine (PDE1 inhibitor, Calbiochem), 80 μM *erythro*-9-(2-Hydroxy-3-nonyl)adenine (PDE2 inhibitor, Calbiochem), 100 nM trequinsin (PDE3 inhibitor, Calbiochem), 10 μM rolipram (PDE4 inhibitor, Calbiochem), 23 μM 4-Hi-6-methoxyquinazoline (PDE5 inhibitor, Calbiochem) for 16 hours prior to stimulation with forskolin (10 μM for one hour). Since these inhibitors specifically inhibit a PDE isoform and have no effect on the other PDE isoenzymes [153, 154], they are extensively used for the identification of the specific PDE isoforms that are involved in different cellular functions. Induction with neurotrophins was performed using 100 ng/ml NGF or 50 ng/ml BDNF, 750 ng/ml of FcTrkB, or 1.35 ug/ml of $Fcp75^{NTR}$ for 1 hour prior to cAMP assay.

For the qualitative and quantitative PKA assay (Promega, Madison, WI), cells were treated with 10 μM forskolin for 30 minutes, lysed in 1% NP-40 buffer with 150 mM NaCl, 50 mM Tris, and 1 mM EGTA, and protein concentration was determined using the Bradford Assay (Biorad, Hercules, CA) according to the manufacturer's

protocol (Promega). Subsequently, one μg of lysate was incubated with PepTag A1 peptide (final concentration 0.08 μg/μL) in 1X PepTag PKA reaction buffer for 30 minutes at room temperature. Phosphorylation of the PepTagA1 peptide results in a change in the overall charge of the peptide from +1 to -1. The reaction was stopped by boiling at 95°C for 10 minutes. One μL of glycerol was added and then samples were loaded into a 0.8% agarose gel and run at 100V for 20 minutes. The phosphorylated peptide migrates towards the positive electrode while the unphosphorylated peptide migrates towards the negative electrode. Bands were excised from the gel, transferred to a microfuge tube and heated at 95°C until melted. 125 μL of hot agarose solution was added to 75 μL of Gel Solubilization solution (Promega), 100 μL of glacial acetic acid, and 150 μL distilled water then the absorbance was read at 570 nm.

2.7. Constructs

For the construction of pm-AKAR2.2 we used the previously described cytoplasmic PKA sensor, AKAR2 [155]. pm-AKAR2.2 consists of a cDNA containing a FRET pair, monomeric enhanced cyan fluorescent protein (ECFP) [156] and monomeric citrine (an optimized version of YFP), fused to forkhead associated domain 1 (FHA1) (Rad53p 22–162), and the PKA substrate sequence LRRATLVD via linkers. A206K mutations were incorporated to ECFP and Citrine by the QuickChange method (Stratagene). The C-terminal sequence from K-Ras KKKKKKSKTKCVIM was added to target the construct to the plasma membrane.

For expression in mammalian cells, the chimaeric proteins were subcloned into a modified pcDNA3 vector (Invitrogen) behind a Kozak sequence as described [155].

For the generation of the C-terminal truncated PDE4A4, PDE4A4B was sub-cloned into p3XFLAG-CMV-14 using plasmid pde46 (GenBank L20965) as template from Met-1 to Iso-721 [157]. A forward (5') primer containing a Hind III restriction site immediately 5' to the initiating Met-1 (ATG) of PDE4A4B and a reverse primer designed to the DNA sequence ending at Iso-721 (ATA) with Bam HI restriction site immediately 3' to Iso-721 was used to amplify Met-1 to Iso-721. The C-terminal was removed simply by amplifying from Iso-721 instead of the final codon at the end of the full-length PDE4A4B. The C-terminally truncated PDE4A4B was cloned in-frame with three FLAG (Asp-Tyr-Lys-Xaa-Xaa-Asp) epitopes (Asp-726, Asp-733 & Asp-740) after the Bam HI restriction site, therefore at the C-terminal of the now truncated PDE4A4B. The stop codon (TAG) after the FLAG epitopes is located immediately after Lys-747. This strategy generates a C-terminal truncate of PDE4A4B from 1-721.

2.8. FRET Imaging

NIH3T3 cells and NIH3T3p75^{NTR} cells were transiently transfected with pm-AKAR2.2, AKAR3, or pm-AKAR3 [158] and imaged within 24 hours of transfection. Cells were rinsed once with HBSS (Cellgro) prior to imaging in HBSS in the dark at room temperature. A Zeiss Axiovert microscope (Carl Zeiss Microimaging, Inc.) with a MicroMax digital camera (Roper-Princeton Instruments) and MetaFluor software (Universal Imaging, Corp.) was used to acquire all images. Optical filters were obtained from Chroma Technologies. CFP and FRET images were taken at 15 second

intervals. Dual emission ratio imaging used a 420/20-nm excitation filter, a 450 nm dichroic mirror and a 475/40 nm or 535/25 nm emission filter for CFP and FRET respectively. Excitation and emission filters were switched in filter wheels (Lambda 10-2, Sutter).

2.9. Peptide Array Mapping

Peptide libraries were synthesized by automatic SPOT synthesis [159]. Synthetic overlapping peptides (twenty-five amino acids in length) were spotted on Whatman 50 cellulose membranes according to standard protocols by using Fmoc-chemistry with the AutoSpot Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany). Membranes were overlaid with 10μg/ml recombinant GST-$p75^{NTR}$ ICD. Bound recombinant GST-$p75^{NTR}$ ICD [160] was detected using rabbit anti-GST (1/2000, Amersham Biosciences) followed by secondary anti-rabbit horseradish peroxidase (1/2500, Dianova) [161]. The procedure and detection of signals is identical to that used for immunoblotting as described. Alanine scanning was performed as described [162].

3. The role of $p75^{NTR}$ in regulating fibrinolysis in the lung.

3.1. Induction of lung fibrosis

Lung fibrosis was induced as described [163]. Briefly, adult mice were anesthetized with an intraperitoneal injection of 2.5% avertin (18μl/g body weight, Aldrich Chemical Company). After the mice became anesthetized, they were injected with Nitro-L-arginine methyl ester (L-NAME, Sigma, 50 μg/g). Thirty minutes after

the first L-NAME injection, mice were given a second L-NAME injection followed immediately by either a single intraperitoneal injection of lipopolysaccharide (LPS, *Escherichia coli* serotype 0127:B8, Sigma, 2μg/g) or a control injection of saline. L-NAME was administered to the animals again 2 and 4 hours after the LPS or saline injection. Thirty minutes following the final L-NAME injection, mice were sacrificed, perfused with phosphate buffered saline (PBS) and lung tissues were isolated. Tissue was either embedded in optimal cutting temperature (OCT) embedding medium and frozen or snap frozen in liquid nitrogen. For the rolipram treatments, mice were administered 5 mg/kg rolipram (Calbiochem) prior to the LPS injection as described. Mice were sacrificed 4.5 hours after LPS or saline administration.

3.2. Immunohistochemistry

Immunohistochemistry was performed as described previously. Antibodies used were sheep anti-human fibrin(ogen) (1/200; US Biologicals) and rabbit anti-PAI-1 (1/500, kind gift of David Loskutoff).

3.3. Immunoblots and quantification fibrin deposition

Immunoblot was performed as described above. Antibodies used were mouse anti-fibrin (1/500; Accurate Chemical & Scientific Corp., NY), rabbit anti-myosin (1/1000; Sigma, Saint Louis, MO), and rabbit anti-PAI-1 (1/5000, kind gift of David Loskutoff). Quantification of fibrin deposition was performed as described above.

3.4. *In situ* zymography

In situ zymography was performed as described above for the sciatic nerve.

4. $p75^{NTR}$ in the CNS.

4.1. Animals

PDE4A-/- mice were a kind gift of Marco Conti and were in a mixed 129/Ola, C57/BL/6 background. C57BL/6J mice were used as controls. Mice were kept on a twelve hour light dark cycle and had access to food and water *ad libitum.* Animal procedures were performed in accordance with the guidelines set by the University of California San Diego Institutional Animal Care and Use Committee and the National Institutes of Health.

4.2. Immunohistochemistry

Immunohistochemistry was performed as described previously. Antibodies used were rabbit polyclonals against PDE4A, PDE4A5, PDE4B, and PDE4D (Fabgennix, 1/100), and rabbit anti $p75^{NTR}$ (9651, 1/300).

4.3. RT-PCR and Real-Time PCR

RT-PCR was performed as described [22]. Primers for *tPA, uPA* and *PAI-1* genes were used as described. Primer sequences were:

PDE4A: 5’ – CGAGCACTACAGTGGTGGAA

3’ - AAAAGGATCAGGCAGGGTCT

PDE4B: 5’ – GTCCCAGGTTGGTTTCATTG

3' – ACACAGGGATGGAATCGAAG

PDE4C: 5' – CTCCTGGCTGACCTCAAGAC

3' – TCCAAGGTATCCAGCAGCTC

PDE4D: 5' – GTCCCATGTGTGACAAGCAC

3' – TCAGTGTCTGACTCGCCATC

GAPDH: 5' - CAAGGCCGAGAATGGGAAG

3' – TGTAGTTTACCCCACTCCGG

Real time PCR was performed using the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) and the POWER SYBR Green PCR kit (Applied Biosystems, Foster City, CA). Results were analyzed with the 7300 System software using the comparative Ct method as described [150]. Data were expressed as ΔΔCt normalized against *GAPDH*.

4.4 Rho activity assays

Two different assays were used to determine Rho activity: the classical Rho-GTP pull down assay and an ELISA based assay. The classic Rho-GTP pull down experiments were performed essentially as described [21, 164] with modifications. CGNs were cultured on poly-d-lysine coated dishes overnight and then treated with recombinant Nogo peptide (4 μM) for 30 minutes. CGNs were then lysed in Rho assay lysis buffer (50mM Tris, pH 7.4, 0.1% Triton X-100, 5 mM $MgCl_2$, 100 mM NaCl, 10% glycerol) and incubated with Rho-GTP binding beads (Upstate) for 45

minutes. Beads were collected by centrifugation, washed in Rho assay lysis buffer 6 times and bound active RhoA was eluted by boiling in 2X SDS buffer. Samples were then run on a 15% Tris-Glycine gel and analyzed via Western Blotting, using an antibody against RhoA.

For the RhoA activity ELISA, HEK293 cells were either mock transfected or transfected with $p75^{NTR}$ alone or $p75^{NTR}$ with a dominant negative mutant of PDE4A5. Thirty six hours after transfection, cells were treated with MAG (25 μg/ml) for thirty minutes, then lysed and analyzed according to the manufacturer's protocol.

4.5. Neurite outgrowth assay

Twenty thousand mouse CGNs were plated in each well of sixteen-well Nunc plates coated with either poly-D-lysine or myelin. Myelin from mouse spinal cord was prepared as described [165] and 1 μg of myelin protein was dried overnight directly onto the plate, as described [166]. Neurons were allowed to extend processes for 24 hours prior to fixation in 4% paraformaldehyde and staining with an anti β-tubulin antibody. Quantification was performed as described [64, 167, 168]. All neurites longer than the length of the cell body were measured and the average neurite length was determined for each condition. At least 100 neurites were counted per condition. All experiments were performed three times in duplicate.

4.6. cAMP assay

cAMP assays were performed as described above, except that two million CGNS were used for each condition.

4.7 Statistics

Statistical significance was calculated using JMP2 or GraphPad Prism Software (GraphPad Software) by unpaired Student's *t* test for isolated pairs or by analysis of variance (one-way ANOVA, Bonferroni post-test) for multiple comparisons. Data are presented as the mean ± SEM.

Chapter 3: The role of $p75^{NTR}$ in regulating fibrin clearance after injury

1. Introduction

Previous work by Katerina Akassoglou demonstrated that fibrin inhibits sciatic nerve regeneration by inhibiting migration of Schwann cells [169] and arresting their differentiation in a proliferating, non-myelinating state [119]. However, the precise molecular mechanisms that regulate fibrin deposition remained unclear. One potential candidate protein to regulate fibrin deposition is $p75^{NTR}$, a single transmembrane receptor upregulated after sciatic nerve injury. In contrast to fibrin, which inhibits both myelination and migration of SCs, $p75^{NTR}$ is a positive regulator of myelination and is required for SC migration [81, 170, 171]. Thus, it is possible that $p75^{NTR}$ antagonizes fibrin functions after sciatic nerve injury. Furthermore, the upregulation of $p75^{NTR}$ after sciatic nerve injury has been shown to depend upon the presence of fibrin [139], providing another potential link between these two proteins. To investigate the possibility of interplay between $p75^{NTR}$ and fibrin after sciatic nerve injury, we examined the effects of $p75^{NTR}$ deletion on fibrin deposition after sciatic nerve crush injury.

2. Fibrin deposition is reduced in *$p75^{NTR}$*-/- mice

As previously reported [139], sciatic nerve crush induces a dramatic increase in fibrin deposition (Fig. 6 c) and $p75^{NTR}$ expression (Fig. 6 d) in wt nerves when compared to uninjured, control nerves (Fig. 6 a, b). Remarkably, both immunohistochemistry (Fig. 6 e) and Western blotting (Fig. 7 a) reveal that *$p75^{NTR}$*-/-

mice show reduced fibrin deposition after injury when compared to wt controls. Quantification of Western blots using densitometry reveals that *p75^{NTR}*-/- mice have threefold less fibrin three days after injury and exhibit a fourfold reduction in fibrin deposition eight days after injury (Fig. 7 a) when compared to wt controls. Confirmation of these results using quantification of fibrin immunostaining also reveals that *p75^{NTR}*-/- mice exhibit a statistically significant decrease in fibrin deposition (Fig. 7 b, $P<0.003$) compared to wt controls. Taken together, these results demonstrate that loss of p75^{NTR} results in decreased levels of fibrin in the sciatic nerve after injury.

3. p75^{NTR} regulates expression of tPA in the sciatic nerve after crush injury

The decreased levels of fibrin in the *p75^{NTR}*-/- sciatic nerve following injury could be the result of either decreased fibrin deposition or increased fibrin clearance. To determine whether the fibrin decrease in the sciatic nerve resulted from decreased in circulating levels of fibrinogen, we analyzed plasma levels of fibrinogen using Western blotting. Immunoblots revealed no differences in circulating fibrinogen between the two genotypes, indicating that the decreased level of fibrin deposited in the sciatic nerve of *p75^{NTR}*-/- mice can not be explained by hypofibrinogenemia (Fig. 8). In light of this finding, we hypothesized that the observed decrease in fibrin in *p75^{NTR}*-/- nerves results from increased fibrin clearance.

Clearance of fibrin occurs as the result of fibrinolysis, a proteolytic degradation process catalyzed by the serine protease, plasmin. High proteolytic activity corresponds to a greater capacity to remove fibrin, and it has been shown that

inhibition or genetic loss of proteolytic activity results in sustained fibrin deposition after injury [117]. We therefore examined whether the decreased fibrin in the *p75^{NTR}*-/- mice reflected an upregulation of proteolytic activity in the sciatic nerve. *In situ* zymography reveals that four days after sciatic nerve crush, *p75^{NTR}*-/- mice do indeed exhibit increased proteolytic activity as judged by the size of the lytic zone (Fig. 9 b), when compared to wt mice (Fig. 9 a). Quantification of the size of lytic zones reveals a statistically significant 2-fold increase in proteolysis in *p75^{NTR}*-/- nerves when compared to wt controls (Fig. 12 e, $P<0.05$). In contrast, uninjured nerves exhibit minimal proteolytic activity (Fig. 9 e), as expected [139]. Similar to wt controls, crushed *p75^{NTR}*-/- sciatic nerves do not show lysis of fibrin in the absence of plasminogen (Fig. 9 c), suggesting that the proteolytic activity is plasminogen-dependent. Furthermore, a specific tPA inhibitor, tPASTOP, blocks the increased proteolytic activity in *p75^{NTR}*-/- mice providing pharmacologic evidence that the increased proteolysis observed in *p75^{NTR}*-/- nerves after injury is due to increased tPA activity (Fig. 9 d).

This result is in accordance with previous work that has shown that the tPA/plasmin system regulates fibrin clearance after nerve injury. However, the mechanisms of tPA regulation after nerve injury had not been identified. p75^{NTR} is strongly activated by withdrawal of axons [172] and its expression correlates with proliferating, non-myelin producing Schwann cells (SCs) [173]. After sciatic nerve injury in wt mice, there is an increase in both p75^{NTR} (Fig. 10 a, red) and tPA (Fig. 10 a, green), when compared to uninjured controls (Fig. 10 b), but there is little colocalization of tPA and p75^{NTR} (Fig. 10 a). Higher magnification images show tPA

positive SCs (Fig. 10 e, green) adjacent to $p75^{NTR}$ positive SCs (Fig. 10 e, red) but very few yellow, $p75^{NTR}$ and tPA co-expressing SCs, suggesting that $p75^{NTR}$ re-expressing SCs do not express tPA. Expression of tPA (Fig. 10 f, red) and $p75^{NTR}$ (Fig. 10 g, red) in Schwann cells is confirmed using double immunofluorescence with the Schwann cell marker, S100 (Fig 10 f, g, green). These results reveal an inverse correlation between $p75^{NTR}$ expression and tPA expression in the sciatic nerve after injury.

4. Genetic loss of tPA rescues the effects of $p75^{NTR}$ deficiency

To confirm genetically that the enhanced proteolytic activity in the *$p75^{NTR}$*-/- mice is due to increased levels of tPA, we generated *$p75^{NTR}$*-/-*tPA*-/- double knockout mice by crossing *$p75^{NTR}$*-/- mice with *tPA*-/- mice. As stated previously, *$p75^{NTR}$*-/- mice show a decrease in fibrin deposition (Fig. 6, 7) and an increase in proteolytic activity (Fig. 9 b) compared to wt control mice after sciatic nerve crush injury (Fig. 9 a and e, respectively). In contrast, *$p75^{NTR}$*-/-*tPA*-/- mice show similar levels of fibrin deposition when compared to injured wt mice and show increased fibrin deposition (Fig. 11 c), when compared to *$p75^{NTR}$*-/- mice (Fig. 11 b). In addition, these mice exhibit no evidence of proteolytic activity (Fig. 12 c and g), indicating that the primary protease responsible for the increased degradation of fibrin observed in the *$p75^{NTR}$*-/- is tPA. As a control, *tPA*-/- mice also show no evidence of proteolytic activity after sciatic nerve crush injury (Fig. 12 d and e), as described previously [139]. Quantification of proteolytic activity reveals a statistically significant increase in proteolysis in mice deficient for $p75^{NTR}$ (Fig. 12 e). The evidence derived from the

genetic depletion of tPA in the *p75^{NTR}*-/- mice (*p75^{NTR}*-/-*tPA*-/- mice, Figs. 11 and 12) are in accordance with the pharmacologic inhibition of tPA activity in the *p75^{NTR}*-/- sciatic nerve using tPASTOP (Fig. 9 d). Overall, these results suggest that the increased proteolytic activity observed in the sciatic nerve of *p75^{NTR}*-/- mice depends upon an upregulation of tPA.

5. *p75^{NTR}*-/- SCs show increased expression of tPA and increased fibrinolysis

Since SCs are a major source for both p75^{NTR} and tPA after injury, and since there appeared to be a negative correlation between p75^{NTR} and tPA expression in SCs in wt injured sciatic nerves, we hypothesized that SCs were the primary cell type in which the regulation of tPA by p75^{NTR} occurred. To confirm that p75^{NTR} inhibits fibrinolytic activity in SCs, we isolated primary SCs from wt and *p75$^{NTR-/-}$* mice and cultured them on a 3-dimensional (3D) fibrin gel. Wt SCs, which express high levels of p75^{NTR}, form a monolayer on the fibrin gel and degrade very little of the gel within the first 48 hours of culture (Fig. 13 a). In contrast, *p75$^{NTR-/-}$* SCs degrade the fibrin gel (Fig. 13 b) and show a 2.7-fold increase of fibrin degradation as measured by the weight of gel remaining after two days in culture (Fig. 13 c). To verify that the increased fibrin degradation observed in *p75$^{NTR-/-}$* SCs was the result of increased tPA activity, we measured tPA activity in wt and *p75^{NTR}*-/- SCs. Results show a 6-fold increase in tPA levels in *p75^{NTR}*-/- SCs, when compared to wt controls (Fig. 13 d, $P<0.01$). These results suggest that p75^{NTR} downregulates tPA activity and blocks fibrin degradation in SCs *in vitro*.

6. Expression of p75^{NTR} inhibits tPA and fibrinolysis

After finding a biological function for p75^{NTR} in the regulation of tPA in *p75$^{NTR-/-}$* SCs and in the sciatic nerve, we used stable and transient transfections of p75^{NTR} as well as siRNA against *p75^{NTR}* to test the properties of p75^{NTR} in heterologous systems. To examine whether overexpression of p75^{NTR} could inhibit fibrin degradation, we first used a NIH3T3 fibroblast cell line stably transfected with high levels of p75^{NTR} (10^5 receptors/cell) [174]. When plated on a 3D fibrin gel, NIH3T3 cells degrade fibrin, as indicated by the clear hole in the center of the gel (Fig. 14 a), while NIH3T3p75^{NTR} cells are unable to degrade fibrin (Fig. 14 b). Quantification of fibrin degradation shows that overexpression of p75^{NTR} in fibroblasts inhibits fibrin degradation by 12-fold (Fig. 14 c, $P<0.001$). In contrast to NIH3T3 cells, which form lytic areas within the fibrin gel (Fig. 15 a), NIH3T3p75^{NTR} cells grow uniformly on fibrin (Fig. 15 b). *In situ* casein zymography reveals that NIH3T3 cells readily degrade the plasmin substrate casein (Fig. 15 c), while NIH3T3p75^{NTR} cells cannot degrade casein (Fig. 15 d), suggesting impaired proteolysis in NIH3T3p75^{NTR} cells. Aprotinin, a general inhibitor of serine proteases such as tPA and uPA, completely inhibits fibrin degradation by NIH3T3 cells (data not shown). However, fibroblasts express and secrete both tPA and uPA, and the use of aprotonin cannot distinguish between a tPA and a uPA effect. To determine whether the inhibition fibrinolysis by p75^{NTR} in NIH3T3 cells results from inhibition of tPA or uPA, we examined the tPA and uPA activity in these cells. We find that tPA activity is significantly decreased in NIH3T3p75^{NTR} cells cultured either on plastic or on fibrin (Fig. 16 a). In contrast, stable over-expression of p75^{NTR} has no effect on uPA activity

(Fig. 16 b). Similar to the results obtained using stably transfected cells, transient over-expression of either FL-p75^{NTR} or p75^{NTR}-ICD in NIH3T3 cells leads to a reduction in tPA activity (Fig. 16 c). Thus, in accordance with our *in vivo* data in the sciatic nerve and our genetic depletion data in SCs, p75^{NTR} inhibits plasminogen activation in fibroblasts via inhibition of tPA activity.

tPA is as an immediate-early gene [175] that is regulated both transcriptionally and at the protein level by inhibitors such as plasminogen activator inhibitor 1 (PAI-1) [176]. To test whether the inhibition of plasmin activity by p75^{NTR} depends upon alterations in tPA or PAI-1 levels, we performed reverse transcription PCR. Indeed, we find that expression of p75^{NTR} leads to a robust downregulation of tPA transcripts (Fig. 17 a), but results in only a moderate decrease of uPA mRNA levels (Fig. 17 a). In addition, mRNA of PAI-1 is also upregulated in NIH3T3p75^{NTR} cells (Fig. 17 a). Real time quantitative PCR shows a 10.1-fold decrease in tPA mRNA, a 4-fold increase in PAI-1 mRNA, and a 2-fold decrease in uPA mRNA in NIH3T3p75^{NTR} cells. The decrease in tPA expression correlates well with the observed decrease in tPA activity observed in cells overexpressing p75^{NTR} (Fig. 16, $P<0.01$). In contrast, upon expression of p75^{NTR}, the 2-fold decrease of uPA RNA does not affect uPA activity (Fig. 16 b). Three days after crush injury, sciatic nerves of *p75^{NTR}*-/- mice show a 4-fold increase in *tPA* RNA when compared to wt nerves (Fig. 17 b). However, we were unable to detect PAI-1 in the sciatic nerve, either using real time PCR, or with immunohistochemistry in either wt or *p75^{NTR}*-/- animals (data not shown), indicating that the regulation of PAI-1 by p75^{NTR} may not play a role in sciatic nerve injury. It remains possible that the regulation of PAI-1 by p75^{NTR} might

have functional significance in other tissues during injury or disease. Overall, these data suggest that expression of $p75^{NTR}$ inhibits the tPA/plasmin system *in vivo* after sciatic nerve injury, as well as *in vitro* in primary SCs and fibroblasts transiently or stably transfected with $p75^{NTR}$.

7. $p75^{NTR}$ Regulates tPA and PAI-1 via a cAMP/PKA Pathway

Several studies have indicated that transcriptional upregulation of tPA depends on elevation of cAMP [177]. Indeed, elevation of cAMP, using dibutyryl-cAMP (db-cAMP), overcomes the inhibitory effect of $p75^{NTR}$, since fibrin lysis is only observed in NIH3T3$p75^{NTR}$ cells treated with db-cAMP (Fig. 18 a). Moreover, elevation of cAMP via the use of the general PDE inhibitor, IBMX, restores tPA activity in NIH3T3$p75^{NTR}$ to the levels seen in untransfected NIH3T3 cells (Fig. 18 b). In contrast, IBMX has minimal effects on the basal levels of tPA in NIH3T3 cells (Fig. 18 b). These data suggest that PDE activity is required for the $p75^{NTR}$–induced tPA decrease and that $p75^{NTR}$ may somehow regulate PDE activity.

Because the cAMP/PKA pathway is known both to enhance tPA transcription [177] and suppress PAI-1 [178] we tested whether the cAMP/PKA pathway influences the $p75^{NTR}$–regulation of tPA and PAI-1 levels. Elevation of cAMP using forskolin increases levels of tPA, while KT5720-induced PKA inhibition decreases tPA RNA levels in NIH3T3 cells (Fig. 19 a). Treatment of NIH3T3$p75^{NTR}$ cells with forskolin also increases both tPA RNA and activity (Fig. 19 a), while forskolin decreases PAI-1 RNA in both NIH3T3 and NIH3T3$p75^{NTR}$ cells (Fig. 19 b). Similar to fibroblasts, elevation of cAMP increases the activity of tPA in both wt and *$p75^{NTR}$*-/- SCs (Fig.

20). Given the finding that elevation of cAMP and activation of PKA in SCs and fibroblasts leads to increased tPA activity, we next examined whether blocking PKA could lead to the opposite effect. Treatment with KT5720, a specific PKA inhibitor, decreases tPA activity in NIH3T3 cells, similar to overexpression of $p75^{NTR}$ (Fig. 18 b). Taken together, these results indicate that in SCs and fibroblasts, high levels of cAMP/PKA activity correlate with increased plasminogen activation and fibrinolysis.

To determine whether neurotrophin ligands have any effect on the regulation of tPA by $p75^{NTR}$, we treated wt primary SCs with various neurotrophins and measured tPA activity. We show here that treatment of SCs with either Brain Derived Growth Factor (BDNF) or Nerve Growth Factor (NGF) has no effect on tPA (Fig. 20). Similarly, treatment of SCs with pro-NGF, the high affinity ligand of $p75^{NTR}$ [38], does not affect tPA activity (Fig. 21). BDNF/TrkB signaling has been shown to regulate tPA in primary cortical neurons [179]. In contrast to cortical neurons, which express high levels of TrkB, SCs are known to express minute levels of TrkB, but high levels of $p75^{NTR}$. The differences in relative levels of expression of $p75^{NTR}$ and Trk receptors likely explain the differences in neurotrophin dependence of the regulation of tPA expression. In support of this, in NIH3T3 and NIH3T3$p75^{NTR}$ cells, which do not express Trk receptors, the $p75^{NTR}$–mediated suppression of tPA activity occurs independent of neurotrophins or serum. Treatment of NIH3T3 or NIH3T3$p75^{NTR}$ cells with either NGF, or BDNF, or cerebrospinal fluid (a major source of pro-neurotrophins), or Fc-TrkB leads to no differences in tPA activity (Fig. 22 a), indicating that the neurotrophins and their precursors do not regulate tPA through $p75^{NTR}$. Similarly, removal of serum does not alter tPA activity in cells

overexpressing $p75^{NTR,}$ indicating that there is no ligand present within serum that is responsible for the $p75^{NTR}$-induced effects (Fig. 22 b). Further evidence that the inhibition of plasminogen activation by $p75^{NTR}$ does not depend on extracellular ligands comes from the fact that in NIH3T3 cells, transient expression of a truncated mutant of $p75^{NTR}$ lacking the neurotrophin binding extracellular domain decreases tPA similar to the full length (FL) $p75^{NTR}$ (Fig. 16 c, $P < 0.05$). Overall our data indicate that neurotrophin/$p75^{NTR}$ signaling is not involved in the regulation of tPA in SCs and fibroblasts and that regulation of tPA by $p75^{NTR}$ occurs independently of neurotrophins.

Upon failing to find any neurotrophin dependence for the inhibition of plasminogen activation by $p75^{NTR}$, we wanted to determine if proteolytic processing of $p75^{NTR}$ plays a role in the inhibition of tPA. To test this possibility, we used an inhibitor of $p75^{NTR}$ proteolysis. Treatment with the gamma-secretase inhibitor led to no differences in the $p75^{NTR}$ induced inhibition of tPA (Fig. 22 b), suggesting that this process is also independent of $p75^{NTR}$ cleavage.

8. $p75^{NTR}$ decreases cAMP

Since the effects of $p75^{NTR}$ were overcome by elevating cAMP and overexpression of $p75^{NTR}$ led to decreased PKA activity, we examined whether expression of $p75^{NTR}$ leads to reduced cAMP levels. Indeed, cAMP is decreased 7.8-fold in NIH3T3 stably overexpressing $p75^{NTR}$ (Fig. 23 a, $P<0.0001$). Similarly, transient expression of $p75^{NTR}$ in NIH3T3 cells also decreases levels of cAMP, but to a lesser extent than the stable NIH3T3$p75^{NTR}$ cells (Fig. 23 b, $P<0.0005$). Western

blot analysis reveals that NIH3T3 cells transiently transfected with $p75^{NTR}$ express ~80% less $p75^{NTR}$ than the stably transfected NIH3T3$p75^{NTR}$ cells, thus likely explaining the differences in extent of cAMP downregulation. After observing a decrease in cAMP upon $p75^{NTR}$ over-expression in NIH3T3 cells, we next investigated whether $p75^{NTR}$ expression also inhibits PKA activity. Indeed, NIH3T3$p75^{NTR}$ cells (Fig. 23 c, lanes 3 and 4) exhibit decreased total PKA activity when compared to NIH3T3 cells (Fig. 23 c, 1 and 2). Since overexpression of $p75^{NTR}$ leads to decreased cAMP and PKA, we next examined whether genetic depletion of $p75^{NTR}$ could increase cAMP levels. Indeed, knockdown of $p75^{NTR}$ expression using siRNA leads to increased cAMP levels in both NIH3T3$p75^{NTR}$ cells (Fig. 24 a, b, $P<0.02$) and primary rat SCs (Fig. 24 c, d, $P<0.03$). In addition to these *in vitro* findings, immunostaining with an antibody against cAMP shows increased cAMP in injured sciatic nerves from $p75^{NTR}$ -/- mice when compared to wt control (Fig. 26 a, b).

Prior literature in neurons has shown that BDNF elevates cAMP exclusively via TrkB [180]. In NIH3T3$p75^{NTR}$ cells, which do not express TrkB, stimulation with NGF or BDNF does not affect the $p75^{NTR}$–mediated suppression of cAMP (Fig. 25). Similarly, inhibition of neurotrophins using the neurotrophin scavenger, Fc-$p75^{NTR}$, or the BDNF scavenger, Fc-TrkB, does not alter cAMP levels in NIH3T3$p75^{NTR}$ cells (Fig. 25). In accordance, transient expression of the ICD of $p75^{NTR}$ decreases cAMP similar to the FL $p75^{NTR}$ in NIH3T3 cells, indicating that the extracellular domain is not required to elicit this effect (Fig. 23 b). Overall, these data suggest a neurotrophin-independent cAMP pathway downstream of $p75^{NTR}$, which consequently leads to decreases in extracellular proteolysis.

Downregulation of cAMP can be mediated either by inhibition of cAMP synthesis via the activation of G_i, a G protein that inhibits adenylyl cyclase, or via the action of PDEs, enzymes that degrade cAMP [181]. Treatment of cells with pertussis toxin (PTX) that blocks interactions between the G_i and G Protein Coupled Receptors (GPCRs) [182] does not rescue the $p75^{NTR}$ – mediated downregulation of cAMP (Fig. 23 a, P>0.5). In contrast, the general PDE inhibitor, IBMX, resulted in significant increase of cAMP in the NIH3T3$p75^{NTR}$ cells, when compared to control NIH3T3$p75^{NTR}$ cells (Fig. 23 a). This result indicates that the $p75^{NTR}$ induced inhibition of cAMP requires phosphodiesterase activity.

There are at least 11 different families of PDEs that differ in their mode of regulation as well as their specificities for cAMP or cGMP. PDE4, PDE7 and PDE8 have been shown to degrade cAMP almost exclusively, while PDE5, PDE6 and PDE9 preferentially degrade cGMP. The other PDEs (PDEs 1, 2, 3, 10 and 11) degrade both cAMP and cGMP with similar affinity but are differentially regulated {reviewed in [181, 183]}. Each of these gene families consists of multiple genes, and due to alternative splicing, there are estimated to be at least 50 different PDE isoforms {reviewed in [181]}. To date, pharmacologic inhibitors of the first 5 families have been developed. To determine which PDE family was responsible for the $p75^{NTR}$ induced decrease in cAMP, we performed a pharmacological screen using the commercially available inhibitors. The results from this screen show that only rolipram, a specific inhibitor of PDE4, significantly increases cAMP levels in NIH3T3$p75^{NTR}$ cells (Fig. 23 a) to the levels of NIH3T3 cells (Fig. 23 a, P=.051), suggesting that the $p75^{NTR}$–induced cAMP decrease is mediated via PDE4.

9. p75^{NTR} interacts with PDE4A4/5 to target cAMP degradation to the membrane

It is well established that recruitment of PDE4 to subcellular structures such as the plasma membrane concentrates the activity of PDEs and reduces PKA activity by enhancing degradation of cAMP [184]. We therefore examined whether p75^{NTR} regulated cAMP via recruitment of PDE4. Co-immunoprecipitation (co-IP) experiments indicate that in NIH3T3p75^{NTR} cells, p75^{NTR} associates with endogenous PDE4A (Fig. 27 a). Immunoblotting with antibodies against the other 3 PDE4 sub-families (PDE4B, PDE4C or PDE4D) reveals no evidence of association between p75^{NTR} and these isoforms (data not shown), indicating that this interaction is PDE4A specific. Based on the molecular weight of PDE4A at 109 kDa, we determined that p75^{NTR} co-IPs with the PDE4A5 isoform. Western blot analysis of whole cell lysates reveals similar levels of expression of PDE4A5 in NIH3T3 cells and NIH3T3 cells overexpressing p75^{NTR} (Fig. 27 b). However, cell fractionation experiments reveal increased PDE4A5 in membrane fractions of NIH3T3p75^{NTR} cells when compared to NIH3T3 cells (Fig. 27 c), indicating increased recruitment of PDE4A5 to the membrane in response to overexpression of p75^{NTR}. Endogenous co-IP from injured wt sciatic nerve (Fig. 28) shows that p75^{NTR} and PDE4A5 interact at endogenous expression levels *in vivo* after sciatic nerve injury. These results indicate that p75^{NTR} recruits PDE4A5 to the membrane and forms a complex that degrades cAMP both *in vivo* and *in vitro*. Our results are in accordance with previous research showing that in the sciatic nerve, reduction of cAMP after injury is attributed primarily to upregulation of PDE4 by SCs, the cells that upregulate p75^{NTR} after nerve injury [185]. To

determine if the inhibition of PDE4 activity would promote fibrin clearance in sciatic nerve after injury, we injected wt mice with rolipram daily for eight days following sciatic nerve crush. Indeed, rolipram treatment reduces fibrin deposition in the sciatic nerve (Fig. 29), revealing that both PDE4 and $p75^{NTR}$ regulate fibrinolysis *in vivo*.

A functional consequence of the recruitment of PDE4A5 to the membrane would be a decrease in membrane-associated cAMP/PKA signaling. To investigate whether $p75^{NTR}$ reduces membrane-associated PKA activity, we modified the genetically encoded A-kinase activity reporter, AKAR2[158] and generated pm-AKAR2.2, a membrane-targeted fluorescent reporter of PKA activity that generates a change in fluorescence resonance energy transfer (FRET) when it is phosphorylated by PKA in living cells (Fig. 30 a). As expected, NIH3T3 cells show a dramatic emission ratio change for the pm-AKAR2.2 in response to forskolin, which activates adenylyl cyclase at the plasma membrane (Fig. 30 b). By contrast, NIH3T3$p75^{NTR}$ cells show an attenuated response, revealing reduced PKA activity at the plasma membrane (Fig. 30 b). Transient transfections of $p75^{NTR}$ confirmed the results observed in the stable NIH3T3$p75^{NTR}$ cells using the latest generation of plasma-membrane specific PKA biosensor pm-AKAR3 [158] (Fig. 31 a). Effective targeting of this construct to the plasma membrane was confirmed using fluorescence microscopy (Fig. 31 b). In addition to the observed decrease in PKA activity at the plasma membrane in cells overexpressing $p75^{NTR}$, we observed a general decrease in PKA activity using an untargeted construct as well (Fig. 31 c, d). These results are in accordance with our previous data showing decreased total intracellular cAMP and

PKA upon overexpression of $p75^{NTR}$. Overall, our results suggest that $p75^{NTR}$ targets cAMP degradation to the membrane via its interaction with PDE4A5.

To verify the specificity of $p75^{NTR}$-PDE4A5 association, a series of mapping studies were conducted using deletion mutants in the ICD of $p75^{NTR}$ [160, 186] or the full length receptor. PDE4A5 interacts with FL $p75^{NTR}$, as well as deletions Δ3, Δ62, Δ83, but not a deletion missing the distal 151 amino acids, Δ151 (Fig. 32), suggesting that the interaction between $p75^{NTR}$ and PDE4A5 occurs in the juxtamembrane region of $p75^{NTR}$ and requires sequences between residues 275 and 343.

To further characterize the interaction, we used a novel peptide array technology that has been previously utilized to define interaction sites in PDE4D5 that allow for bind to RACK1 and to β-arrestin [161, 187, 188]. Screening a library of overlapping 25-mer peptides that scanned the sequence of PDE4A4 for binding to a GST fusion protein of $p75^{NTR}$ encoding the entire ICD [186] identified three potential sites of direct interaction between $p75^{NTR}$-ICD and PDE4A4 (Fig. 33). These potential sites of direct interaction include the LR1 domain of PDE4A4, whose sequence is unique to the PDE4A subfamily (peptides 40 and 41, aa191-220), and also to a sequence within the catalytic domain (peptides 135 and 136, aa671-700). However, the strongest interaction is observed with a sequence within the extreme C-terminal region of PDE4A4 (peptides 172 and 173, aa856-885), which is not present in other PDE4s and may explain the specificity observed for the $p75^{NTR}$-PDE4A4/5 interaction.

Alanine scanning mutagenesis of this extreme C-terminal region shows that substitution of C862 abolishes the interaction of $p75^{NTR}$ with the 173 peptide that is

unique to PDE4A (Fig. 34). Furthermore, co-immunoprecipitations using a truncated C-terminus of PDE4A4 reveal that the extreme C-terminus of PDE4A4 is required for the interaction between PDE4A4 and $p75^{NTR}$ (Fig. 35). The $p75^{NTR}$ interacting sequences within the LR1 and C-terminal domains are highly conserved between the human PDE4A4 and the rodent PDE4A5. Indeed, peptide array screening for PDE4A5 reveals direct interaction with $p75^{NTR}$ similar to that seen for PDE4A4 (data not shown). Overall, these results suggest that the interaction of $p75^{NTR}$ with PDE4A4/5 is direct and that sequences within the juxtamembrane region of $p75^{NTR}$ and the unique C-terminal region of PDE4A4/5 are required for the interaction (Figs. 33 - 35).

To examine whether $p75^{NTR}$ could potentially interact with PDE4A5 directly, in a cell free system, we performed *in vitro* pull down assays using recombinant proteins. A GST fusion protein of $p75^{NTR}$ encoding the entire ICD [186] interacts with both recombinant PDE4A5 and its human homolog, PDE4A4 (Fig. 36). In contrast, GST- $p75^{NTR}$ does not interact with recombinant PDE4D3 (Fig. 36). These results are in accordance with the co-immunoprecipitation data in cells that show that $p75^{NTR}$ specifically interacts with the PDE4A5 isoform.

Given the specificity of the interaction between $p75^{NTR}$ and a single PDE isoform, PDE4A5, the regulation of cAMP by $p75^{NTR}$ would only be predicted to occur in cell types that also express PDE4A5. In support of this, a stable HEK293 cell line overexpressing $p75^{NTR}$ does not exhibit decreased cAMP levels either basally, or in response to forskolin treatment (Fig. 38). To confirm that overexpression of $p75^{NTR}$ does not lead to decreased cAMP in cells that do not express PDE4A5, we performed transient transfections of $p75^{NTR}$ or GFP into HEK293 cells. Similar to stable

transfections, transiently transfected $p75^{NTR}$ has no effect on forskolin stimulated levels of cAMP (Fig 39). Taken together, these results indicate that PDE4A5 is required for the $p75^{NTR}$ mediated decrease in cAMP.

10. $p75^{NTR}$ regulates plasminogen activation and fibrin deposition in a model of lipopolysaccharide (LPS)-induced pulmonary fibrosis

Since expression of $p75^{NTR}$ is expressed in a wide variety of cell types and can inhibit fibrinolysis in both fibroblasts and SCs, we hypothesized that the role of $p75^{NTR}$ as a modulator of fibrinolysis *in vivo* is not restricted to the nervous system, but rather extends to other tissues that express both $p75^{NTR}$ and PDE4A5 after injury or disease. Since $p75^{NTR}$ is expressed in the lung [105, 143] and PDE4A4 is the PDE4 isoform that is specifically upregulated in lung disease [189], we hypothesized that $p75^{NTR}$-PDE4A4/5 interactions may play a functional role in the lung as well. To test this hypothesis, we compared the levels of fibrin in the lung of wt and *$p75^{NTR}$-/-* mice in a model of LPS-induced lung fibrosis [163]. LPS-treated wt mice showed widespread extravascular fibrin deposition (Fig. 40 b) and decreased proteolytic activity after LPS treatment (Fig. 40 e), when compared to saline-treated wt mice (Fig. 40 a, d). In contrast, *$p75^{NTR}$-/-* mice show a 2.6-fold decrease of fibrin immunoreactivity (Fig. 40 c and Fig. 41 d) and increased proteolytic activity (Fig. 40 f). Overall, these results show that *$p75^{NTR}$-/-* mice exhibit increased proteolytic activity and less fibrin deposition after LPS-induced lung fibrosis.

Previous studies have established that decreased proteolytic activity in the lung after injury depends upon the upregulation of PAI-1 [126]. Loss of PAI-1 protects

from pulmonary fibrosis in LPS-induced airway disease, hyperoxia, and bleomycin-induced fibrosis [190]. Since $p75^{NTR}$ increases PAI-1 (Fig. 17 a), we examined whether $p75^{NTR}$ regulates expression of PAI-1 *in vivo*. PAI-1 is upregulated in LPS-treated wt mice (Fig. 41 b), when compared to saline-treated wt mice (Fig. 41 a). In contrast, LPS-treated *$p75^{NTR}$*-/- mice show similar immunoreactivity for PAI-1 (Fig. 41 c) as saline-treated wt mice (Fig. 41 a), suggesting that $p75^{NTR}$ is required for the upregulation of PAI-1 after lung injury. Western blot analysis confirms this decrease in PAI-1 in the lungs of LPS treated *$p75^{NTR}$*-/- mice when compared to LPS treated wt mice (Fig. 41 e).

Similar to its effects in the sciatic nerve, inhibition of PDEs reduces fibrin deposition in the lung as well (Fig. 42 a, b). This reduced fibrin deposition results from the attenuation of PAI-1 upregulation in the lung after LPS injection (Fig. 42 c), suggesting the involvement of PDE4 in $p75^{NTR}$-mediated inhibition of fibrinolysis *in vivo*. Taken together, our data show that $p75^{NTR}$ increases fibrin deposition via a PDE4-mediated inhibition of plasminogen activation in both LPS-induced lung fibrosis and sciatic nerve crush injury. These data suggest a role for $p75^{NTR}$/PDE4 signaling as a general regulator of plasminogen activation and fibrinolysis at sites of injury.

To investigate whether the regulation of tPA by $p75^{NTR}$ occurs in the absence of injury or disease, and whether it can occur within the CNS, we compared tPA activity in the cerebellum of ten day old WT and *$p75^{NTR}$*-/- mice. Genetic loss of $p75^{NTR}$ results in increased proteolytic activity in the cerebellum (Fig. 43 a, b).

Moreover, primary cerebellar granule neurons (CGNs) derived from *p75^{NTR}*-/- mice show an increase in tPA RNA (Fig. 43 c) when compared to wt controls.

11. Summary

We have demonstrated that p75^{NTR} inhibits proteolytic activity and fibrin degradation during peripheral nerve injury and pulmonary fibrosis via a novel p75^{NTR}-PDE4A5-cAMP pathway. This pathway is mediated via the neurotrophin-independent recruitment of PDE4A4/5 to the p75^{NTR}. Our results suggest the following model for the role of p75^{NTR} in the regulation of plasminogen activation (Fig. 44): 1) Injury induces upregulation of p75^{NTR} in cell types both within and outside of the nervous system. 2) Once expressed, p75^{NTR} interacts with PDE4A4/5 directly to promote targeted degradation of cAMP and inhibition of PKA activity. 3) The resulting lowered level of cAMP allows upregulation of PAI-1 and inhibits tPA. Overall, these effects result in an anti-fibrinolytic environment characterized by decreased extracellular proteolysis. 4) The decreased proteolytic activity perpetuates fibrin deposition at sites of p75^{NTR} upregulation after injury (for example, in the sciatic nerve and the lung).

12. Acknowledgments

Chapter 3, in part, has been published and appears in The Journal of Cell Biology 2007. Sachs, Benjamin; Baillie, George; McCall, Julianne; Passino, Melissa; Schachtrup, Christian; Wallace, Derek; Dunlop, Allan; MacKenzie, Kirsty; Klussmann, Enno; Lynch, Martin; Sikorski, Shoana; Nuriel, Tal; Tsigelny, Igor;

Zhang, Jin; Houslay, Miles; Chao, Moses; Akassoglou, Katerina, The Rockefeller University Press 2007. The dissertation author was the primary investigator and author of this paper.

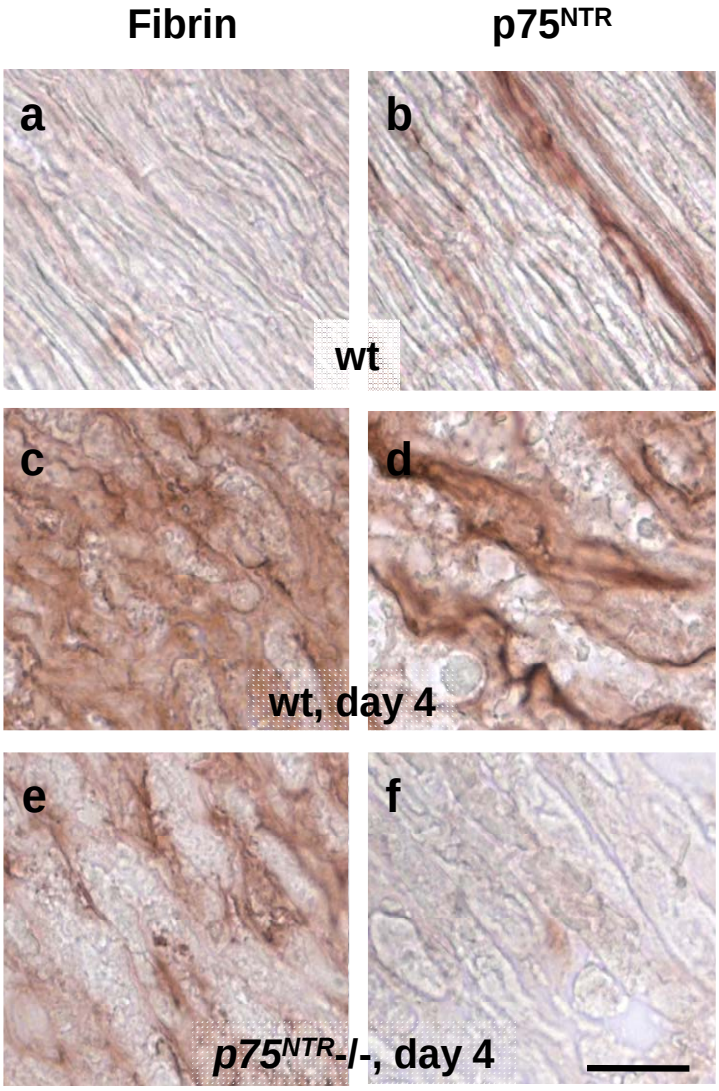

Figure 6. Fibrin and p75^{NTR} immunohistochemistry in sciatic nerves. Immunohistochemistry for fibrin on uninjured wt (a) and 4 days after sciatic nerve crush injury wt (c) and *p75^{NTR}-/-* mice (e). Immunohistochemistry for p75^{NTR} on uninjured wt (b) and 4 days after sciatic nerve crush injury wt (d) and *p75^{NTR}-/-* mice (f). Representative images are shown from n=20 wt and n=20 *p75^{NTR}* -/- mice. Experiment performed by Katerina Akassoglou.

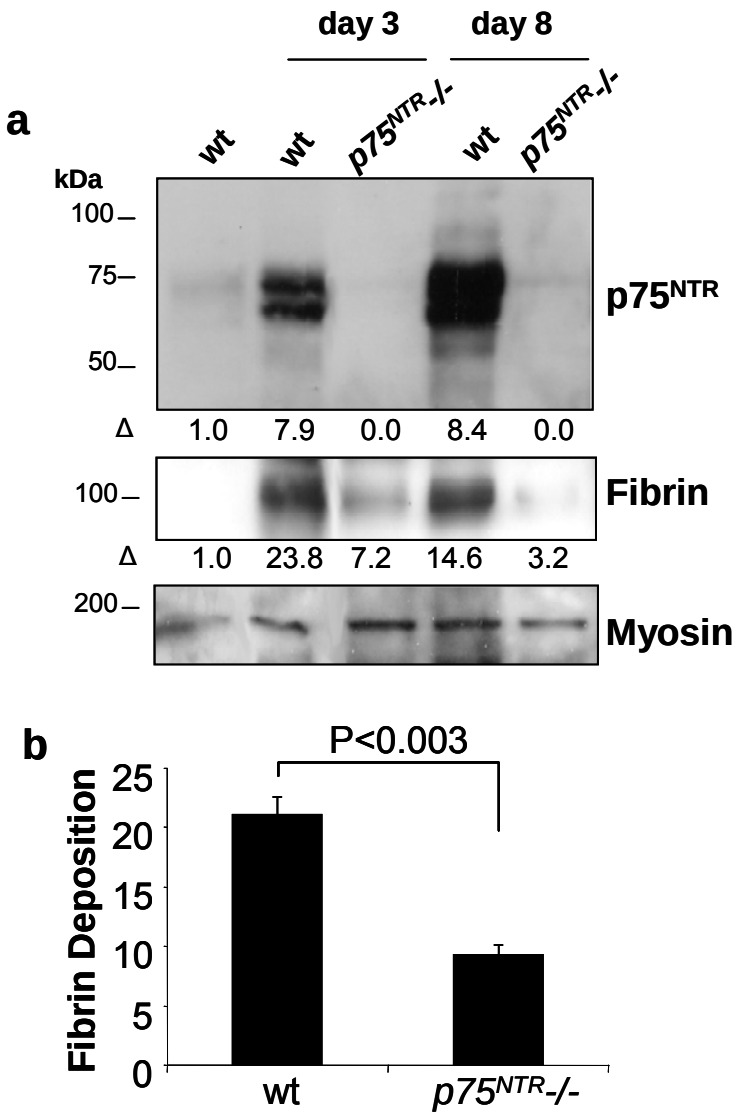

Figure 7. p75^{NTR} upregulation and fibrin deposition after sciatic nerve injury. (a) Western blot for p75^{NTR} and fibrin on sciatic nerve extracts from uninjured wt, and wt and *p75^{NTR}*-/- mice 3 and 8 days after injury. Myosin serves as loading control. Western blots were performed 3 times. A representative blot is shown. (b) Quantification of fibrin deposition shows significant decrease for fibrin in *p75^{NTR}*-/- mice (n=5), when compared to wt mice (n=4).

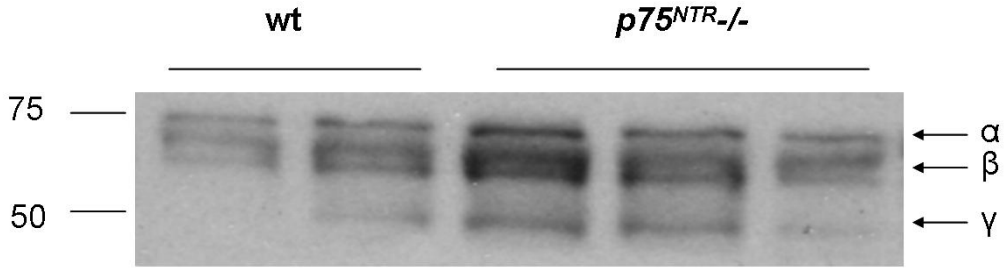

Figure 8. Loss of p75^{NTR} does not decrease circulating fibrinogen.
Western blot analysis of plasma isolated from wt and *p75^{NTR}*-/- mice reveals no decrease in circulating fibrinogen levels in animals deficient for p75^{NTR}, indicating that the effects of p75^{NTR} deletion on fibrin deposition do not result from hypofibrinogenemia.

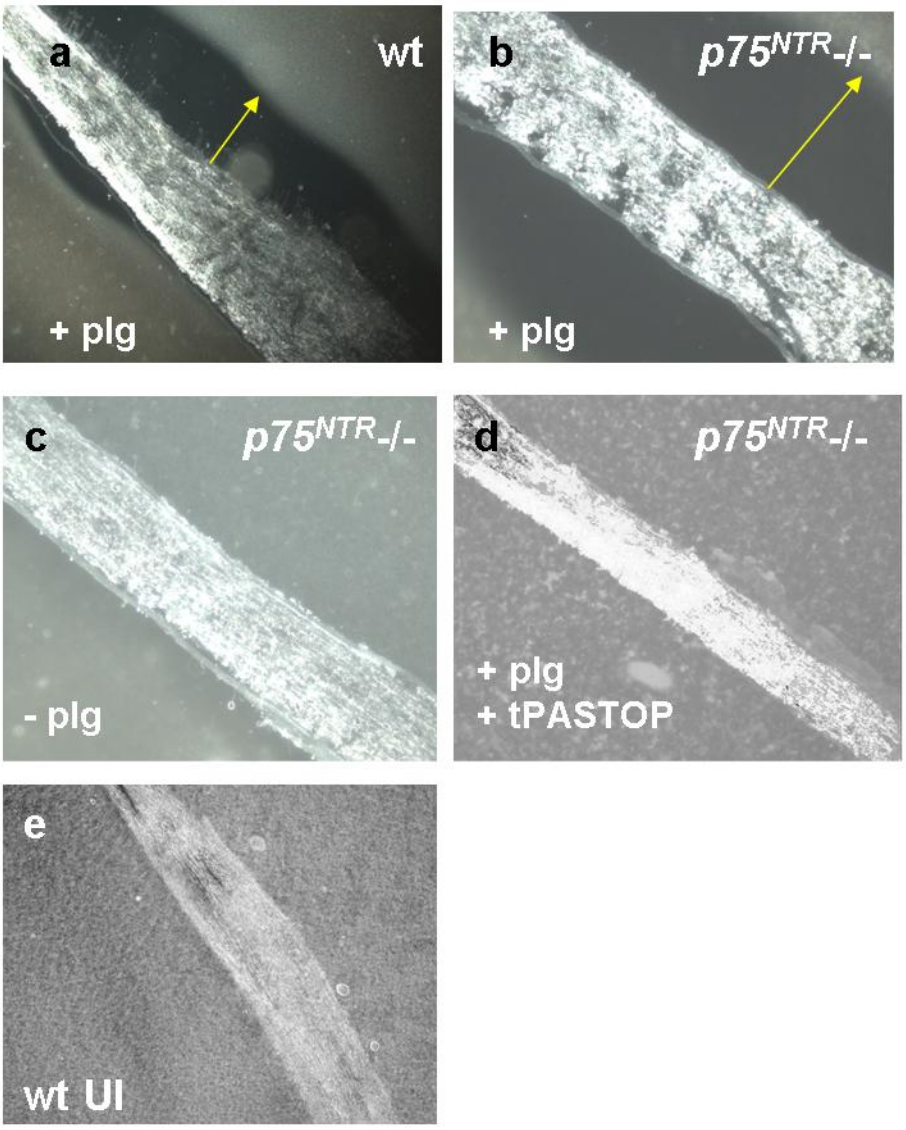

Figure 9. p75^{NTR} regulates tPA activity in the sciatic nerve after crush injury.
In situ zymography in the presence of plasminogen on wt (a) and *p75^{NTR}*-/- (b) mice and in the absence of plasminogen (c) or in the presence of plasminogen and tPASTOP (d) in *p75^{NTR}*-/- mice. Arrows indicate the lytic zone. (e) Uninjured wt sciatic nerve exhibits minimal proteolytic activity. Zymographies have been performed on n=10 wt and n=10 *p75^{NTR}* -/- mice. Scale bar = 400 μm. Experiment performed by Katerina Akassoglou.

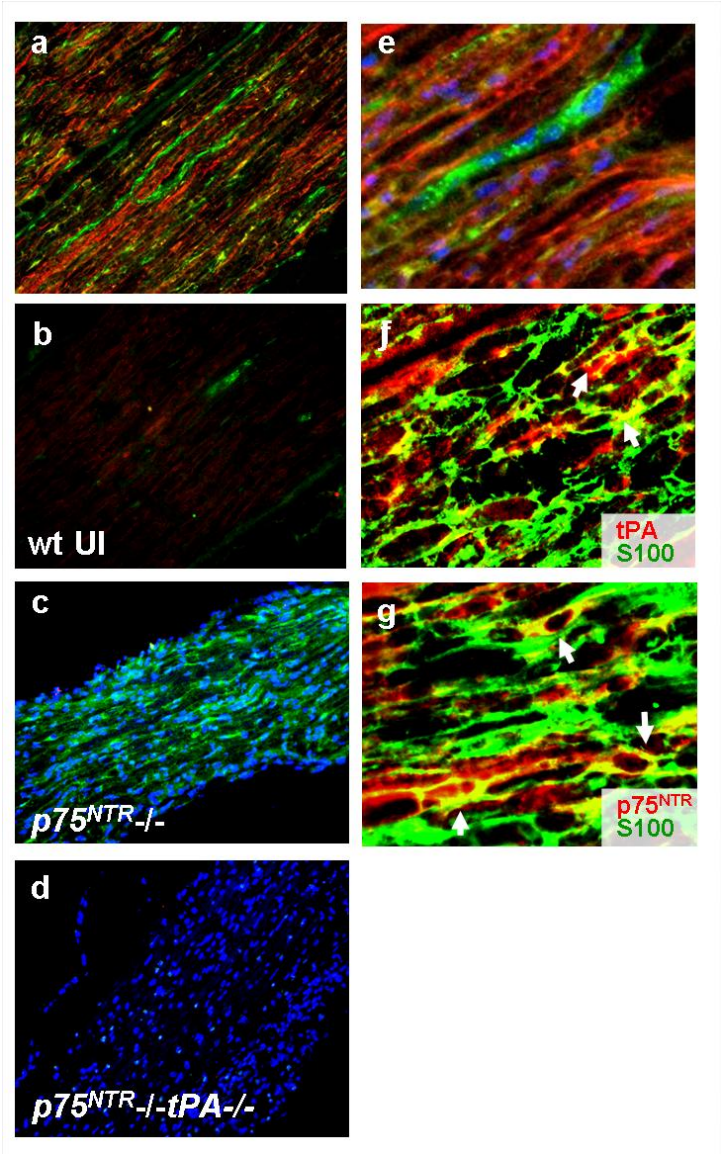

Figure 10. p75^{NTR} regulates expression of tPA in the sciatic nerve after crush injury.
Double immunofluorescence for tPA (green) or p75^{NTR} (red) on injured wt (a, e), *p75^{NTR}*-/- (c) and *p75^{NTR}*-/-*tPA*-/- mice (d). Uninjured wt sciatic nerve exhibits minimal tPA and p75^{NTR} immunoreactivity (b). Representative images are shown. tPA (f) and p75^{NTR} (g) expression in SCs was verified by double immunofluorescence with an S100 (SC marker) antibody. Arrows indicate double –positive cells (f and g, yellow). The experiment was repeated at 2 different timepoints (4 and 8 days after crush injury) in n=4 mice per genotype per timepoint and representative images are shown. Scale bar = 150 µm (a-d), 20 µm (e-g).

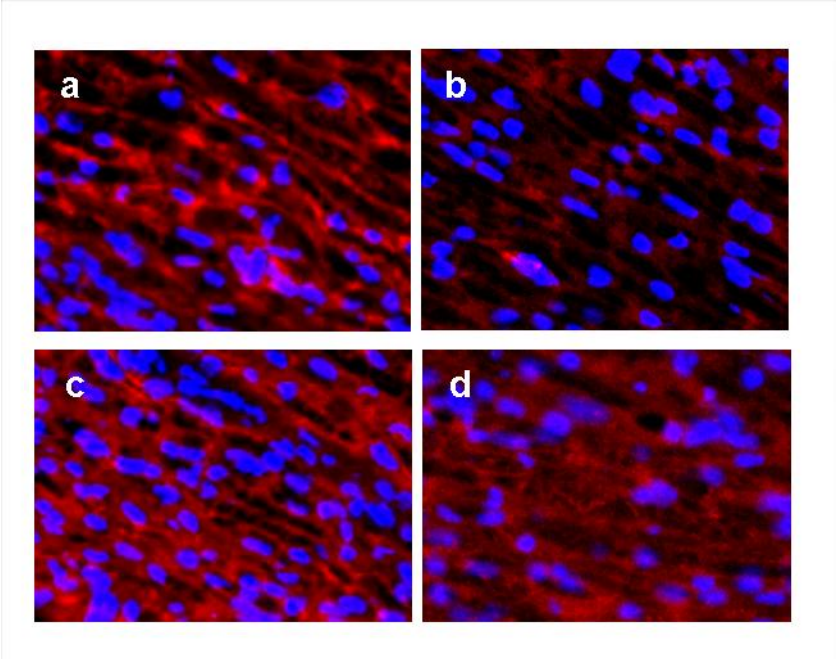

Figure 11. Loss of tPA rescues the effects of p75^{NTR} deficiency on fibrin deposition in the sciatic nerve.
Increased fibrin deposition in the crushed sciatic nerve of *p75^{NTR}*-/-*tPA*-/- mice (n=5) (c), when compared to crushed *p75^{NTR}*-/- sciatic nerve (n=20) (b). Wild-type (n=20) (a) and *tPA*-/- (n=5) (d) nerves are used for control. Scale bar = 50 µm.

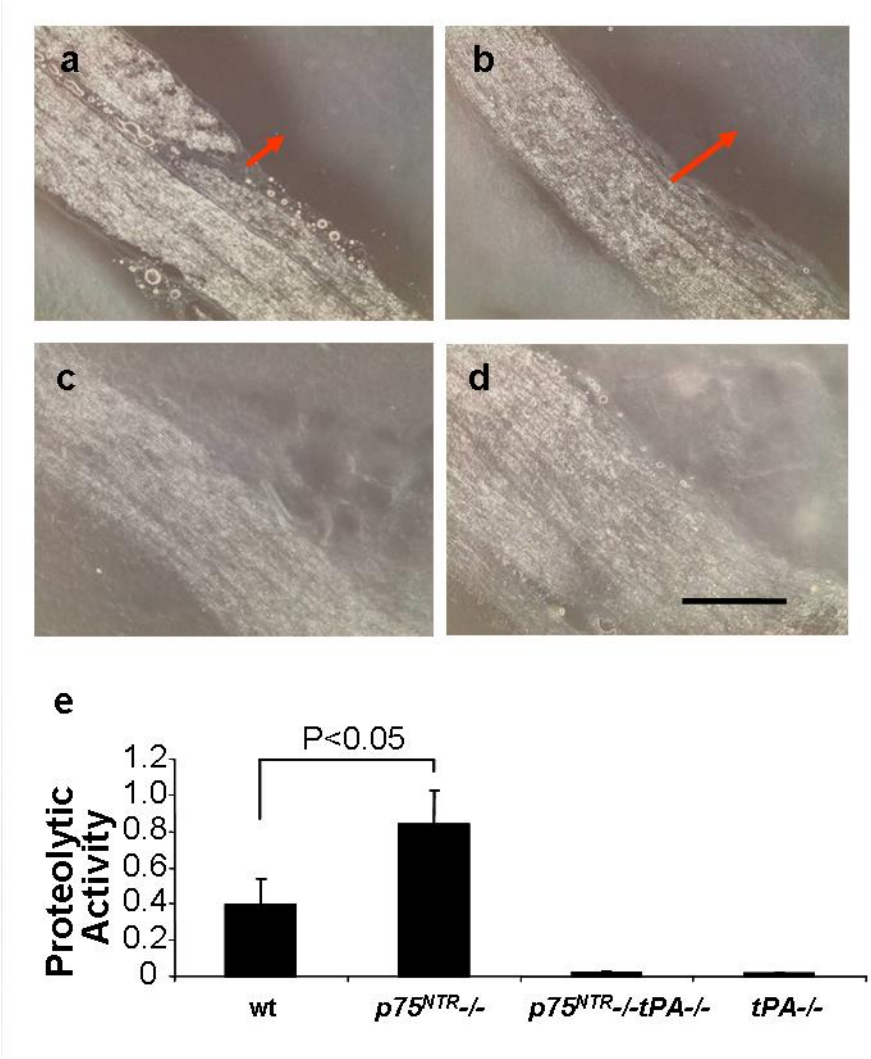

Figure 12. Genetic loss of tPA rescues the effects of p75^{NTR} deficiency on proteolytic activity in the sciatic nerve.
In situ zymography shows lack of proteolytic activity in the crushed *p75^{NTR}*-/- *tPA*-/- sciatic nerves (n=5) (c), when compared to crushed *p75^{NTR}*-/- sciatic nerves (n=20) (b). Crushed wt (a) and *tPA*-/- (d) nerves are used for control. (e) Quantification of proteolytic activity 4 days after crush injury shows statistically significant increase for proteolytic activity in *p75^{NTR}* -/- mice. Quantification results are based on n=5 *p75^{NTR}* -/-, n=5 *p75^{NTR}*-/- *tPA*-/-, n=5 *tPA*-/- and n=4 wt mice. Scale bar 300 µm (a-d).

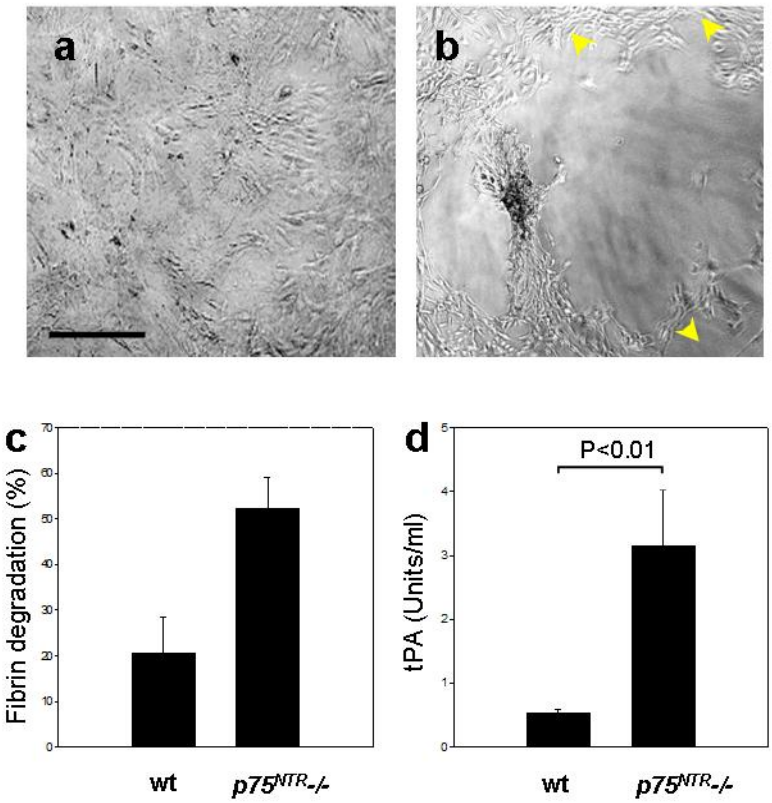

Figure 13. p75^{NTR}–mediated regulation of tPA and fibrinolysis in SCs.
Primary SC cultures on a 3D fibrin gel from wt (a) or *p75^{NTR}*-/- mice (b). Arrowheads indicate the border of fibrin degradation. Quantification of fibrin degradation (c) and tPA activity (d) from wt and *p75^{NTR}*-/- SCs. Experiments were performed 3 times in duplicates. Representative images are shown. Scale bar = 130 μm. Experiment performed by Katerina Akassoglou.

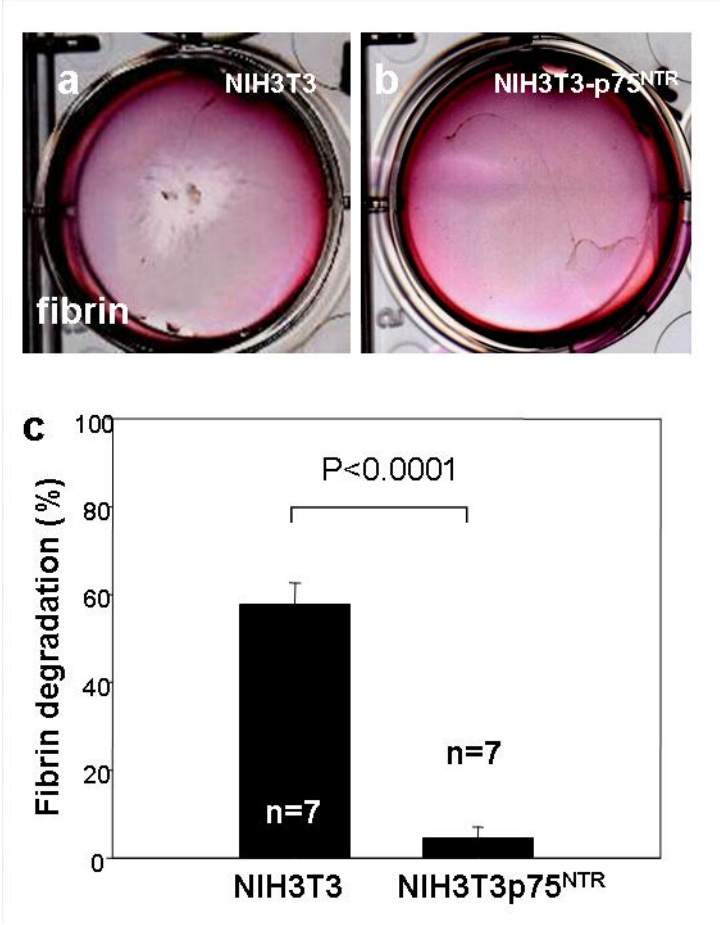

Figure 14. Expression of $p75^{NTR}$ inhibits fibrin degradation in fibroblasts.
3D fibrin gel degraded by NIH3T3 (a), but not by NIH3T3$p75^{NTR}$ cells (b). (c) Quantification of fibrin degradation. Experiments were performed 7 times in duplicates. Scale bar = 1.2 cm (a, b).

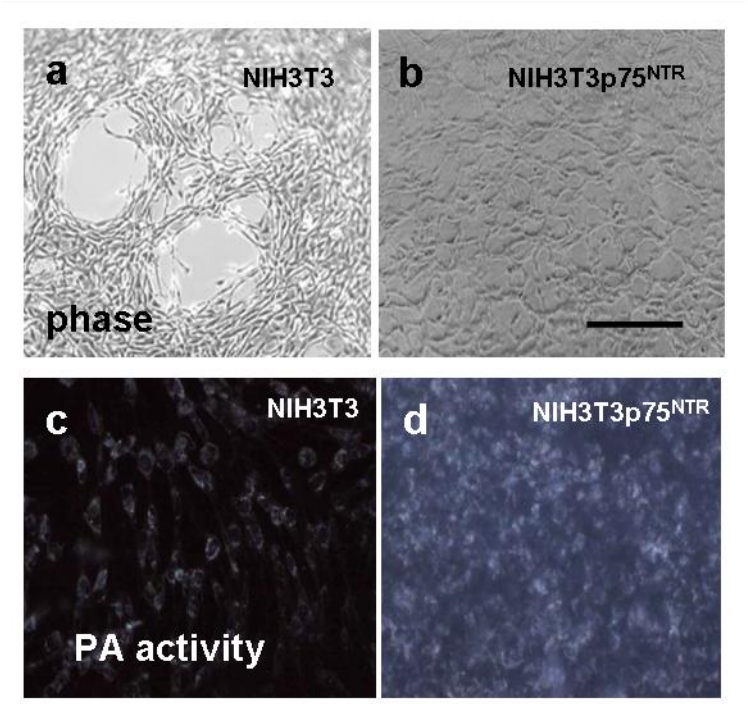

Figure 15. Expression of p75^{NTR} inhibits fibrin degradation and plasminogen activation in fibroblasts.
Phase contrast microscopy shows lytic zones in NIH3T3 (a), but not in NIH3T3p75^{NTR} cultures (b). Zymography shows degradation of casein by NIH3T3 cells (c), while NIH3T3p75^{NTR} cells do not degrade casein (d). Scale Bar = 130 μm (a-d)

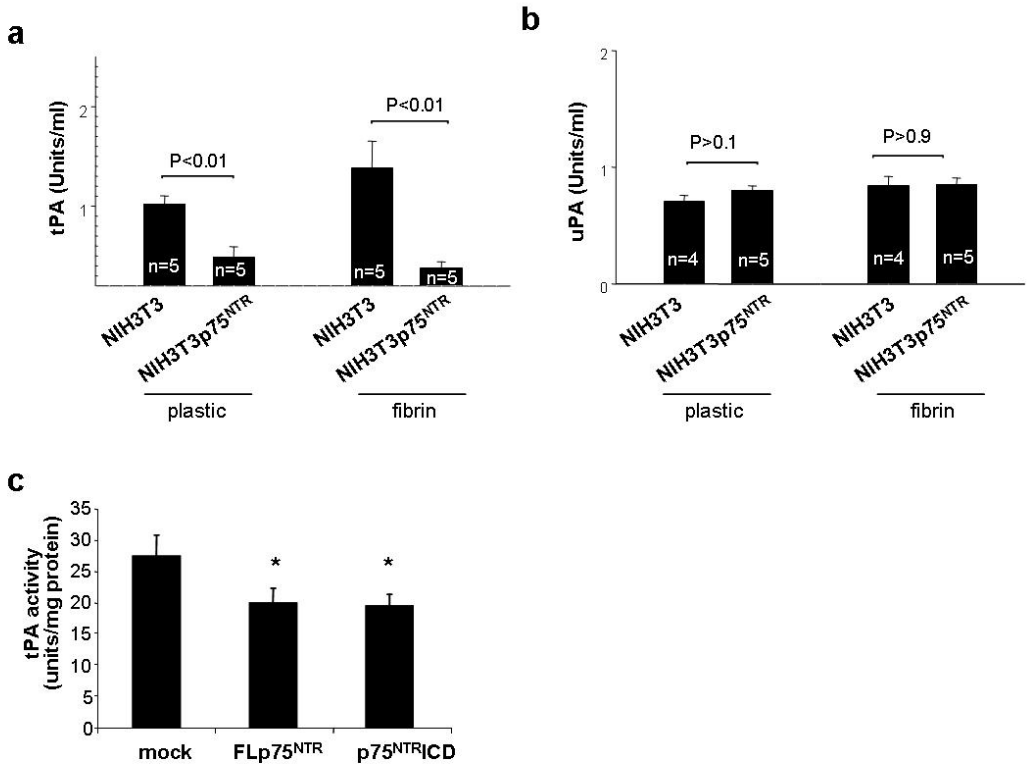

Figure 16. Expression of p75^{NTR} leads to inhibition of tPA activity, but has no effect on uPA activity.

Quantification of tPA (a) and uPA (b) activity in supernatants from NIH3T3 and NIH3T3p75^{NTR} cells cultured on either plastic or 3-dimensional fibrin gels. Experiments were performed 5 times in duplicates. Transient transfections of either FL-p75^{NTR} or p75^{NTR}-ICD into NIH3T3 cells also significantly decreases tPA activity (c).

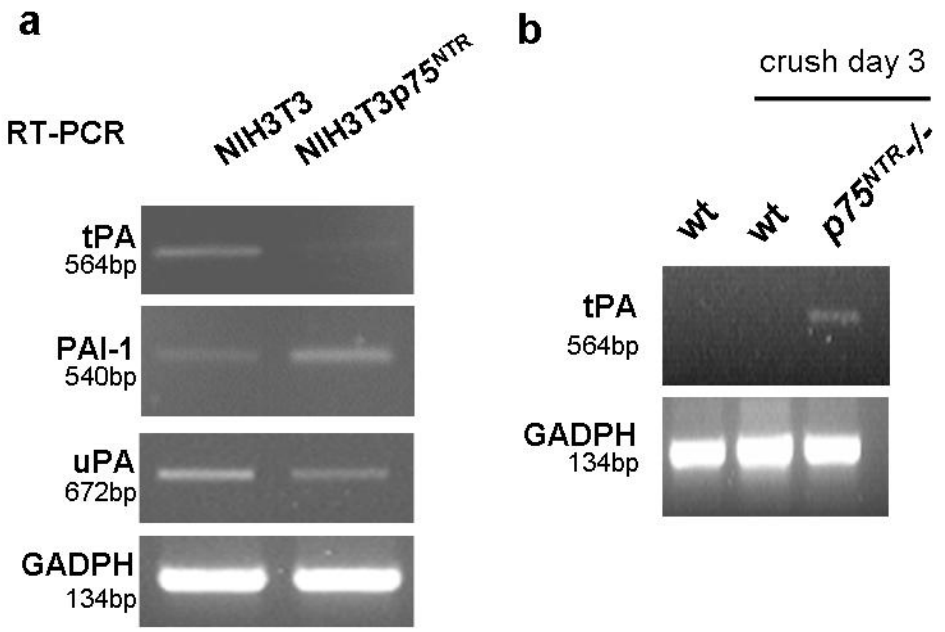

Figure 17. p75^{NTR} inhibits tPA expression and increases PAI-1 expression.
(a) RT-PCR analysis for *tPA*, *PAI-1*, *uPA* and *GAPDH* on cDNA derived from NIH3T3 and NIH3T3p75^{NTR} cells. (b) RT-PCR analysis for *tPA* and *GAPDH* on cDNA derived from uninjured wt, and wt or *p75^{NTR}*-/- mice three days after nerve injury.

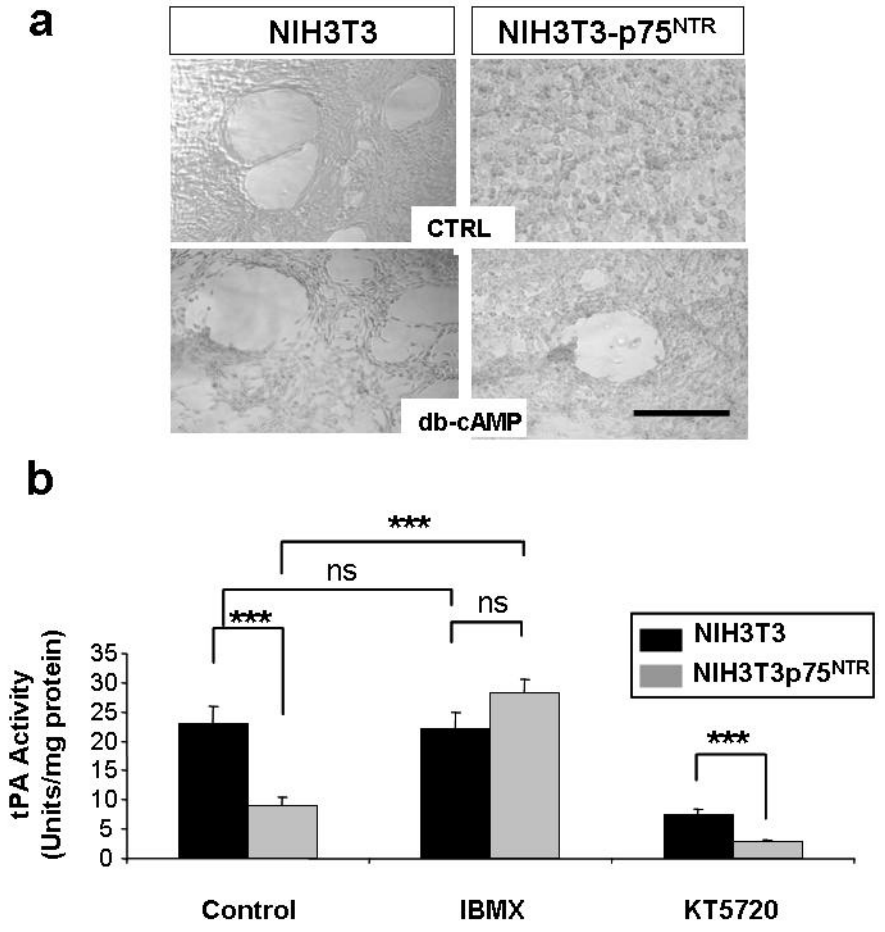

Figure 18. Elevation of cAMP in cells overexpressing $p75^{NTR}$ overcomes the $p75^{NTR}$ induced inhibition of fibrin degradation and tPA activity.
(a) db-cAMP induces fibrinolysis in NIH3T3$p75^{NTR}$ cells. (b) IBMX increases tPA activity of NIH3T3$p75^{NTR}$ cells to the levels of NIH3T3 cells. Inhibition of PKA by KT5720 shows decrease of tPA activity in both NIH3T3 and NIH3T3$p75^{NTR}$ cells ($P<0.0001$).

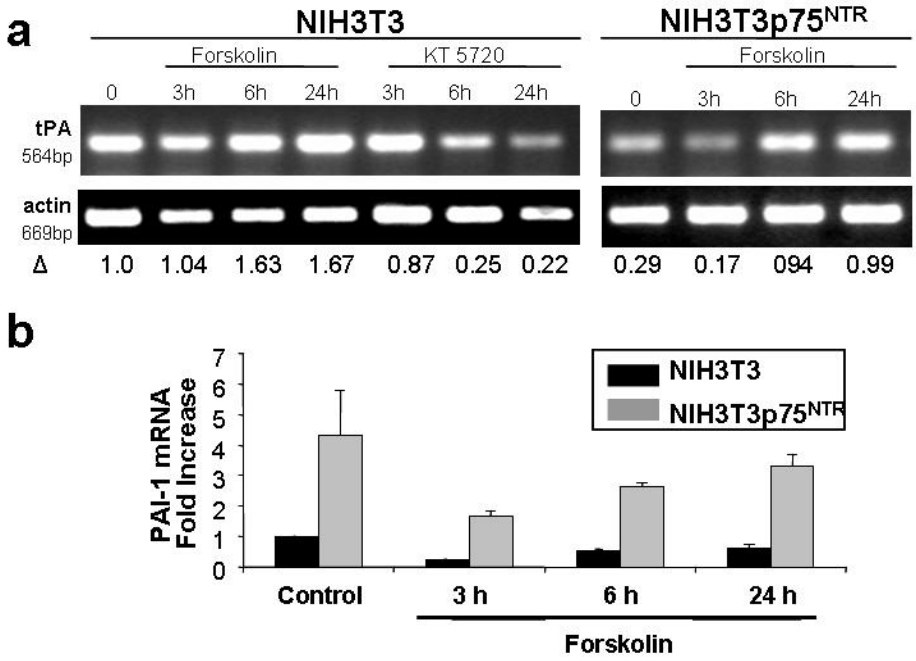

Figure 19. Elevation of cAMP increases tPA and decreases PAI-1 in fibroblasts overexpressing p75^{NTR}

(a) Forskolin increases *tPA* mRNA in NIH3T3 and NIH3T3p75^{NTR} cells. Inhibition of PKA by KT5720 decreases tPA transcript. (b) Quantification of PAI-1 mRNA changes by Real Time PCR shows a 4-fold increase of PAI-1 mRNA in NIH3T3p75^{NTR} cells compared to NIH3T3 cells.

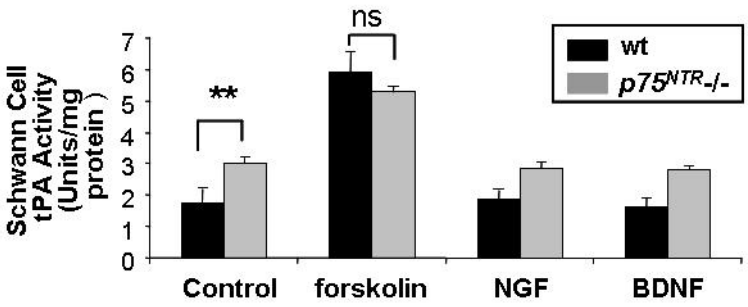

Figure 20. Elevation of cAMP in SCs increases tPA activity, but neurotrophins have no effect.
Forskolin increases tPA activity in both wt ($P<0.001$) and $p75^{NTR}$-/- ($P<0.00001$) SCs. NGF and BDNF do not affect activity of tPA ($P>0.8$ and $P>0.3$, respectively). Experiments were performed three times in duplicate. Bar graphs represent means ± SEM (statistics by ANOVA or *t* test).

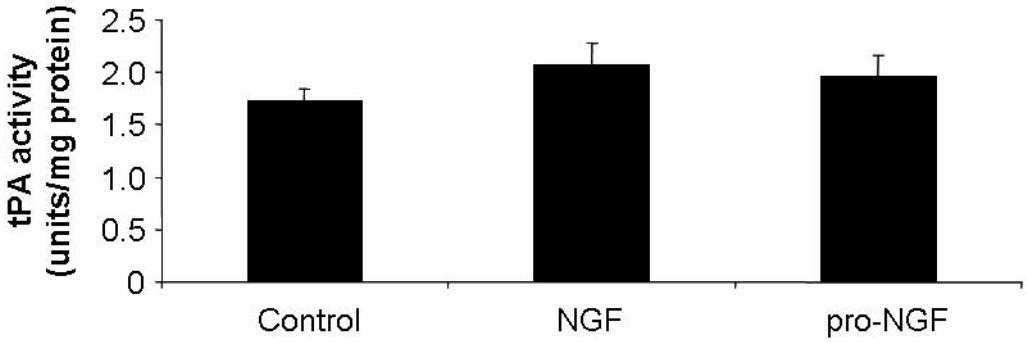

Figure 21. The high affinity ligand for p75^{NTR}, pro-NGF, has no effect on SC tPA activity.

tPA activity assay on wt SCs treated with either NGF or pro-NGF leads to no observed difference in tPA activity. Experiment was performed two times in duplicate. Bar graphs represent means ± SEM

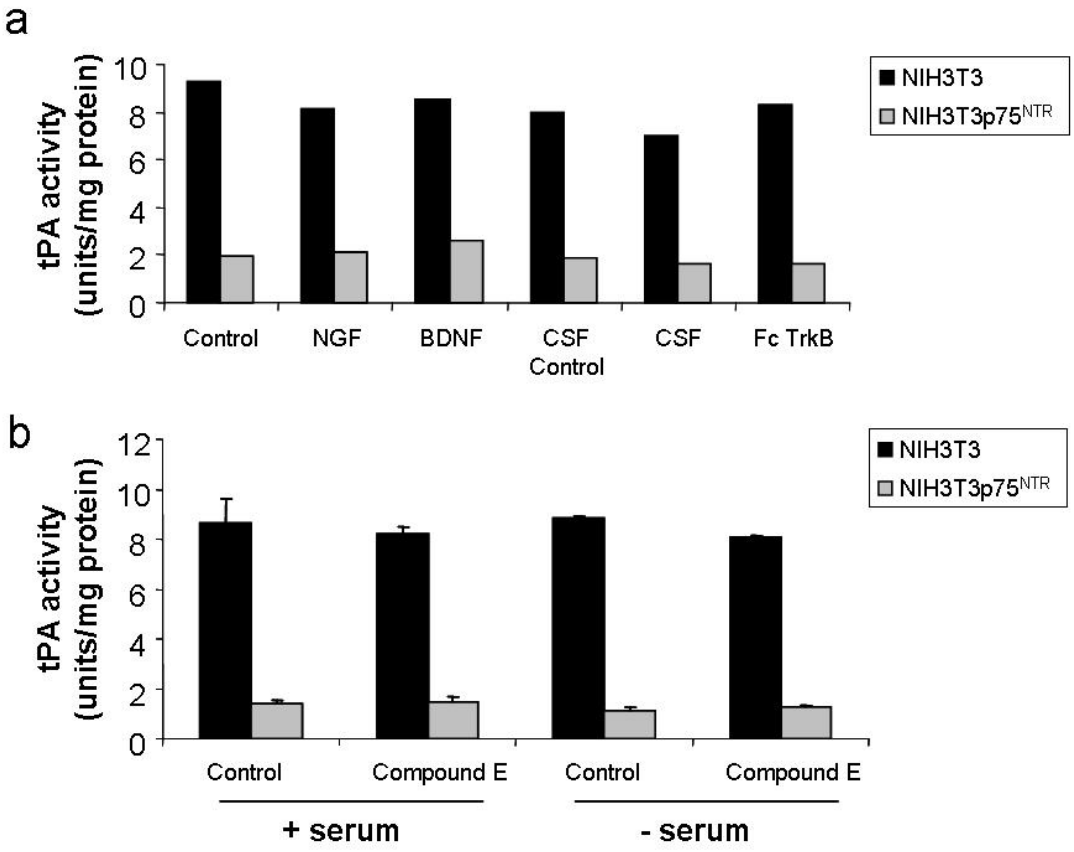

Figure 22. The inhibition of tPA by $p75^{NTR}$ is independent of neurotrophins, serum, and $p75^{NTR}$ RIP.
tPA activity assay on NIH3T3 and NIH3T3$p75^{NTR}$ cells with NGF (50 ng/ml), BDNF (100 ng/ml), cerebrospinal fluid (CSF), CSF control, or Fc-TrkB reveals no differences in tPA activity with any of the treatments (a). Removal of serum or treatment with the gamma secretase inhibitor, Compound E, also does not alter tPA activity in either NIH3T3 or NIH3T3$p75^{NTR}$ cells (b). Experiments were performed three times in duplicate. Bar graphs represent means ± SEM.

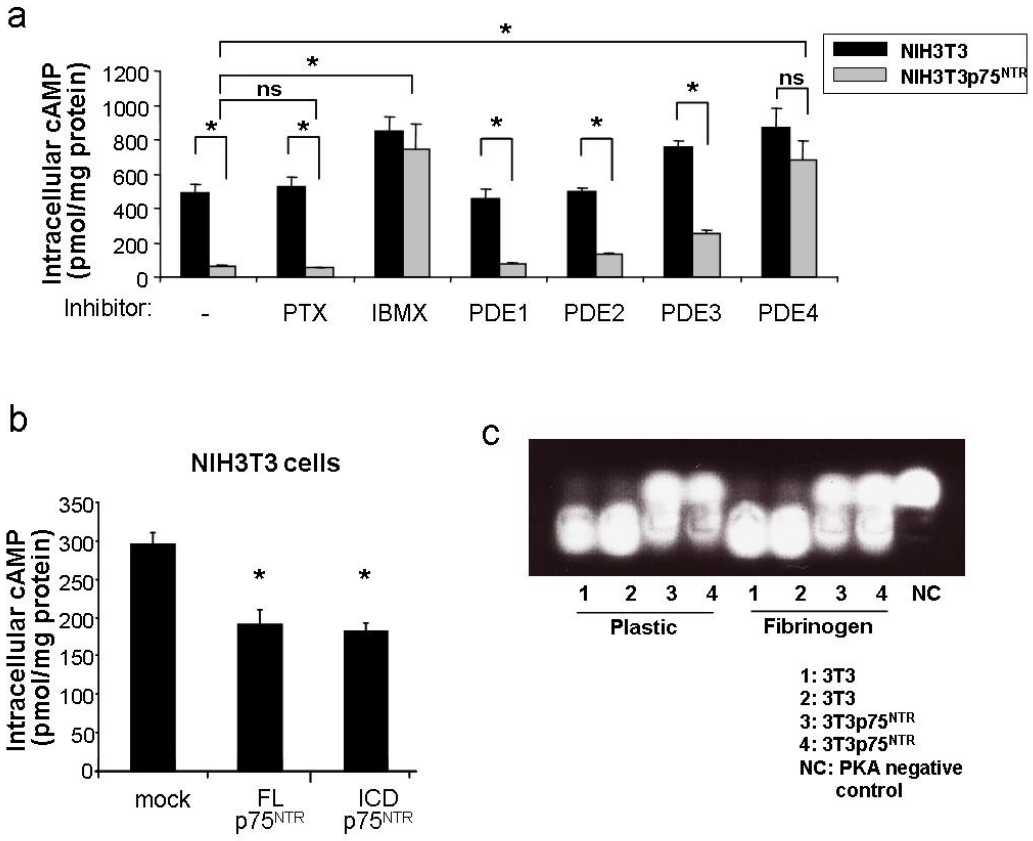

Figure 23. Expression of p75^{NTR} inhibits cAMP/PKA activity via a PDE4 dependent mechanism.

(a) Treatment with PTX, IBMX, specific inhibitors for PDE1, PDE2, PDE3, and PDE4 (rolipram) shows that only IBMX (IC50 for PDE4 2-50 μM) and rolipram (IC50 for PDE4 0.8 μM) ($P<0.0001$) increase levels of cAMP in NIH3T3p75^{NTR} cells. Experiments were performed at least 5 times in duplicates. (b) Transient transfection of FL-p75^{NTR} or p75^{NTR}-ICD decreases intracellular levels of cAMP in NIH3T3 cells. (c) Overexpression of p75^{NTR} leads to decreased PKA activity in addition to decreased cAMP levels. *$P<0.0001$, **$P<0.05$, ***$P<0.01$, NS: not significant.

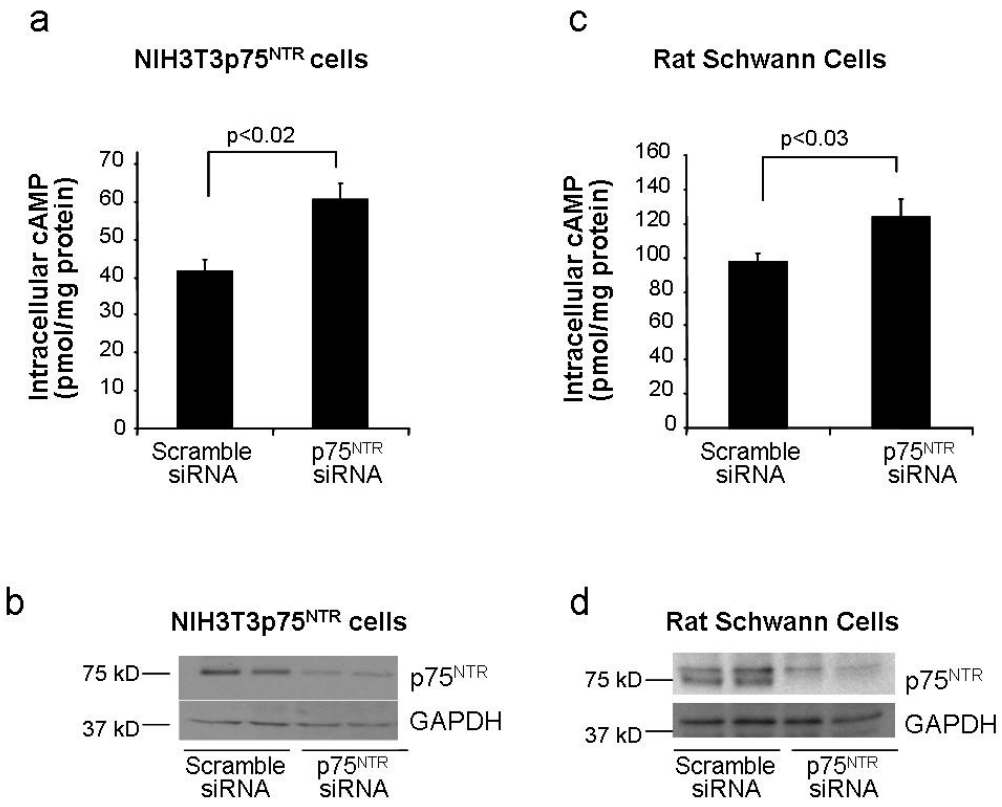

Figure 24. Knockdown of p75^{NTR} in SCs and in NIH3T3p75^{NTR} cells increases intracellular cAMP.

siRNA mediated knockdown of p75^{NTR} levels in NIH3T3p75^{NTR} cells leads to increased levels of cAMP (a). siRNA mediated knockdown of p75^{NTR} in primary rat Schwann cells leads to increased levels of cAMP (b). Western blots depicting p75^{NTR} levels after siRNA knock down in duplicate samples of NIH3T3p75^{NTR} cells (b) and SCs (d). Experiments were performed four times in duplicate. Bar graphs represent means ± SEM (statistics by ANOVA or *t* test).

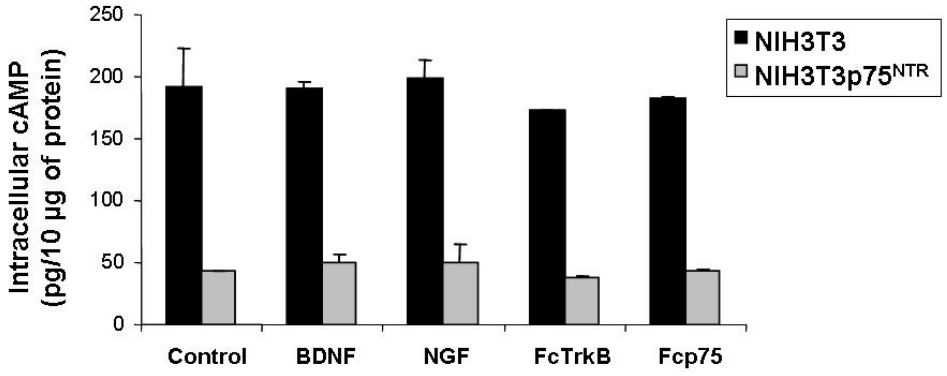

Figure 25. p75^{NTR} decreases intracellular cAMP in a neurotrophin-independent manner.
Expression of p75^{NTR} is sufficient for the reduction of intracellular cAMP (control). Addition of neurotrophins, such BDNF or NGF or inhibition of neurotrophins in the cell culture medium either by Fc-TrkB or Fc-p75^{NTR} does not affect the levels of intracellular cAMP in either NIH3T3 or NIH3T3p75^{NTR} cells. Experiments were performed five times in duplicate.

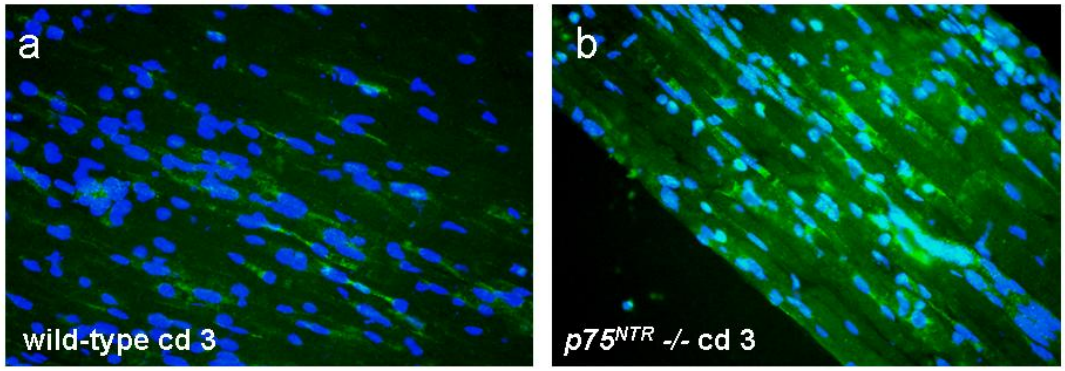

Figure 26. Genetic deletion of p75^{NTR} leads to increased cAMP levels in crushed sciatic nerve.
Immunostaining crushed sciatic nerve sections three days after injury with a cAMP specific antibody reveals increased cAMP immunoreactivity in the absence of p75^{NTR} (b) when compared to cAMP levels in wt nerves (a).
.

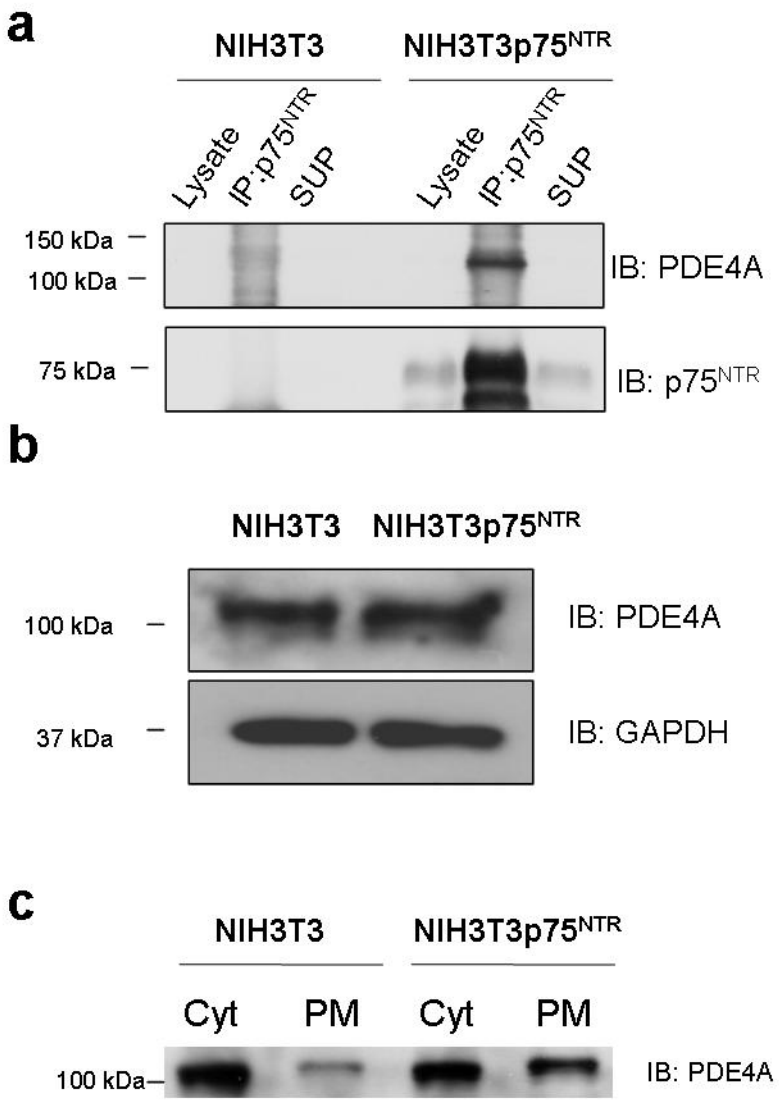

Figure 27. p75^{NTR} interacts with PDE4A5 and recruits it to the membrane. Co-immunoprecipitation experiments reveal an interaction between p75^{NTR} and PDE4A5 (a). Western blotting reveals equal levels of PDE4A5 expression in lysates of NIH3T3 cells and NIH3T3p75^{NTR} cells (b). Cell fractionation western blot analysis reveals increased recruitment of PDE4A5 to the plasma membrane in cells overexpressing p75^{NTR} (c).

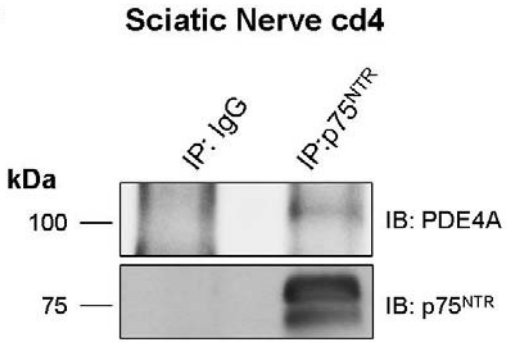

Figure 28. $p75^{NTR}$ interacts with PDE4A5 in injured sciatic nerve.
Co-immunoprecipitation of $p75^{NTR}$ and PDE4A5 in crushed wt sciatic nerve. Sciatic nerve lysates were immunoprecipitated with rabbit and $p75^{NTR}$ antibody (9992), and probed with an antibody specific for PDE4A. The band observed at ~109 kDa corresponds to PDE4A5. The rabbit $p75^{NTR}$ antibody was a kind gift of Moses Chao.

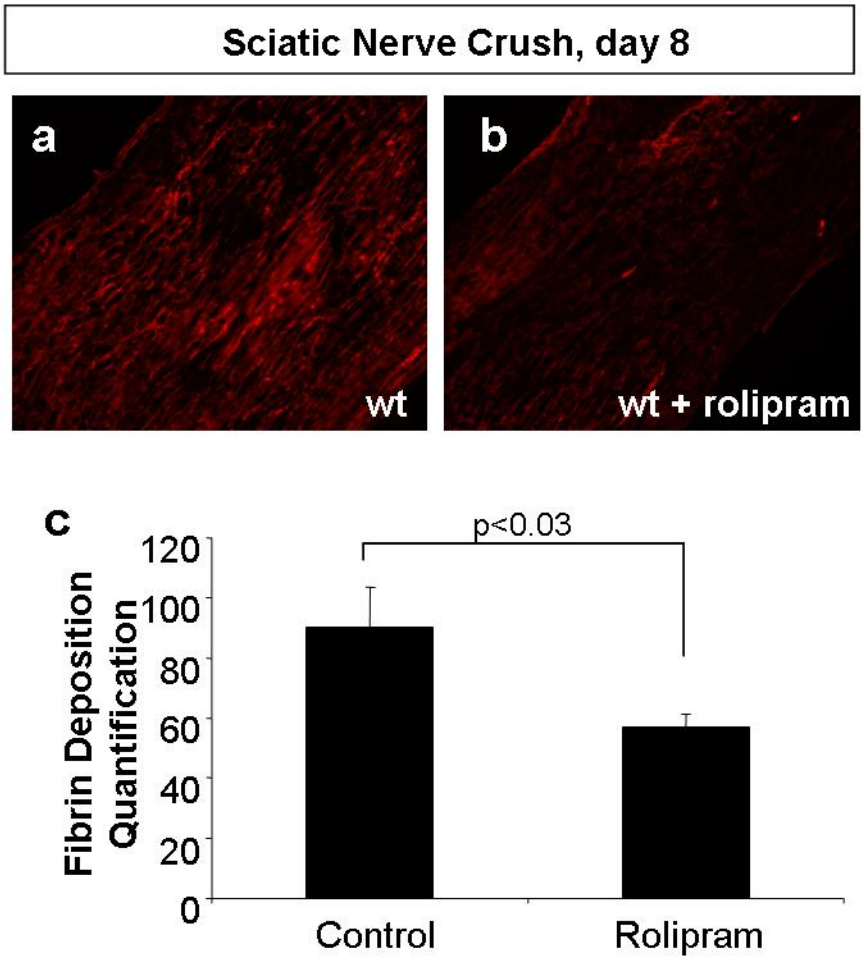

Figure 29. Inhibition of PDE4 with rolipram reduces fibrin deposition after sciatic nerve injury *in vivo*.
Rolipram treatment leads to decreased levels of fibrin deposition in wt nerves 8 d after sciatic nerve crush injury (red staining, a), when compared with untreated wt nerves (b). Quantification reveals a statistically significant reduction of fibrin deposition in response to rolipram treatment (c). Quantification of the sciatic nerve samples is based on $n = 9$ wt and $n = 9$ wt+rolipram-treated mice.

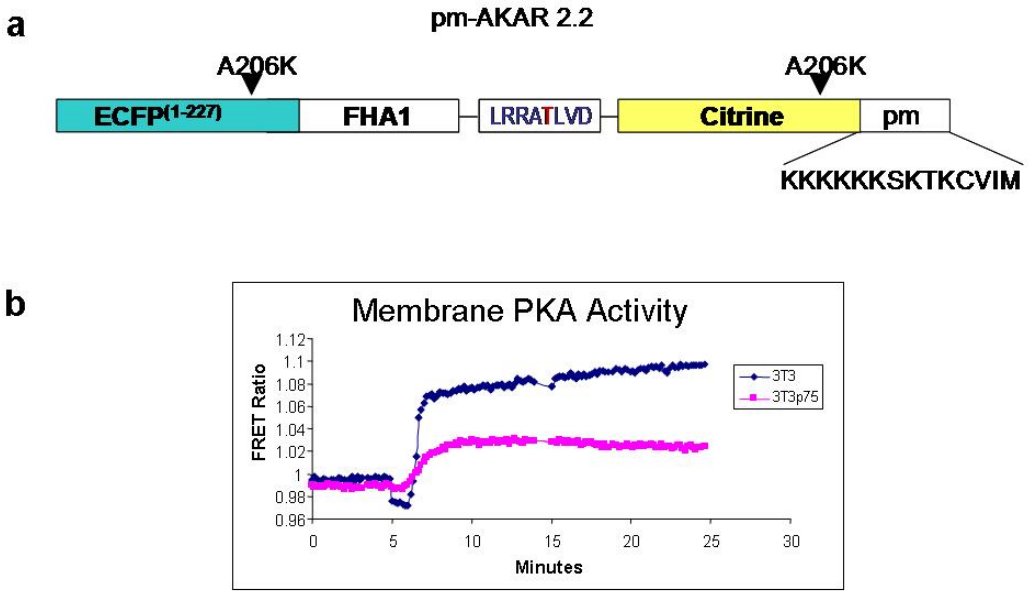

Figure 30. FRET analysis of membrane PKA activity in NIH3T3 cells
Schematic diagram of pm-AKAR2.2 (a). Overexpression of $p75^{NTR}$ in NIH3T3 cells leads to reduced PKA activity at the membrane in response to forskolin (b). Experiments were performed 3 times in triplicate. The pm-AKAR2.2 construct was a kind gift of Jin Zhang.

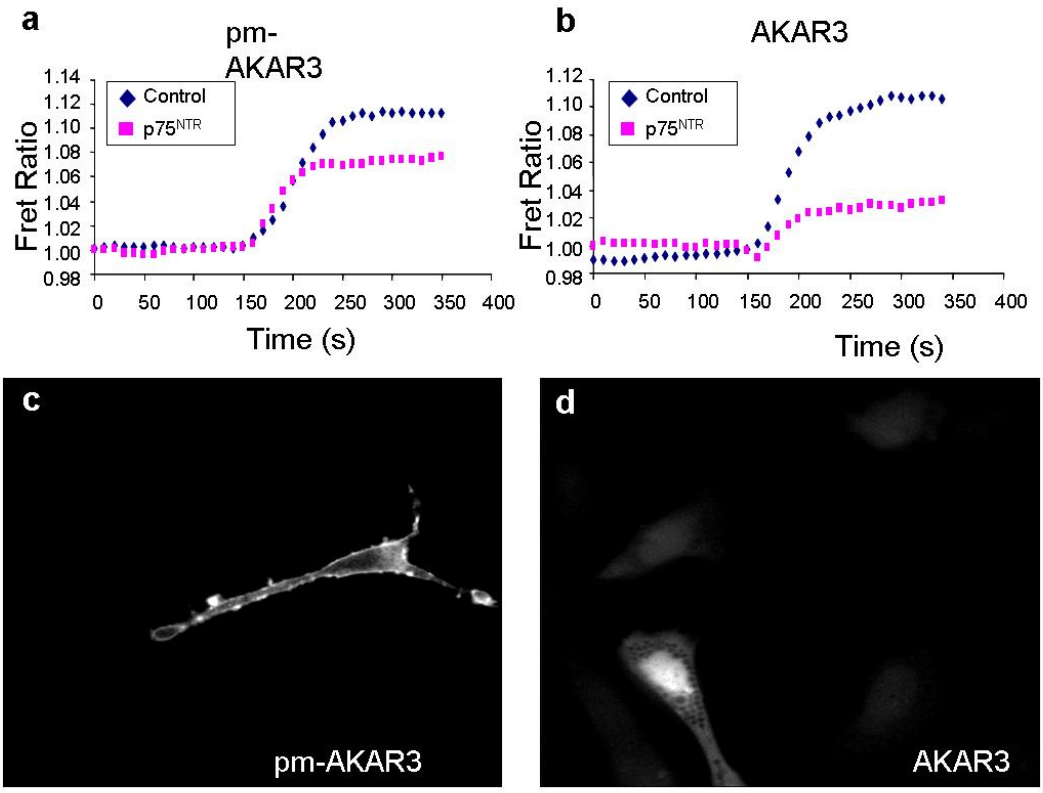

Figure 31. FRET analysis of p75^{NTR} inhibition of PKA activity in live cells.
FRET emission ratio change of control NIH3T3 cells and NIH3T3cells transiently transfected with p75^{NTR} and co-transfected with the pm-AKAR3 (a) or AKAR3 (b) in response to forskolin, a potent activator of adenylyl cyclase. Images show the localization for pm-AKAR3 (c) and AKAR3 (d). pm-AKAR3 localizes at the membrane (d). Experiments were performed three times in triplicate. The pm-AKAR3 and AKAR3 constructs were kind gifts of Jing Zhang.

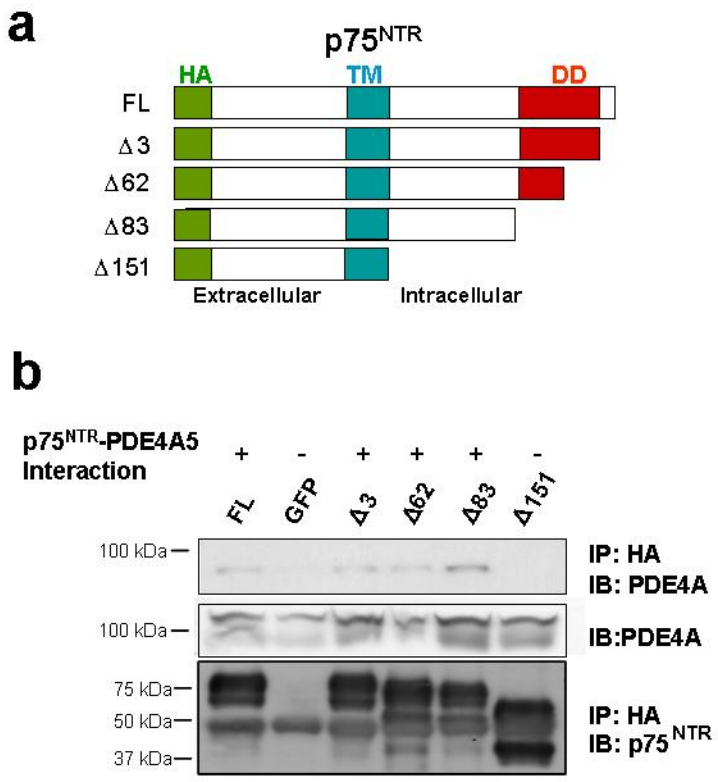

Figure 32. The juxtamembrane domain of p75^{NTR} is required for interaction with PDE4A5.

Schematic representing p75NTR deletion mutant constructs used (a). Co-immunoprecipitation experiments using transiently transfected PDE4A5 and HA-tagged p75^{NTR} deletion mutants reveal that while p75^{NTR}- FL, Δ3, Δ62, and Δ83 interact with PDE4A5, Δ151 does not (b). p75^{NTR} deletion constructs were a kind gift of Moses Chao. PDE4A5 construct was a kind gift of Miles Houslay.

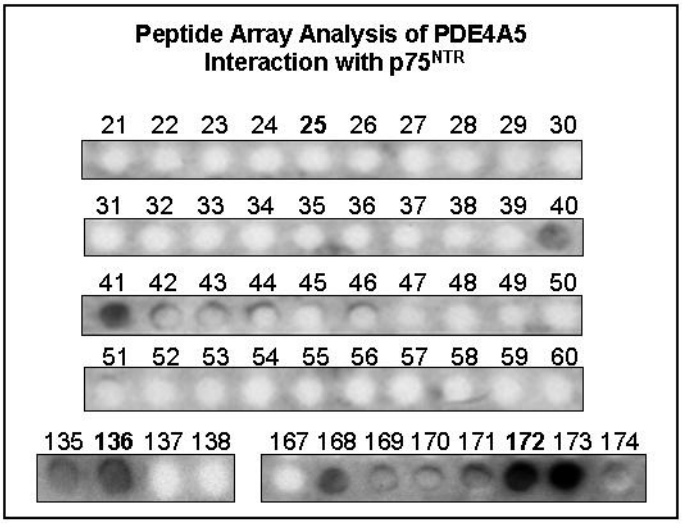

Figure 33. $p75^{NTR}$ can interact with three distinct domains of PDE4A5.
Peptide array mapping analysis of the interaction between $p75^{NTR}$-ICD and PDE4A5 reveals that sequences within the LR1 (peptides 40 and 41), the catalytic (peptides 135 and 136), and the extreme C-terminal (peptides 172 and 173) domains of PDE4A5 can interact with $p75^{NTR}$-ICD. Experiment was performed in the laboratory of Miles Houslay.

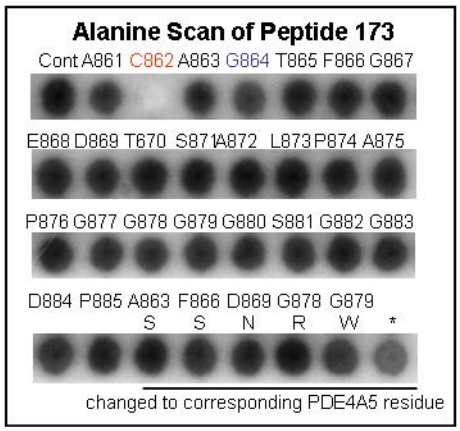

Figure 34. Alanine scanning mutagenesis of the C-terminal domain of PDE4A4. Alanine mutagenesis experiments reveal that C862 or PDE4A4 is required for the interaction between $p75^{NTR}$-ICD and PDE4A4. Mutating specific residues of PDE4A4 to the corresponding residue in PDE4A5 leads to no differences in the interaction between $p75^{NTR}$-ICD and PDE4A4/5. Experiment was performed in the laboratory of Miles Houslay.

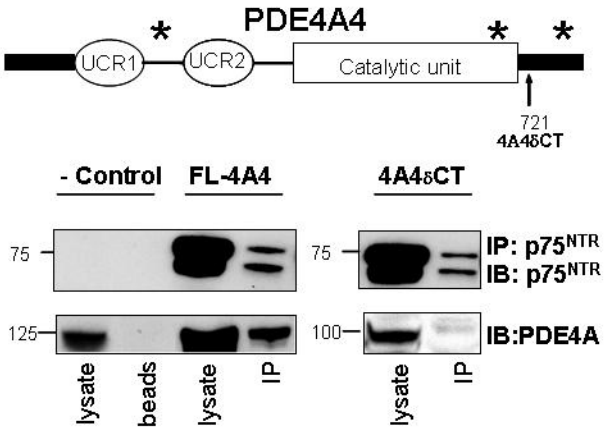

Figure 35. The C-terminus of PDE4A4 is required for its interaction with $p75^{NTR}$.

Co-immunoprecipitation analysis of FL-PDE4A4 or PDE4A4δCT with $p75^{NTR}$ reveals that only FL-PDE4A4, and not PDE4A4δCT interacts with $p75^{NTR}$, demonstrating the importance of the C-terminus of PDE4A4 in mediating this interaction. Experiment was performed in the laboratory of Miles Houslay.

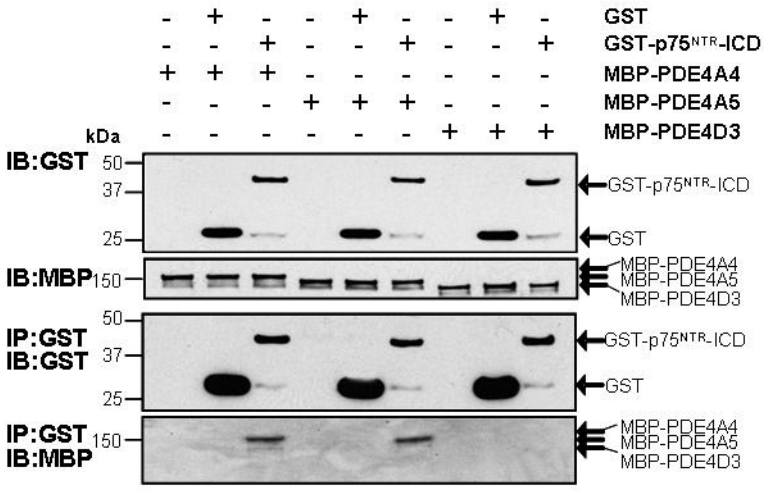

Figure 36. p75^{NTR} interacts directly with PDE4A4 and PDE4A5
In vitro co-immunoprecipitation analysis reveals that PDE4A4 and PDE4A5 both interact directly with p75^{NTR} in a cell free system. In contrast, PDE4D3 does not interact with p75^{NTR} directly. Experiment was performed in the laboratory of Miles Houslay.

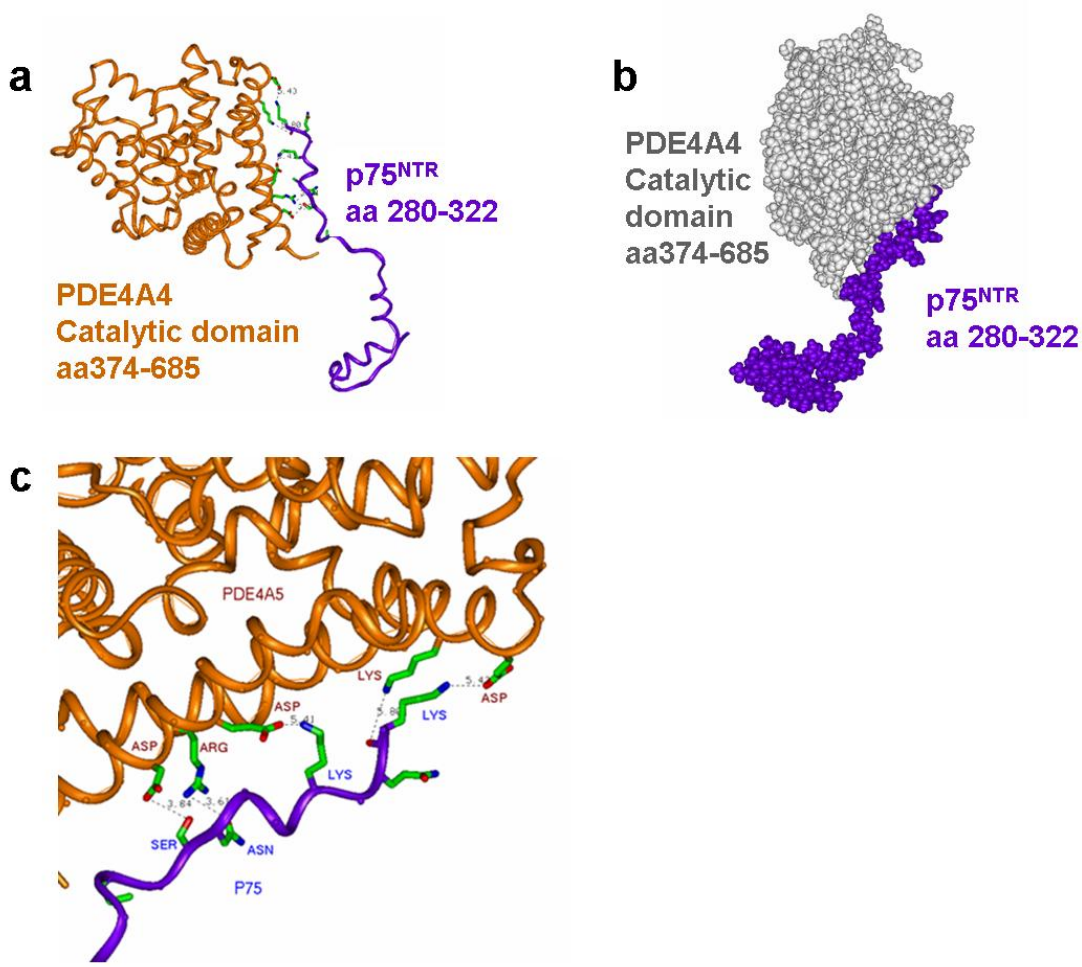

Figure 37. Computational model of the potential interaction between p75^{NTR} and the catalytic domain of PDE4A4.
Computer simulated docking ribbon (a) and CPK (b) models of the catalytic domain of PDE4A with the p75^{NTR} ICD. Higher magnification ribbon model of the interaction reveals potential sites of interaction (c). The residues of PDE4A shown to interact with p75^{NTR} ICD *in silico* are found to be within the same interacting sequences identified *in vitro* using peptide arrays and deletion mutagenesis co-IPs. Computer modeling analysis was performed by Igor Tsigelny.

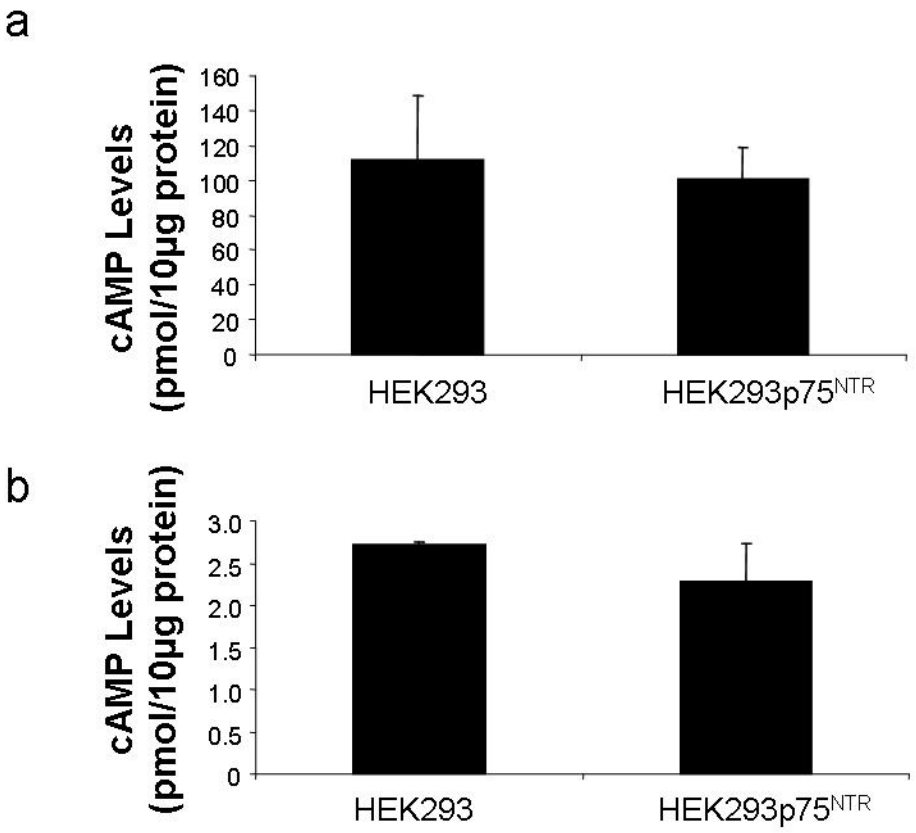

Figure 38. Stable overexpression of $p75^{NTR}$ into cell lines that do not express PDE4A5 does not lead to decreased cAMP levels.
To determine if expression of $p75^{NTR}$ leads to significant decreases in cAMP in other cell types, we measured cAMP levels in HEK293 cells that had been stably transfected with $p75^{NTR}$. Overexpression of $p75^{NTR}$ in these cells, which do not express endogenous PDE4A5, does not lead to decreases in either basal (b) or forskolin stimulated (a) cAMP levels. HEK293$p75^{NTR}$ cells were a kind gift of Davide Comoletti.

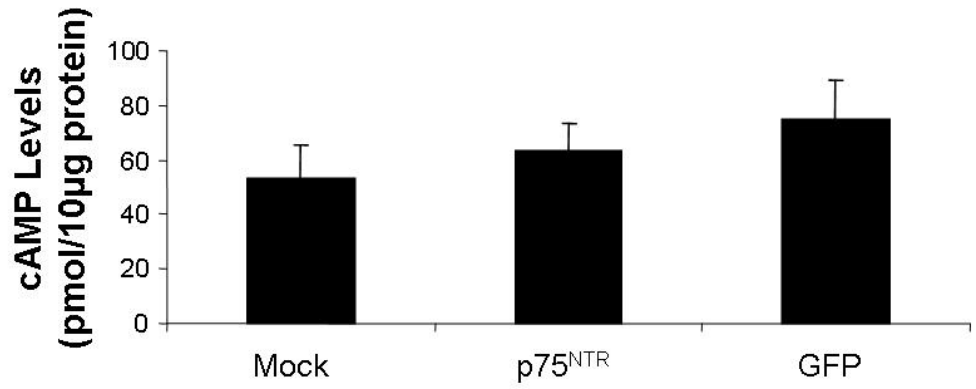

Figure 39. Transient overexpression of $p75^{NTR}$ in HEK293 cells does not lead to decreased cAMP.
cAMP ELISA on lysates from HEK293 cells transiently transfected with $p75^{NTR}$, GFP, or mock transfected. Results reveal no significant changes in cAMP levels in response to $p75^{NTR}$ expression.

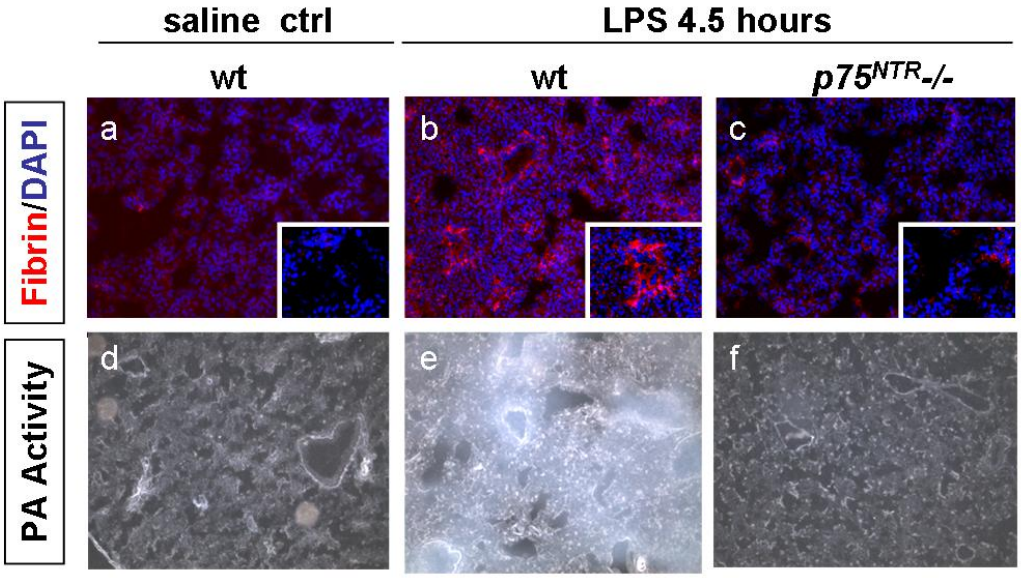

Figure 40. p75^{NTR} regulates fibrin clearance in the lung.
LPS induces fibrin deposition (red) in the wt lung (b), when compared with the saline-injected lung (a). Lungs derived from *p75$^{NTR–/–}$* mice show less fibrin deposition (c). In situ zymography after 3 h of incubation shows clearance of casein in the lung of saline-injected wt (d), when compared with LPS injected wt lung (e). Lung from LPS-treated *p75$^{NTR–/–}$* mouse shows enhanced proteolytic activity (f), when compared with the wt mouse (e). Scale bar: 150 µm (a–c), 75 µm (a–c, inset), 200 µm (d–f).

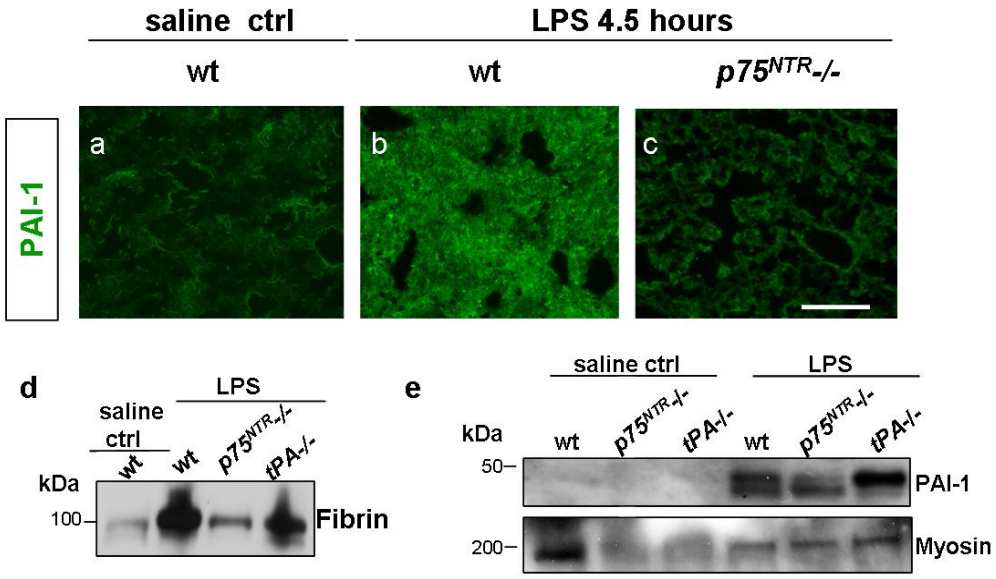

Figure 41. p75^{NTR} regulates PAI-1 expression in the lung.
Immunoreactivity for PAI-1 is increased in wt lung derived from LPS-treated mouse (b), when compared with saline-treated control (a). Lung from LPS-treated *p75$^{NTR-/-}$* mouse shows decreased PAI-1 (c), when compared with the wt LPS-treated mouse (b). (d) Western blot of fibrin precipitation from the lung shows an up-regulation of fibrin in the LPS-treated wt lung, when compared with the *p75$^{NTR-/-}$* lung. (e) Western blot for PAI-1 in the lung shows a decrease of PAI-1 in the *p75$^{NTR-/-}$* lung, when compared with the wt lung. Images are representative of n = 10 wt and n = 9 *p75$^{NTR-/-}$* mice. Western blots have been performed for n = 4 wt and n = 4 *p75$^{NTR-/-}$* mice. Scale bar: 150 µm.

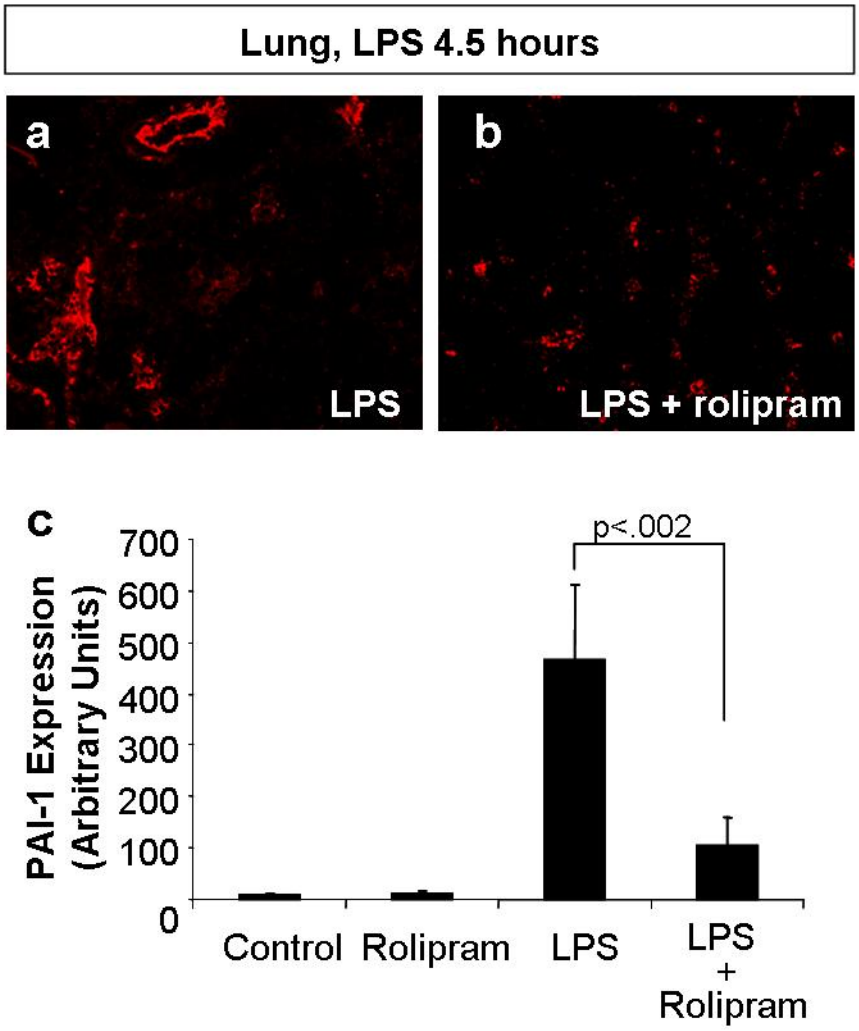

Figure 42. Inhibition of PDE4 with rolipram decreases fibrin deposition and PAI-1 induction after LPS induced lung injury *in vivo*.
Fibrin deposition (red) is decreased in the lung in rolipram treated mice after induction of LPS-induced acute lung injury (b), when compared with mice treated with LPS alone (a). Quantification shows a 34% decrease in fibrin rolipram vs. control treated wt lungs after LPS-induced lung fibrosis (not shown). Quantitative real time PCR of PAI-1 transcripts show that rolipram treatment attenuates LPS-induced PAI-1 upregulation, but does not affect basal levels of PAI-1 in the lung, in the absence of LPS (c). Quantification of the lung samples is based on $n = 7$ LPS treated mice, $n = 4$ LPS+rolipram treated mice, $n = 5$ rolipram treated mice, and $n = 7$ control untreated mice.

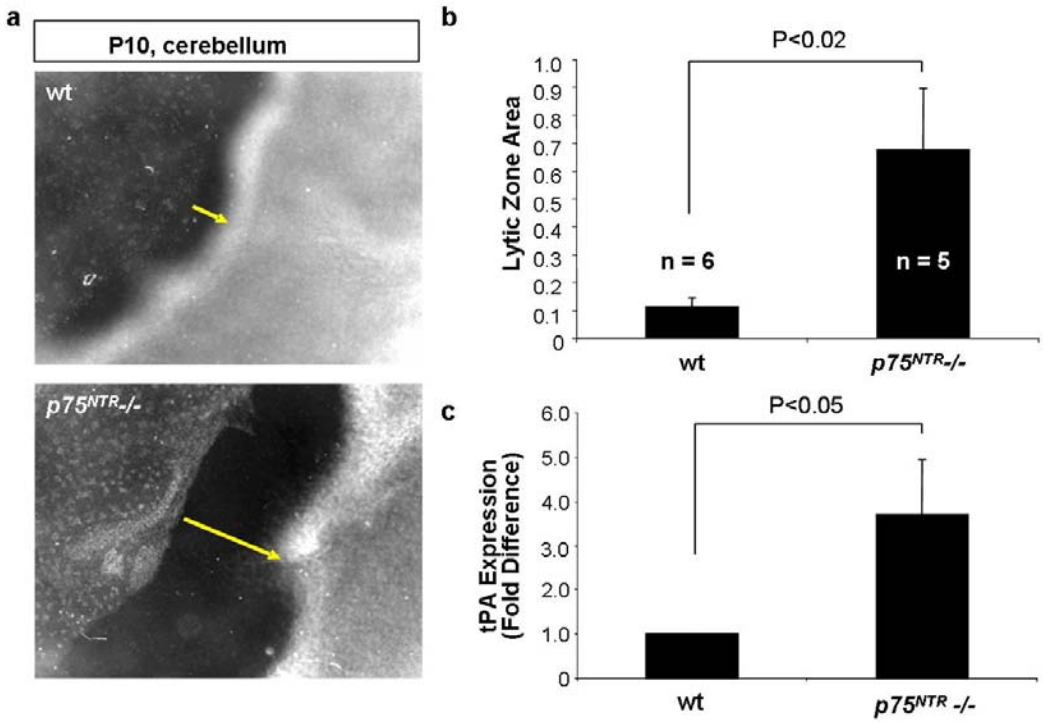

Figure 43. Loss of p75^{NTR} leads to an increase in tPA mRNA levels and proteolytic activity in the CNS.
In situ zymographies on cerebella isolated from ten day old wt (n = 6) and *p75*$^{NTR-/-}$ (n = 5) mice reveal enhanced proteolytic activity in *p75*$^{NTR-/-}$ cerebella compared to wt (a). Quantification reveals a statistically significant 7 fold increase in proteolysis in the absence of p75^{NTR} (b). Quantitative real-time PCR analysis of mRNA isolated from primary CGNs from wt and *p75*$^{NTR-/-}$ animals revealed a fourfold increase in tPA levels in *p75*$^{NTR-/-}$ neurons (c).

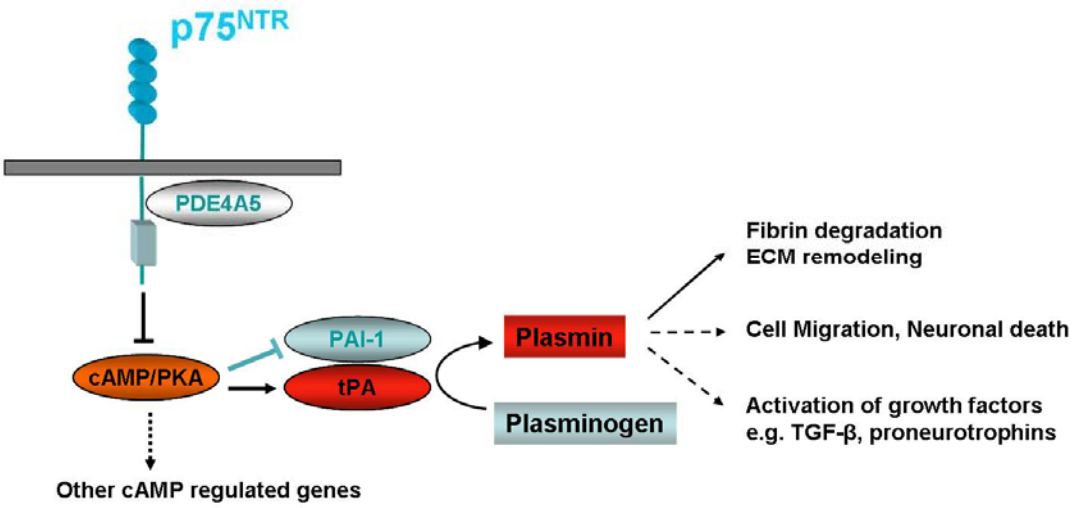

Figure 44. Proposed model for the role of p75^{NTR} in the cAMP-mediated plasminogen activation.
Upon re-expression after injury, p75^{NTR} interacts with PDE4A4/5 to induce localized degradation of cAMP and a corresponding reduction in PKA activity. The resulting decrease in cAMP/PKA activity reduces expression of tPA and increases PAI-1, leading to reduced plasmin generation and inhibition of plasmin-dependent extracellular proteolysis, fibrin degradation, and ECM remodeling. Because plasmin can proteolytically modify nonfibrin substrates, such as growth factors and cytokines, this mechanism may be upstream of various cellular functions. In addition, given the major role of cAMP in transcription, it is possible that p75^{NTR} may regulate expression of other cAMP-dependent genes as well.

Chapter 4: Regulation of cAMP by $p75^{NTR}$ in the CNS

1. Introduction

The identification of a direct interaction between $p75^{NTR}$ and PDE4A5 provides a mechanism whereby $p75^{NTR}$ decreases cAMP and inhibits fibrinolysis. However, this novel finding also gave rise to a new question – what other functions might $p75^{NTR}$ regulate via the cAMP pathway? Cyclic AMP is a ubiquitous second messenger within the central nervous system (CNS) with widespread functions in physiological processes such as chemosensation [191] and long term potentiation [192]. In addition, cAMP is one of the major second messengers utilized by a number of neurotransmitters, including the biogenic amines, serotonin and dopamine, which play important roles in mood regulation and the reward pathway {reviewed in [193]}. Tight regulation of cAMP signaling is vital to maintaining proper CNS function. Dysregulation of cAMP signaling underlies a variety of CNS disorders, including depression [194] and addiction [195], and plays a major role in the inhibition of axonal regeneration after injury {reviewed in [196]}. One of the primary means of regulation of cAMP levels within the central nervous system occurs at the level of cAMP degradation, a process catalyzed by PDEs {reviewed in [197]}. The various PDE isoforms differ in both their cellular distribution, means of regulation and subcellular compartmentalization. PDEs have long been recognized as an attractive therapeutic target due to their unique roles as regulators of cAMP levels in the CNS.

cAMP mediates its effects through a number of downstream effectors. Perhaps the best characterized downstream effector of cAMP is the cAMP dependent protein

kinase, PKA. In the absence of cAMP, PKA is held inactive by the binding of two regulatory subunits to the two catalytic subunits of the enzyme. Upon cAMP binding to the regulatory subunit, a conformational change results and allows the dissociation of the regulatory subunits from the catalytic subunits. Once this occurs, PKA is free to phosphorylate its many downstream targets. One such target is the cAMP responsive element binding protein (CREB), a 43 kDa phosphoprotein transcription factor that when phosphorylated at serine 119, promotes transcription of target genes by binding to cAMP responsive elements (CREs) in promoters. CREs were first identified in the somatostatin gene promoter by Marc Montminy in 1986 [198], who also purified CREB the following year. Since its discovery, CREB has been implicated in the transcriptional regulation of numerous genes, including arginase I [199], interleukin – 6 [200, 201], BDNF [202], and tPA [177]. Our results identify the regulation of cAMP by $p75^{NTR}$ as a novel mechanism for the regulation of tPA [203]; however, we were quite interested in finding additional functions for $p75^{NTR}$-PDE4A5-cAMP-PKA signaling.

One potential candidate for an additional function regulated by the $p75^{NTR}$-PDE4A5 interaction is the inhibition of neurite outgrowth. $p75^{NTR}$ has been previously implicated in the inhibition of neurite outgrowth via its role as the signal transducing component of the Nogo Receptor complex [20]. *In vitro* studies have demonstrated that elevation of cAMP in neurons, either via the addition of cAMP analogs or via priming with neurotrophins, results in the disinhibition of neurite outgrowth by myelin, a known neurite outgrowth inhibitor. In addition, rolipram, an inhibitor of type four phosphodiesterases (PDEs), the enzymes responsible for the

breakdown of cAMP, has also been shown to overcome inhibition of neurite outgrowth [166, 204]. These results have been verified *in vivo* by a series of experiments which have demonstrated that elevation of cAMP in the spinal cord after injury leads to improved functional recovery and enhanced axonal regeneration [205-207]. Unfortunately, rolipram has been shown to have deleterious side effects, including nausea, and thus has limited therapeutic value. To decrease unwanted side effects, novel inhibitors of PDE4s, with greater specificity for specific isoforms must be developed. In order to target the proper isoforms most effectively, a greater understanding of the functions and means of regulation of specific PDE4s in the CNS is required.

Although the elevation of cAMP via the inhibition of PDE4s has been shown to be effective in overcoming the inhibition of neurite outgrowth and axonal regeneration [205], the mechanisms by which cAMP exerts its effects are not completely understood. Several cAMP – dependent genes have been identified as being important for axonal regeneration after spinal cord injury and overcoming the inhibition of neurite outgrowth, including arginase I [199] and interleukin-6 [201]. It is possible that PDE4A, via its regulation of cAMP, plays a role in regulating transcription of key genes that function during axonal regeneration. However, the role of PDE4s and cAMP in axonal regeneration likely extends beyond cAMP-dependent gene regulation.

The fact that CNS axons fail to regenerate after injury has been attributed to the presence of a variety of inhibitors present in the gliotic scar. Many inhibitors of axonal regeneration have been identified, including the myelin inhibitors (such as

MAG, OMGP, and Nogo), semaphorins, ephrins, and chondroitin sulfate proteoglycans {reviewed in [208]}. Although, these inhibitors act through different receptor systems, their signaling pathways converge on the Rho pathway. Blocking Rho signaling has shown a great deal of promise in promoting regeneration both *in vitro* and *in vivo* {reviewed in [209]}, highlighting the importance of this pathway. It has also been shown in leukocytes that elevation of cAMP leads to Rho inactivation via phosphorylation by PKA [210]. Our finding that the interaction between $p75^{NTR}$ and PDE4A5 regulates tPA activity in the cerebellum strongly suggests that $p75^{NTR}$-PDE4A5 interactions regulate cAMP in the CNS as well. We wanted to verify this hypothesis experimentally and to determine the biological significance of $p75^{NTR}$-PDE4A5 signaling in the CNS.

2. Expression of PDE4A in the cerebellum

After finding that genetic depletion of $p75^{NTR}$ leads to increased tPA activity in the cerebellum, we hypothesized that this increased tPA resulted from loss of $p75^{NTR}$-PDE4A5-mediated inhibition of cAMP. However, despite our evidence that $p75^{NTR}$ interacts with PDE4A5 outside of the CNS to decrease cAMP, we had not yet established that this same mechanism occurs in the CNS. To verify that these $p75^{NTR}$-PDE4A5 interactions do occur within the CNS to regulate cAMP, we first examined whether $p75^{NTR}$ and PDE4 exhibit overlapping patterns of expression within the brain. Immunohistochemistry reveals robust expression of both $p75^{NTR}$ (Fig. 45 a) and PDE4A (Fig. 45 b) in Purkinje cells and in the granule cell layer of the cerebellum. In contrast, PDE4B and PDE4D are expressed at much lower levels within the

cerebellum (Fig. 45 c, d). PDE4C, which is reportedly not expressed within the CNS, was not examined. Real time PCR analysis of RNA isolated from primary cerebellar granule neurons (CGNs) reveals that PDE4A is the predominant PDE4 isoform expressed by CGNs (Fig. 46), indicating that PDE4A and $p75^{NTR}$ are co-expressed in these neurons.

3. $p75^{NTR}$ regulates cAMP in the CNS via an interaction with PDE4A5

Since both $p75^{NTR}$ and PDE4A are expressed in the granule layer, we hypothesized that $p75^{NTR}$-PDE4A interactions might regulate cAMP within granule neurons. However, our initial immunostainings in whole cerebellum only revealed expression of PDE4A, not specifically PDE4A5. In order to determine if CGNs express the PDE4A5 isoform, we performed immunocytochemistry with an antibody specific for this PDE4A5. Results indicate that PDE4A5 is expressed within CGNs (Fig. 47), and provide evidence that $p75^{NTR}$ and PDE4A5 may have the opportunity to interact in these cells to regulate cAMP. Indeed, primary isolated CGNs from *$p75^{NTR}$*-/- animals exhibit a twofold increase in the levels of cAMP in CGNs when compared to wt controls (Fig. 48). Inhibition of PDE4s using rolipram increased levels of cAMP specifically in cells expressing $p75^{NTR}$ (Fig. 48), indicating that the $p75^{NTR}$ induced decrease in cAMP observed in CGNs requires PDE4 activity. In contrast, stimulation of cAMP synthesis with forskolin elevated cAMP in wt and *$p75^{NTR}$*-/- CGNs but did not change the relative differences between the genotypes (Fig. 48). To determine whether the regulation of cAMP by $p75^{NTR}$-PDE4A5 also occurs *in vivo*, we performed immunohistochemistry with an antibody against cAMP. Cerebella isolated

from ten day old *p75^{NTR}-/-* mice exhibit elevated levels of cAMP (Fig. 49 b) when compared to control wt cerebella (Fig. 49 a). Finally, co-immunoprecipitation experiments reveal that p75^{NTR} and PDE4A5 do indeed form a complex within the cerebellum (Fig. 49 c). Taken together, these results demonstrate that interactions between p75^{NTR} and PDE4A5 occur in the CNS to regulate cAMP signaling.

4. PDE4A is required for Nogo – induced Rho activation in CGNs

Upon finding that p75^{NTR}-PDE4A5 interactions regulate cAMP in CGNs, we next sought to determine the functional consequences of cAMP regulation in these cells. Elevation of cAMP has been shown to overcome myelin induced inhibition of neurite outgrowth in CGNs, a process that is dependent upon RhoA activation. Inhibition of PDE4s with rolipram has been shown to overcome myelin induced inhibition of neurite outgrowth [205], but the specific isoform of PDE4 that plays a role in the inhibition of neurite outgrowth remains unknown. To determine whether PDE4A regulates myelin induced RhoA activation in neurons, we isolated CGNs from WT and *PDE4A-/-* mice and treated them with recombinant Nogo peptide (4μM). Rho-GTP pull down assays reveal that stimulation with Nogo induced Rho activation in wt CGNs as expected, but not in *PDE4A-/-* neurons (Fig. 50), indicating that PDE4A is required for RhoA activation in CGNs. Although the elevation of cAMP has been shown to inhibit RhoA in leukocytes [210], this effect had not been evaluated in neurons. Pretreatment with forskolin attenuated Nogo induced RhoA activation in wt CGNs (Fig. 50), revealing that in neurons, as in leukocytes, cAMP/PKA signaling can inhibit RhoA activation.

The results obtained using the *PDE4A-/-* mice implicate PDE4A in the activation of RhoA by ligands of the NogoR/p75^{NTR} receptor complex. However, whether RhoA activation requires PDE4A5 specifically, or whether other isoforms may play a role cannot be determined genetically with the *PDE4A-/-* mouse. In order to determine whether PDE4A5 is the specific isoform that regulates RhoA activation, we compared p75^{NTR} dependent RhoA activation in HEK293 cells transfected with p75^{NTR} along with a dominant negative PDE4A5 construct (DN-PDE4A5). As expected, treating HEK293 cells overexpressing p75^{NTR} with MAG results in an increase in RhoA activation. In contrast, HEK293 cells overexpressing both p75^{NTR} and DN-PDE4A5 do not increase RhoA activation in response to MAG, indicating that PDE4A5 activity is required for this effect (Fig. 51).

5. PDE4A is required for myelin inhibition of neurite outgrowth in CGNs

To determine whether PDE4A regulates myelin inhibition of neurite outgrowth, CGNs were isolated from WT and *PDE4A-/-* mice. CGNs were cultured either on poly-d-lysine or myelin coated dishes and allowed to extend processes for 24 hours. After 24 hours, cells were fixed and stained with β-tubulin. Fluorescence images were taken and the length of each neurite was measured. We observed no observed differences in neurite length between wt and *PDE4A-/-* CGNs cultured on poly-d-lysine. Myelin inhibited neurite outgrowth by approximately 30% in wt CGNs, compared to wt CGNs grown on poly-d-lysine. In contrast, outgrowth of *PDE4A-/-* CGNs was not inhibited by myelin, indicating that the inhibition of neurite outgrowth by myelin requires *PDE4A*.

6. Summary

In order to determine whether the $p75^{NTR}$-PDE4A5-cAMP signaling pathway might be active within the CNS in addition to the PNS and lung, we first performed a series of experiments demonstrating expression of PDE4A5 within the cerebellum. Immunostainings with specific antibodies revealed expression of PDE4A within the cerebellum (Fig. 45) and PDE4A5 in primary isolated cerebellar granule neurons (Fig. 47). Real time PCR analysis indicated that PDE4A is the predominant PDE4 family member present within CGNs (Fig 46). After co-localizing $p75^{NTR}$ and PDE4A5 to the cerebellum, we used co-immunoprecipitation studies to demonstrate that $p75^{NTR}$ and PDE4A5 form a complex in the CNS (Fig. 48). Subsequent analysis of $p75^{NTR}$ deficient CGNs reveals that expression of $p75^{NTR}$ decreases intracellular cAMP in the CNS, similar to its effects in peripheral tissues. As one of the major functions for cAMP within the CNS is in the regulation of neurite outgrowth, we next wanted to determine if $p75^{NTR}$-PDE4A interactions played a role in this process. Previous research has demonstrated roles for $p75^{NTR}$ and PDE4 in the inhibition of neurite outgrowth [61, 205], but no study has investigated the specific role of PDE4A. To this end, we isolated primary CGNs from wt and *PDE4A-/-* animals and compared their neurite outgrowth. In contrast to wt CGNs, *PDE4A-/-* CGNs neurites were not inhibited by the inhibitory substrate, myelin. Finally, we went on to establish that the lack of myelin inhibition of *PDE4A-/-* neurons likely depends upon a failure to activate RhoA in the absence of PDE4A. Overall, these studies identify a mechanism by which the interaction between $p75^{NTR}$ and PDE4A5 contribute to neurite outgrowth

inhibition via the regulation of RhoA and identify PDE4A as the specific phosphodiesterase important for neurite outgrowth inhibition.

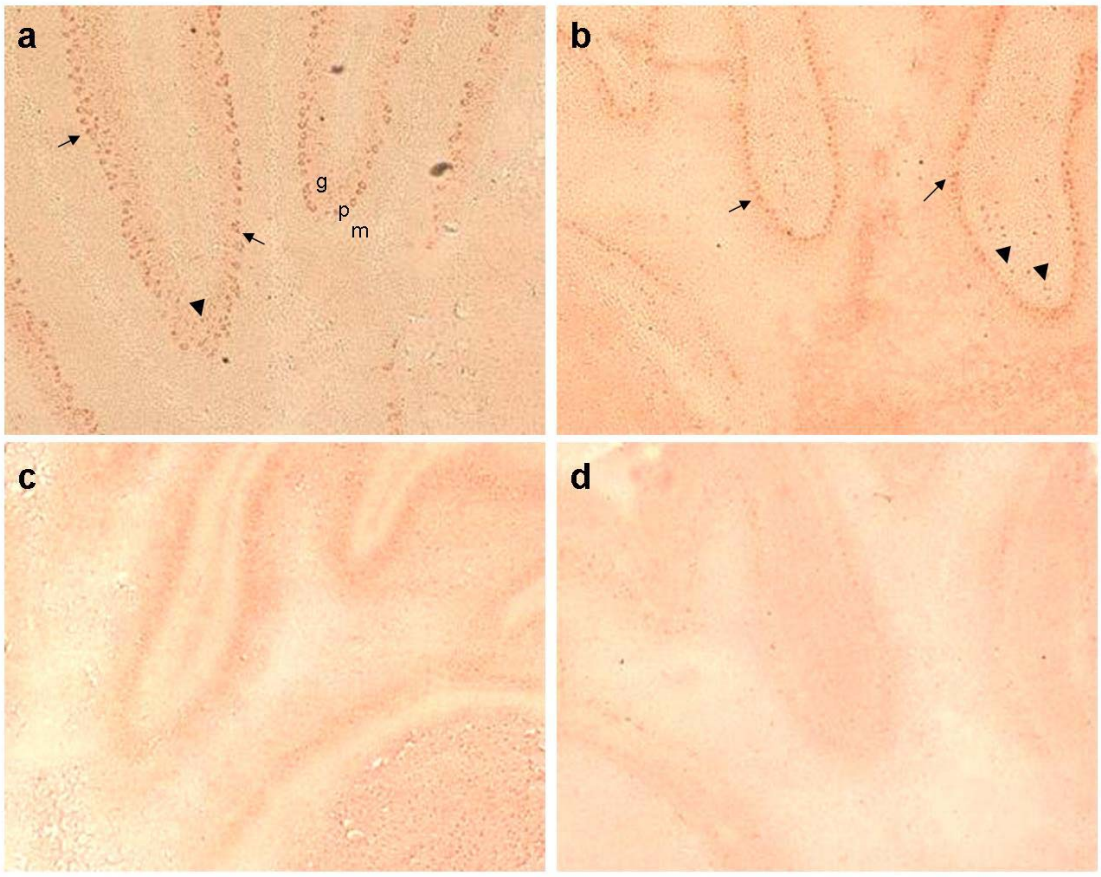

Figure 45. $p75^{NTR}$ and PDE4A are both expressed within the cerebellum. Immunohistochemistry for $p75^{NTR}$ (a) and PDE4A (b) reveals expression within Purkinje cells and the granule layer of the cerebellum. In comparison, levels of PDE4B (c) and PDE4D (d) are much lower than PDE4A. In (a), 'p' denotes the Purkinje layer, the middle layer of the cortex, characterized by large neuronal cell bodies. The 'g' denotes the granule cell layer, which is the deepest layer of the cortex, and consists of small neurons called granule cells. The 'm' refers to the molecular layer, which is the most superficial layer and primarily consists of axons and dendrites. Arrows indicate positive stained Purkinje cells, and arrowheads indicate positive stained granule cells.

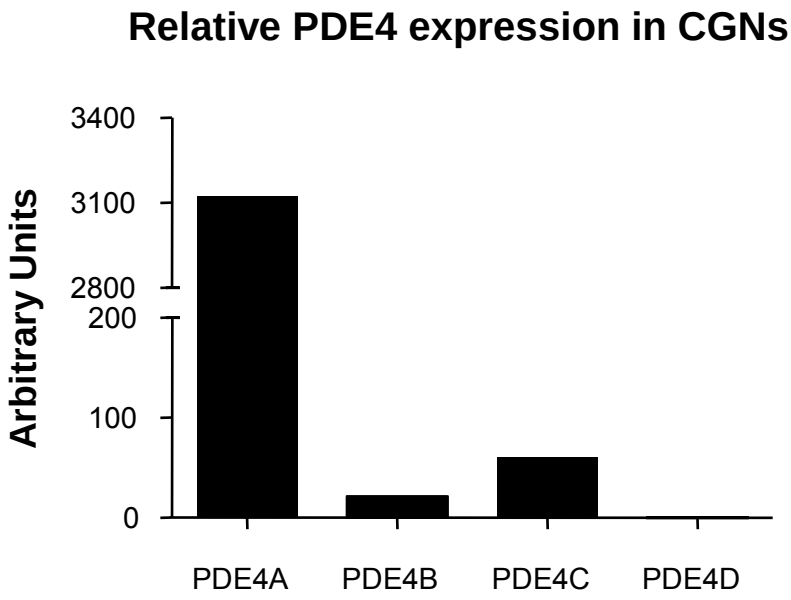

Figure 46. PDE4A is the predominant PDE4 isoform expressed in CGNs.
RNA was isolated from wildtype CGNs and reverse transcribed to cDNA. Real time PCR was performed using primers specific for the various PDE4 isoforms. Results indicate that PDE4A is the predominant PDE4 gene in CGNs.

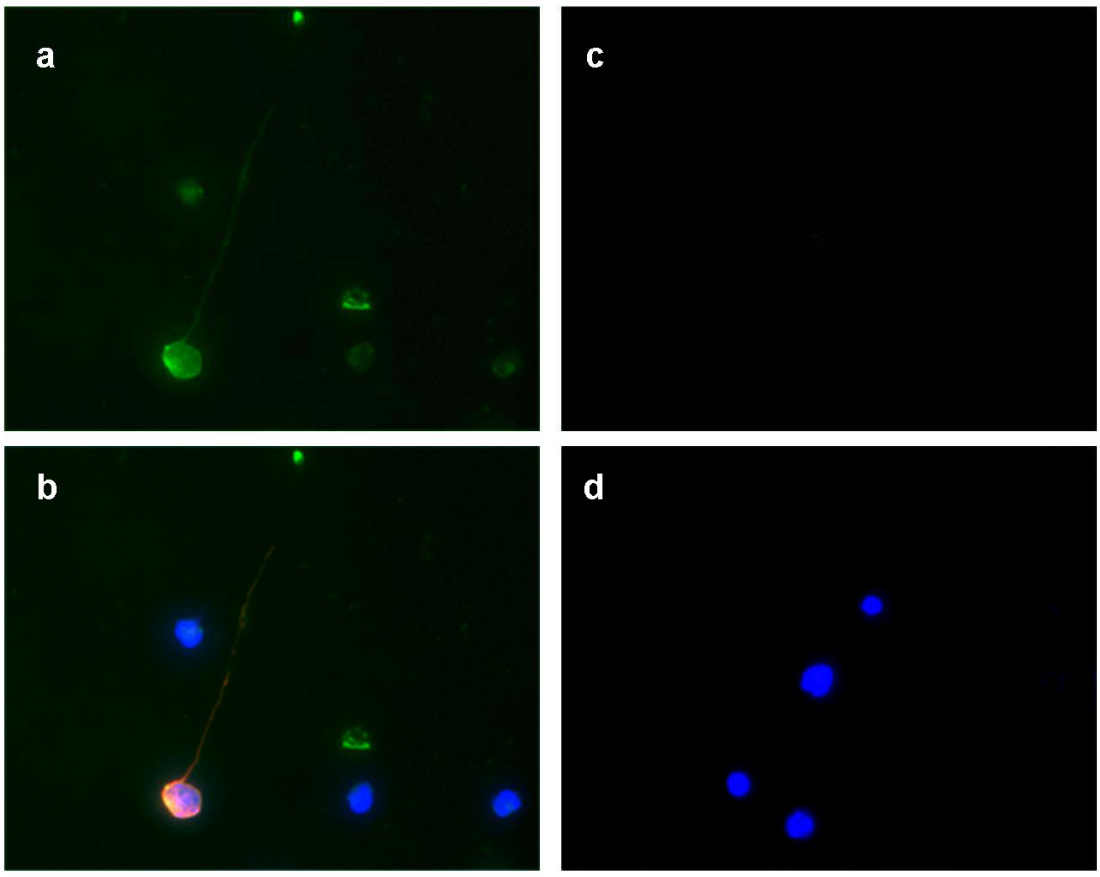

Figure 47. PDE4A5 is expressed by CGNs.
Immunocytochemistry with an antibody specific for PDE4A5 reveals immunoreactivity for PDE4A5 in wt CGNs (a). Counterstaining with DAPI and β-tubulin (b). Omission of primary antibody reveals minimal background staining (c, d).

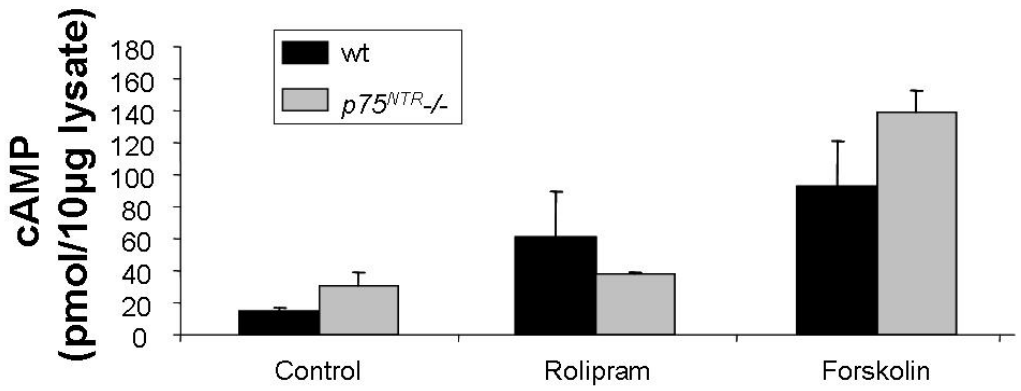

Figure 48. p75^{NTR} decreases cAMP via PDE4 in CGNs
Primary isolated CGNs from wt and *p75^{NTR}-/-* animals were assayed for cAMP levels. *p75^{NTR}-/-* CGNs exhibited twofold higher basal levels of cAMP when compared to wt controls. Treatment with rolipram elevated cAMP levels in wt animals, but not in *p75^{NTR}-/-* animals. Forskolin raised cAMP levels in both genotypes, but *p75^{NTR}-/-* CGNs treated with forskolin still exhibit higher cAMP when compared to wt controls.

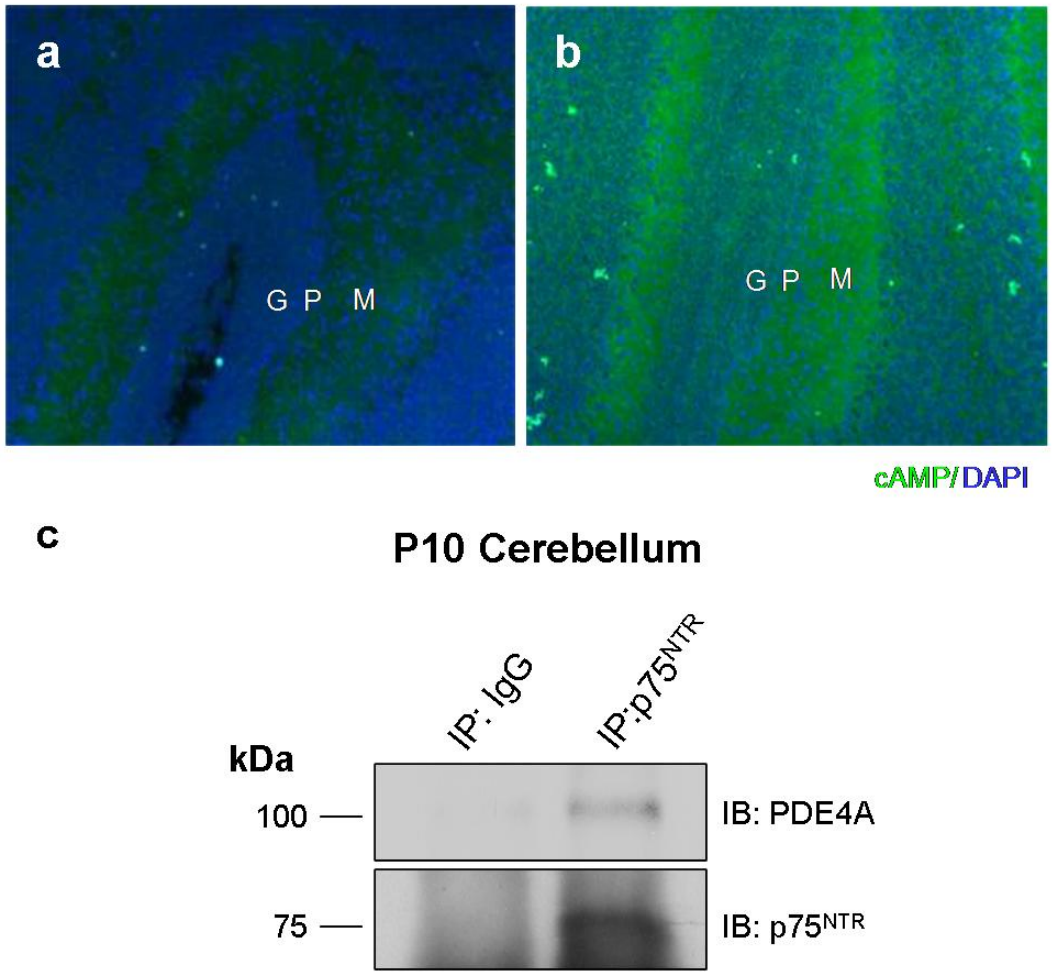

Figure 49. Genetic depletion of p75^{NTR} leads to increased cAMP levels in the cerebellum *in vivo* via its interaction with PDE4A5.
Immunohistochemistry with an antibody specific for cAMP reveals increased cAMP immunoreactivity in *p75^{NTR}*-/- cerebella (b) when compared to wt controls (a). Co-immunoprecipitation experiment from lysates taken from cerebella reveals that PDE4A5 and p75^{NTR} form a complex within the cerebellum (c).

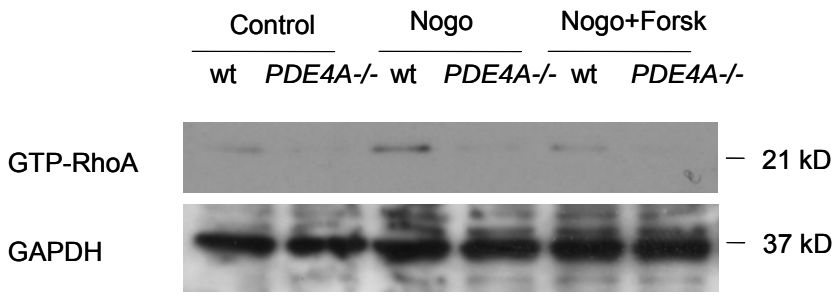

Figure 50. *PDE4A-/-* neurons do not activate RhoA in response to stimulation by Nogo-A.
Primary CGNs were serum starved then stimulated with Nogo-A for 30 minutes prior to lysis and immunoprecipitation of GTP-bound (active) RhoA. CGNs were pretreated with 10 μM forskolin for 30 minutes prior to treatment with Nogo-A. Experiment was performed by Diane Bushman.

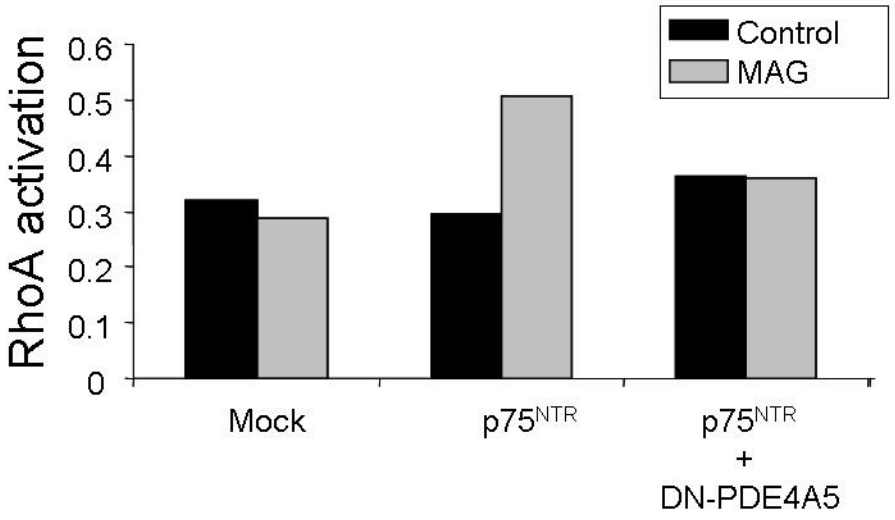

Figure 51. Dominant negative PDE4A5 prevents MAG induced Rho activation. Expression of $p75^{NTR}$ into HEK293 cells allows the activation of RhoA by MAG. Co-expression of DN-PDE4A5 prevents the activation of RhoA by MAG, indicating a role for active PDE4A5 in regulating RhoA activity in response to myelin ligands.

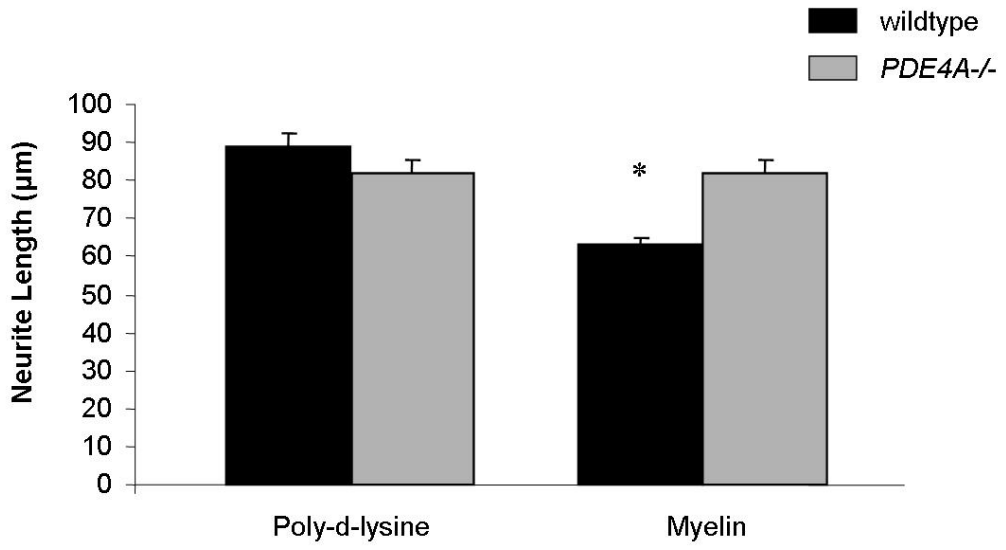

Figure 52. Genetic depletion of PDE4A prevents myelin induced inhibition of neurite outgrowth.
Wt and *PDE4A-/-* CGNs were plated either on poly-D-lysine (PDL) or myelin and allowed to extend processes overnight. Cells were then fixed and stained with an anti β-tubulin antibody. Neurites were measured and average neurite length was compared between groups. The experiment was repeated 4 times and at least 100 neurites were counted per condition. Experiments were performed by Benjamin Sachs and Diane Bushman. Additional counting was performed by Xiaolin Tan.

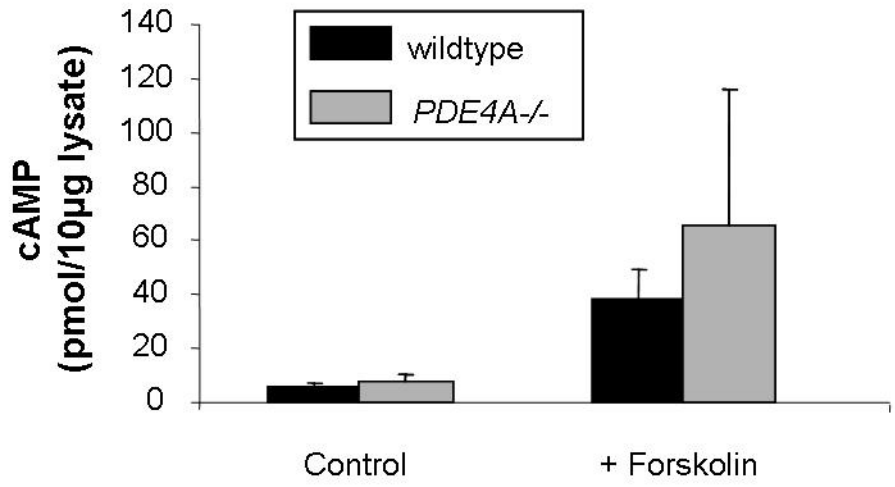

Figure 53. Genetic depletion of PDE4A does not lead to significant increases in total intracellular cAMP.
Analysis of total intracellular cAMP levels in wt and *PDE4A-/-* CGNs reveals no significant increase in either basal or forskolin stimulated cAMP levels. We do observe a slight trend toward increased cAMP in the absence of PDE4A, but this result is not statistically significant. Experiment was performed by Diane Bushman.

Chapter 5. Discussion

In the preceding chapters, I have presented the investigation of $p75^{NTR}$ function and signaling that I have performed for my Ph.D research. These studies have identified a novel signaling pathway downstream of $p75^{NTR}$ involving the direct recruitment of a specific PDE4 isoform to the membrane, resulting in decreased intracellular cAMP. We have demonstrated that this signaling cascade plays a role in scar resolution after sciatic nerve injury and LPS-induced lung fibrosis. We have extended these findings by demonstrating that the regulation of cAMP by $p75^{NTR}$ also occurs within the CNS, where it modulates RhoA activity and neurite outgrowth and has the potential to regulate CNS nerve regeneration after injury. In the following chapter, I will discuss the importance of these findings in the context of nerve regeneration and airway disease and will discuss the therapeutic potential of pharmacologic targeting of this novel $p75^{NTR}$-PDE4A5-cAMP pathway.

1. $p75^{NTR}$ and the regulation of plasminogen activation

The demonstration that $p75^{NTR}$ inhibits plasminogen activation and fibrin clearance after sciatic nerve injury and LPS induced lung fibrosis is the first evidence that $p75^{NTR}$ regulates extracellular matrix remodeling during tissue repair. Our data indicate that $p75^{NTR}$ upregulation after injury can be a pathogenic event resulting in the perpetuation of scar formation, potentially delaying tissue repair. Based on these findings, it is possible that blockade of $p75^{NTR}$ upregulation or signaling may be therapeutic in promoting recovery from lung or sciatic nerve injury. Although my dissertation research focused on injury models outside of the CNS, it is possible that

the increased expression of $p75^{NTR}$ by neurons, glia, and brain endothelial cells could regulate the temporal and spatial pattern of tPA expression during brain injury or inflammation as well. In support of this, we have demonstrated that the regulation of tPA by $p75^{NTR}$ does occur within the CNS under physiological conditions. Future research will determine if this regulation occurs in the CNS during disease pathology and will define the contribution of $p75^{NTR}$ mediated inhibition of plasminogen activation to tissue repair in CNS injury models.

The work presented in this dissertation has focused on the role of tPA/plasmin exclusively as it pertains to fibrin degradation and scar resolution. However, tPA/plasmin also exhibit non-fibrinolytic functions in neurodegeneration [211], synaptic plasticity [212], and long term potentiation [213] via proteolytic processing of a variety of other substrates. For example, tPA and/or plasmin have been reported to cleave TGF-β [214], laminin [215], and phosphacan [216], thus raising the possibility that $p75^{NTR}$ may also regulate TGF-β dependent functions and participate in ECM remodeling of non-fibrin substrates. In addition, tPA has been reported to cleave the pro-neurotrophins and catalyze their conversion to the mature neurotrophins [217]. By regulating the proteolytic processing of pro-neurotrophins, $p75^{NTR}$ might promote the availability of its own high affinity ligands and decrease mature neurotrophin signaling through Trk receptors. tPA has also been reported to exert non-proteolytic effects, such as the activation of microglia [218]. It is likely that by modulating tPA expression, $p75^{NTR}$ could regulate non-proteolytic functions of tPA as well. Future research will determine the biological relevance of $p75^{NTR}$ mediated inhibition of tPA in non-fibrinolytic and non-proteolytic functions of tPA.

Given the dependence of $p75^{NTR}$ functions on the availability of different ligands and co-receptors [219], further analysis will be necessary to determine the effects of $p75^{NTR}$ on extracellular proteolysis and ECM remodeling in different cellular systems. Our study indicates that expression of $p75^{NTR}$ can inhibit tPA in the absence of neurotrophin ligands and/or in the absence of serum. Constitutive expression of $p75^{NTR}$ has previously been shown to signal in a neurotrophin-independent manner to induce neuronal apoptosis [69] and activation of Akt [68]. Similarly, the regulation of tPA identified here is an effect of expression of $p75^{NTR}$ that does not depend on neurotrophin signaling. Future work will examine the effects of non-neurotrophin ligands that bind directly to $p75^{NTR}$, such as β-amyloid [39], or ligands of co-receptors of $p75^{NTR}$, such as Nogo, MAG, and OMgp [61], on the regulation of plasminogen activation by $p75^{NTR}$.

Although it is possible that either an unidentified ligand or a ligand of a co-receptor of $p75^{NTR}$ plays a role in the inhibition of plasminogen activation by $p75^{NTR}$, it is also possible that this effect is completely ligand independent. In this case, it would still prove beneficial to understand the molecular event or events that trigger the activation of this signaling pathway. We believe that the induction of $p75^{NTR}$ expression after injury serves as the catalyst that activates the $p75^{NTR}$-PDE4A5 pathway. However, the molecular mechanisms that underly this injury-induced upregulation of $p75^{NTR}$ are not well understood. It has been shown that in the absence of fibrin, the upregulation of $p75^{NTR}$ after sciatic nerve injury is reduced [119], indicating that fibrin itself may participate in the upregulation of $p75^{NTR}$. If this is the case, it would provide evidence for an interesting mode of positive feedback

regulation in which fibrin inhibits its own clearance by stimulating the inhibition of fibrinolysis. Future research will determine the precise mechanisms leading to upregulation of $p75^{NTR}$ after injury as well as the specific contribution of fibrin to this process.

2. $p75^{NTR}$ and the regulation of cAMP

The finding that expression of $p75^{NTR}$ decreases levels of cAMP via an interaction with PDE4A5 provides the first evidence for the regulation of cAMP by $p75^{NTR}$. Previous studies have shown that BDNF, but not NGF, increases cAMP in neurons via TrkB. Our study is the first to directly examine the role of $p75^{NTR}$ in the regulation of cAMP by using genetic depletion, siRNA knockdown or upregulation of the $p75^{NTR}$. Our results suggest that $p75^{NTR}$ might exert the opposite function as TrkB receptors by recruiting PDE4A4/5 and decreasing cAMP. It is also possible that by decreasing cAMP and inhibiting tPA, $p75^{NTR}$ expression may lead to decreased levels of mature BDNF (described above), thus inhibiting the elevation of cAMP by BDNF-TrkB.

We have found that expression of $p75^{NTR}$ leads to recruitment of PDE4A5 to the membrane and decreases total levels of cAMP. In addition to decreasing total cellular cAMP, it is likely that targeting cAMP degradation to sites of $p75^{NTR}$ expression contributes to the compartmentalization of cAMP via increasing its degradation in specific microdomains. We provide support for this with our data showing reduced membrane associated PKA activity in cells overexpressing $p75^{NTR}$ (Figs. 30, 31). Future research with targeted PKA and cAMP biosensors will shed

light upon more specific deficits in cAMP signaling that result from membrane recruitment of PDE4A5 to $p75^{NTR}$. Compartmentalization of PDEs represents a major mechanism that regulates intracellular specificity of cAMP signaling [184, 220]. Previous studies showed that β_2-adrenergic receptors target degradation of cAMP to the membrane via recruitment of multiple PDE4 isoforms, such as PDE4B1, PDE4B2 and PDE4Ds [221]. Our finding of interaction between $p75^{NTR}$ and PDE4A4/5 represents the first example of recruitment of a single PDE4 isoform to a transmembrane receptor. In contrast to the interaction of β_2-adrenergic receptors with PDE4s, which is mediated via β-arrestin, our study suggests that the interaction of $p75^{NTR}$ with PDE4A4/5 could be potentially mediated by direct binding to PDE4A domains, such as the C-terminal domain that is unique to this sub-family. In support of this, we do not detect an interaction between $p75^{NTR}$ and β-arrestin in NIH3T3$p75^{NTR}$ cells (data not shown). Future research will determine whether other scaffolding proteins play a role in the recruitment of PDE4A5 to $p75^{NTR}$ or the formation of a stable complex between these two proteins.

It remains to be determined whether the recruitment of PDE4A5 to $p75^{NTR}$ can occur in the presence of other $p75^{NTR}$ interactors. Future research will establish if the $p75^{NTR}$-PDE4A5-cAMP pathway signals simultaneously with other $p75^{NTR}$ pathways, or if interactions between $p75^{NTR}$ and other intracellular mediators preclude the recruitment of PDE4A5. Conversely, it is not clear from our results whether the interaction between $p75^{NTR}$ and PDE4A5 has any effect upon the interaction between $p75^{NTR}$ and other intracellular signaling molecules. Similarly, it is unclear whether

PDE4A5 interacts with $p75^{NTR}$ when $p75^{NTR}$ is contained within co-receptor complexes with either Trks, sortilin, or the Nogo receptor.

3. Potential additional targets of $p75^{NTR}$ –cAMP signaling

In addition to inhibiting plasminogen activation, the regulation of cAMP by $p75^{NTR}$/PDE4A5 interactions may have important consequences for expression of other cAMP/PKA dependent genes. cAMP/PKA signaling through CREB is one of the most extensively studied mechanisms for the regulation of gene transcription by elevation of cAMP. However, cAMP can regulate gene transcription via PKA-independent mechanisms {reviewed in [222]}, and PKA can has numerous CREB-independent effects in the CNS {reviewed in [223]}. For example, PKA phosphorylates DARPP-32 and converts it into an inhibitor of protein phosphatase – 1 (PP-1), and thus plays an important role in dopamine signal transduction within the brain {reviewed in [224]}. PKA also phosphorylates synapsin – 1 to modulate neuronal activity [225], promotes tau phosphorylation [226], and inhibits sonic hedgehog signaling within the CNS [227, 228]. Interestingly, PKA has also been shown to phosphorylate $p75^{NTR}$ and regulate its localization to lipid rafts [229], indicating that $p75^{NTR}$ may regulate its own subcellular localization via the inhibition of PKA. Through its regulation of cAMP and PKA, $p75^{NTR}$ may thus influence a wide range of CNS functions. Moreover, since cAMP-analogues decrease expression of $p75^{NTR}$ [230], it is possible that $p75^{NTR}$ by decreasing cAMP contributes to the positive regulation of its own expression. Future research will determine whether the

expression of $p75^{NTR}$ leads to differences in the phosphorylation state of other PKA substrates *in vitro* and *in vivo*.

Elevation of cAMP can activate signaling through non-PKA pathways as well. For example, cAMP induces cell signaling via the regulation of cyclic nucleotide gated channels [231] and by activating exchange proteins activated by cAMP (Epacs), guanine nucleotide exchange proteins known to regulate integrin mediated cell adhesion, cardiac myocyte gap junction formation, and vascular endothelial cell barrier formation {reviewed in [232]}. Epacs have been shown to regulate differentiation of both adipocytes [233] and neurons [234] and to play a role in a pathophysiological processes including fibrosis [235]. Within the CNS, Epacs have been implicated in depression and suicide [236]. Future research will determine whether $p75^{NTR}$ expression leads to decreased Epac signaling or alterations in the activity of cyclic nucleotide gated channels, and whether this regulation plays functional roles in pathophysiology.

4. $p75^{NTR}$ in tissue repair

Given that $p75^{NTR}$ functions not only as a regulator of plasminogen activation, but also as a regulator of other cellular functions such as apoptosis and differentiation, its role in tissue injury will likely depend upon the relative spatial and temporal contributions of cell differentiation and extracellular proteolysis in the progression of tissue repair. For example, we recently showed that in the absence of plasminogen, the effects of $p75^{NTR}$ in tissue repair are protective due to its beneficial effects in cell differentiation [22]. Similarly, in a vascular injury model that depends on uPA and not

on tPA-mediated fibrinolysis [237, 238], $p75^{NTR}$ also appears to be protective due to the induction of apoptosis of the smooth muscle cells that drive lesion development in this model of vascular injury [102].

4.1. $p75^{NTR}$ in sciatic nerve injury

In injury models that depend on apoptosis, differentiation, and extracellular remodeling, it is likely that $p75^{NTR}$ will play multiple, sometimes opposing roles. One example of this is the process of sciatic nerve regeneration, in which $p75^{NTR}$ plays dual, opposing roles by sustaining fibrin deposition [203], and also promoting myelination [91]. Since fibrin deposition is present primarily during the first week after sciatic nerve injury, $p75^{NTR}$ expression may initially delay repair in the sciatic nerve via sustaining fibrin deposition. Indeed, one week after injury genetic deletion of $p75^{NTR}$ does not affect myelination in the sciatic nerve [133]. By contrast, two weeks after injury, a timepoint where fibrin has been degraded in the sciatic nerve by tPA[119, 139], $p75^{NTR}$ plays a positive role in myelination [91] . My dissertation research did not investigate the regulation of plasminogen activation by $p75^{NTR}$ later than 8 days after injury, as most fibrin is cleared by this point. It would be interesting to determine whether the increased plasminogen activation observed in *$p75^{NTR}$-/-* animals persists after fibrin has been cleared. If so, the identification of additional substrates for tPA-plasmin may reveal additional roles for $p75^{NTR}$ after sciatic nerve injury. Similarly, we did not investigate the timecourse of $p75^{NTR}$ and PDE4A5 interaction after sciatic nerve injury and did not determine if the presence of fibrin promotes the interaction between $p75^{NTR}$ and PDE4A5. A closer examination of the

consequences of $p75^{NTR}$ deletion at different timepoints after peripheral nerve injury would likely shed light upon the relative contributions of extracellular matrix remodeling and remyelination during the course of tissue repair.

4.2. $p75^{NTR}$ and PDE4 in spinal cord injury

Prior research has identified one mechanism by which $p75^{NTR}$ inhibits neurite outgrowth - via its role as a co-receptor for NogoR [61]. Conversely, BDNF/TrkB signaling overcomes the inhibition of nerve regeneration by myelin proteins via inhibition of PDE4 [189]. However, the specific isoform of PDE4 that is inhibited remains undetermined. The general PDE4 inhibitor, rolipram, has also been shown to overcome inhibition of neurite outgrowth and promote functional recovery in the spinal cord after injury [105], but again its precise target is unknown. It is interesting to speculate that PDE4A interacting with $p75^{NTR}$ could play an inhibitory role in nerve regeneration by competing with neurotrophin signaling via Trk receptors. Some circumstantial evidence supports this idea as PDE4A has been detected as the predominant PDE4 isoform at the corticospinal tract [239]. We propose that PDE4A5 may play a major role in the inhibition of neurite outgrowth and axonal regeneration via its inhibition of cAMP. Elevation of cAMP has been shown to overcome the inhibition of neurite outgrowth by myelin [166, 204]. However, the molecular mechanisms underlying this effect remain incompletely understood.

Elevation of cAMP has been hypothesized to improve neurite outgrowth and axonal regeneration via two distinct mechanisms {reviewed in [240]}. One mechanism depends upon the increased transcription of cAMP dependent genes, while

the other involves potential crosstalk with the RhoA signaling pathway. Our results provide the first experimental evidence that elevation of cAMP in neurons inhibits RhoA activation. Thus, we propose that the inhibition of cAMP by $p75^{NTR}$ provides a novel mechanism for the regulation of RhoA activity and the inhibition of neurite outgrowth by $p75^{NTR}$ (Fig. 56). As RhoA activation has been shown to prevent neurite outgrowth and CNS repair [209], we believe that our data identify a novel means by which $p75^{NTR}$ may inhibit neurite outgrowth and nerve regeneration in the CNS. In addition to its role in regulating RhoA activation, it is possible that the regulation of plasminogen activation by $p75^{NTR}$ may also have implications for nerve regeneration. Work by Christian Schachtrup in our lab has identified fibrin as an inhibitor of neurite outgrowth [133], and suggests a potential functional role for the inhibition of nerve regeneration by fibrin *in vivo*. As $p75^{NTR}$ inhibits fibrin clearance, it is possible that upregulation of $p75^{NTR}$ may contribute to the inhibitory environment after spinal cord injury by perpetuating fibrin deposition. Our results, taken together, identify $p75^{NTR}$-PDE4A5 signaling may regulate neurite outgrowth and nerve regeneration through at least two distinct, cAMP dependent pathways.

4.3 $p75^{NTR}$ and PDE4A in respiratory disease

Inhibition of PDE4 is considered a major pharmacologic target for lung pathogenesis, especially for Chronic Obstructive Pulmonary Disease (COPD) [189]. However, all the currently identified inhibitors of PDE4 target the common catalytic domain and thus inhibit all the isoforms resulting in unwanted side-effects. The identification of the specific PDE4 isoform that contributes to COPD pathogenesis

would allow for more directed therapeutic intervention. Interestingly, PDE4A4 is the PDE4 isoform that is specifically upregulated in COPD [189]. Similar to PDE4A4, $p75^{NTR}$ has also been previously linked to lung pathology [105, 143]. NGF/$p75^{NTR}$ signaling has been suggested to enhance local neurogenic inflammation to exacerbate pulmonary disease [104]. Our study suggests an additional pathway for $p75^{NTR}$ as a regulator of expression of PAI-1 and a mediator of fibrosis. Expression of $p75^{NTR}$ in the lung is detected in epithelial and smooth muscle cells [241]. Similar to $p75^{NTR}$, PAI-1 is expressed by bronchial epithelial cells [190] and its expression results in an antifibrinolytic environment within the airway wall, resulting in sustained fibrin deposition. Fibrin participates in lung pathogenesis as a regulator of inflammation and airway remodeling. It is therefore possible that $p75^{NTR}$–mediated regulation of PAI-1 via PDE4 could influence inflammatory and tissue repair processes in pulmonary disease. Our research, which implicates both $p75^{NTR}$ and PDE4A4/5 in lung pathology, suggests that targeting the $p75^{NTR}$/PDE4A4 interaction could result in the specific inhibition of a single PDE4 isoform in the treatment of pulmonary disease.

5. Implications for drug discovery

5.1. $p75^{NTR}$ as a drug target

One property of $p75^{NTR}$ that makes it an especially appealing drug target is the fact that it is specifically upregulated in diseased or injured tissue. Because of this, blocking $p75^{NTR}$ would be predicted to interfere primarily with pathological processes and have minimal effects on physiological processes. However, given the fact that $p75^{NTR}$ plays multiple, sometimes opposing roles in tissue repair, it is difficult to

predict the overall effect of blocking $p75^{NTR}$ function. The research performed for my dissertation has identified regulation of cAMP as a novel pathogenic pathway downstream of $p75^{NTR}$. We showed that $p75^{NTR}$-mediated suppression of cAMP regulates extracellular matrix remodeling in sciatic nerves and lungs [170]. It is noteworthy that the inhibitory functions of $p75^{NTR}$ in axonal regeneration [61] are disinhibited by elevation of cAMP in neurons, either via the addition of cAMP analogues or via priming with neurotrophins [115]. We propose that selective targeting of the $p75^{NTR}$-PDE4A5-cAMP pathway may lead to significant improvement over currently available strategies to block $p75^{NTR}$ or PDE4 function.

5.2. Current pharmacologic tools to inhibit $p75^{NTR}$

Three major classes of pharmacologic agents have been developed to manipulate $p75^{NTR}$ function: 1) agents that knock down expression of $p75^{NTR}$, 2) agents that target the extracellular domain of $p75^{NTR}$, and 3) cell permeable inhibitors that target $p75^{NTR}$-ICD. The first class of $p75^{NTR}$-modulating agents consists of DNA and RNA based strategies that alter levels of $p75^{NTR}$ expression. Lentiviral and adenoviral delivery vectors have been used to drive the expression of full length $p75^{NTR}$ [22, 61, 68], $p75^{NTR}$ –ECD [61], and $p75^{NTR}$-ICD [22, 68] *in vitro*, but the use of these agents *in vivo* has not been reported. siRNA has been utilized *in vitro* to demonstrate roles for $p75^{NTR}$ in differentiation [22], survival of carcinoma cells [242], migration [170], inhibition of neurite outgrowth [243], and proliferation [115]. *In vivo* knockdown of $p75^{NTR}$ with antisense oligonucleotides reduces inflammation and demyelination in an animal model of multiple sclerosis [89]. While this class of

$p75^{NTR}$ modifying agents has great potential as research tools, technical difficulties and ethical concerns hinder their ability to be used as therapeutic agents. Furthermore, altering expression levels of $p75^{NTR}$ is likely to affect all aspects of $p75^{NTR}$ signaling, rather than regulating specific $p75^{NTR}$ dependent pathways, and might be predicted to exert some deleterious side effects.

The second major class of $p75^{NTR}$–blocking agents consists of small molecules, peptide inhibitors and antibodies that block the interaction of $p75^{NTR}$ with its extracellular ligands, the neurotrophins. A cyclic decapeptide has been developed that mimics the $p75^{NTR}$ binding loop of NGF. This decapeptide blocks NGF binding to $p75^{NTR}$ and prevents ligand-dependent keratinocyte apoptosis *in vitro* [244]. Antibodies that block neurotrophin binding to $p75^{NTR}$ have also been reported [245, 246]. For example, REX, an antibody generated against the rat extracellular domain of $p75^{NTR}$, inhibits the ability of $p75^{NTR}$ to promote myelination both *in vitro* and *in vivo* [245]. Treatment of mice with REX decreases myelin sheath thickness in sciatic nerve axons via the inhibition of BDNF binding to $p75^{NTR}$ [81]. Screenings of small molecule libraries have identified compounds that can inhibit the pro-apoptotic effects of pro-NGF binding $p75^{NTR}$ and promote survival signaling *in vitro* [247] but have not yet been evaluated *in vivo*. This second class of pharmacologic agents does not carry with it the ethical concerns of DNA based techniques and also affords some degree of specificity as agents can be designed that selectively block the association of $p75^{NTR}$ with specific ligands. However, given the fact that $p75^{NTR}$ can induce signaling in the absence of ligand, it is likely that agents targeted against the extracellular domain will only be effective against a subset of $p75^{NTR}$ functions.

The third strategy to inhibit $p75^{NTR}$ signaling relies upon preventing the association between $p75^{NTR}$ and specific intracellular signaling mediators. This strategy utilizes cell permeable peptides to competitively inhibit interactions of $p75^{NTR}$ with its downstream partners. Tat-pep5, a peptide that competitively inhibits the interaction between $p75^{NTR}$-ICD and Rho-GDI, was designed based on results from deletion mutagenesis co-immunoprecipitation experiments that identified the sequences mediating the interaction between $p75^{NTR}$ and Rho-GDI [21]. Treatment with Tat-pep5 prevents the co-immunoprecipitation of Rho-GDI and $p75^{NTR}$ and consequently prevents both $p75^{NTR}$ induced activation of RhoA and $p75^{NTR}$-dependent inhibition of neurite outgrowth *in vitro* [21]. Tat-pep5 can also block $p75^{NTR}$-induced differentiation of hepatic stellate cells, a RhoA dependent process that plays an important role in liver fibrosis [22]. Thus, the *in vivo* application of this peptide or similarly acting molecules may prove beneficial for treatment of disorders such as spinal cord injury and liver fibrosis.

Targeting the interaction between $p75^{NTR}$ and its other intracellular signaling partners may also provide novel opportunities for molecular intervention in disease states. For example, we have shown that the interaction between $p75^{NTR}$ and PDE4A5 has been shown to play a role after sciatic nerve injury and during LPS-induced lung fibrosis [203]. It is possible that blocking the interaction between $p75^{NTR}$ and PDE4A5 may lead to improved recovery after sciatic nerve injury, or during lung fibrosis. It is also possible that blocking this specific interaction would preserve other $p75^{NTR}$ dependent functions (Fig. 52). For example, in the context of sciatic nerve repair, by blocking the recruitment of PDE4A5 to $p75^{NTR}$, one might prevent the

inhibitory role of $p75^{NTR}$ in fibrin clearance, but retain the role of $p75^{NTR}$ as a positive regulator of myelination. The identification of additional disease states are characterized by $p75^{NTR}$ and PDE4A4/5 co-expression will likely lead to additional opportunities for $p75^{NTR}$-PDE4A4/5 based therapies. Applying this technique to different $p75^{NTR}$ dependent signaling pathways via blocking other $p75^{NTR}$ interactors may prove beneficial to an even wider range of $p75^{NTR}$-based treatments. A greater understanding of the contribution of specific $p75^{NTR}$ dependent pathways to individual disease states would greatly expedite this process.

5.3. PDE4A5 as a drug target

Like $p75^{NTR}$, PDE4 is also an exciting drug target. However, up until recently, there has been little differentiation between the various PDE isoforms in terms of specific roles in disease processes. Recent evidence has described important biological functions for PDE4D in ischemic stroke [248] and heart failure [249] and for PDE4B in schizophrenia [250]. My dissertation research identifies a biological function for PDE4A4/5 as a molecular mediator of $p75^{NTR}$/cAMP signaling involved in the regulation of plasminogen activation and fibrinolysis in sciatic nerve injury and lung fibrosis and thus identifies PDE4A5 as a potential drug target for these pathologies.

Pharmacological targeting of PDEs has so far focused on generating inhibitors of the catalytic domain (Figure 55). Since the catalytic domain is highly homologous between the different isoforms of PDE4, currently available PDE4 inhibitors are nonselective and are consequently associated with side effects that limit their clinical

applications. The identification of the unique C-terminal region of PDE4A4/5 as the domain required for recruitment to $p75^{NTR}$ may provide a novel means to target this specific isoform (Figure 55). The unique C-terminal region of PDE4A4/5 is not shared by other PDE4 isoforms and thus provides the potential for the generation of an isoform-specific inhibitor for PDE4A (Fig. 55). Rolipram, a general inhibitor of PDE4s, has deleterious side effects due to inhibition of PDE4D, including nausea, when given to patients and thus has a limited therapeutic value. Specific inhibitors of particular PDE4 isoforms could decrease unwanted side effects may potentially have impoved therapeutic value in both diseases of the lung and the nervous system. Our recent studies suggest that it might be possible to specifically inhibit the activation of the PDE4A isoform by perturbing its subcellular compartmentalization via inhibition of the binding of $p75^{NTR}$ to the unique C-terminal region of PDE4A. A greater understanding of the biological functions of PDE4A and the cell types and disease processes in which $p75^{NTR}$ and PDE4A4/5 are co-expressed will likely lead to additional targets for $p75^{NTR}$–PDE4A4/5-based therapies.

Taken together, we have identified a novel cAMP-dependent signaling pathway initiated by $p75^{NTR}$ that specifically regulates plasminogen activity and scar formation after sciatic nerve and lung injury. Though $p75^{NTR}$ is responsible for a variety of cell survival and death decisions, our data has revealed an unrecognized property of this receptor to regulate the degradation of cAMP. This property provides a potential mechanism to account for how $p75^{NTR}$ acts at sites of injury to promote ECM remodeling. The impact of high levels of $p75^{NTR}$ expression upon inhibition of extracellular proteolysis indicates that the detrimental effects of $p75^{NTR}$ extend beyond

cell growth and axon inhibition. Finally, the dramatic inhibitory effect of $p75^{NTR}$ signaling on plasminogen activation suggests that the $p75^{NTR}$/PDE4A4 interaction represents a novel target for therapeutic intervention in both neuronal and non-neuronal tissues.

6. Future Studies

6.1. *In vivo* roles for PDE4A

The major findings of my dissertation research include a novel role for $p75^{NTR}$ in the regulation of cAMP and plasminogen activation via an interaction with the specific phosphodiesterase isoform, PDE4A5. As PDE4A5 is required for the effects of $p75^{NTR}$, it would be worthwhile to investigate whether genetic deletion of PDE4A5 leads to similar effects as deletion of $p75^{NTR}$. Unfortunately, *PDE4A5-/-* mice do not exist and the *PDE4A-/-* mice are on a mixed background and are thus not suitable for use in many *in vivo* experiments, due to strain differences in responses to injury models, including spinal cord injury [251]. Our lab is currently working on backcrossing the *PDE4A-/-* mice onto a C57BL/6 background so that they can be studied in animal models of injury and disease. Once backcrossed, *PDE4A-/-* could be analyzed after sciatic nerve crush and LPS administration and their phenotype compared to that of the $p75^{NTR}$*-/-*. The results from these experiments could establish the role of PDE4A, but would lack the specificity required to demonstrate a definitive role for PDE4A5. Further experiments using siRNA strategies directed against the specific PDE4A5 splice variant would be required to determine PDE4A5's role in sciatic nerve injury and LPS induced lung fibrosis.

Another injury model that would be particularly interesting to examine in these mice is the model of spinal cord injury. Inhibition of PDE4s has been shown to promote functional recovery after spinal cord injury; however, the specific PDE4 isoform that contributes to the inhibition of nerve regeneration has not been identified. Interestingly, PDE4A is the PDE4 gene that is most highly expressed within the cortico-spinal tract, one of the major axon tracts affected by spinal cord injury. It would be of great importance to determine if PDE4A, or more specifically, PDE4A5, plays a functional role in axonal regeneration in the CNS.

Given the fact that there are a large number of PDE isoforms with very similar functions, it is quite possible that other isoforms can compensate for genetic loss of PDE4A. It would be important to determine the extent of compensation by other PDE4 isoforms in the absence of PDE4A by examining expression levels of other PDE4 isoforms as well as total cellular PDE activity. As a complimentary approach to genetic depletion of PDE4A, it would be useful to generate siRNA constructs against PDE4A such that PDE4A could be knocked down transiently, thus decreasing the likelihood of significant compensation from alternative isoforms. Finally, the generation of PDE4A5-specific inhibitors, knockout mice, or siRNA constructs would greatly accelerate efforts to assign specific functions to this isoform.

6.2. *In vitro* roles for PDE4A

Prior to evaluating the *PDE4A-/-* mouse in a mouse models of spinal cord injury, once the *PDE4A-/-* mouse has been backcrossed, it will be important to confirm many of the results already obtained. For example, the experiments

performed in in primary CGNs should be repeared to ensure that the observed differences in RhoA activation and neurite outgrowth inhibition are due to the absence of PDE4A, rather than due to nonspecific strain differences. In addition, it will be interesting to repeat the cAMP assays on fully backcrossed CGNs to determine whether or not genetic depletion of PDE4A leads to increased intracellular cAMP.

6.3. Additional roles for $p75^{NTR}$ and PDE4A5 in response to LPS

The LPS induced lung fibrosis model that was employed in my dissertation research is a useful model of acute lung injury. However, the injury induced by systemic injection of LPS is not restricted to pulmonary tissue, but rather induces fibrin deposition in other tissues as well, including the heart, kidney, and adrenals [163]. It would be interesting to determine if genetic depletion of $p75^{NTR}$ or PDE4A would lead to similar effects in these other tissues. Furthermore, at higher doses, LPS injections can be fatal, and are used as a model of sepsis [252]. The investigation of whether *$p75^{NTR}$-/-* or *PDE4A-/-* mice exhibit decreased mortality in response to LPS injection may reveal the importance of $p75^{NTR}$-PDE4A5 regulated fibrinolysis in sepsis.

In addition to identifying novel tissues in which $p75^{NTR}$-PDE4A5 signaling regulate tissue remodeling and fibrin clearance after acute endotoxic shock, it would also be interesting to examine the role of $p75^{NTR}$-PDE4A5 in chronic models of lung inflammation. One common model of chronic airway inflammation involves the exposure of mice to aerosolized LPS for several hours daily over the course of one or two months [190]. This model, which is studied as a model for asthma, is

characterized by fibrin deposition. Genetic depletion of PAI-1 promotes airway remodeling after this chronic LPS treatment [190], suggesting that factors that regulate PAI-1, such as $p75^{NTR}$ and PDE4A5, may also influence tissue repair. An investigation of *$p75^{NTR}$-/-* and *PDE4A-/-* mice after chronic LPS inhalation would reveal whether $p75^{NTR}$ and PDE4A5 play a pathogenic role in this model of lung disease as well.

6.4. Additional roles for the regulation of cAMP by $p75^{NTR}$/PDE4A5

Given the multitude of functions regulated by cAMP, it would be of great interest to determine additional processes that are regulated by the $p75^{NTR}$/PDE4A5 mediated inhibition of cAMP/PKA. For example, the phosphorylation of $p75^{NTR}$ by PKA has been shown to induce its translocation to lipid rafts [229]. As we have shown that $p75^{NTR}$-PDE4A5 interactions inhibit PKA activity at the plasma membrane [203], the site of PKA phosphorylation of $p75^{NTR}$, it would be interesting to determine if preventing $p75^{NTR}$ – PDE4A5 interactions either with a pharmacologic inhibitor, or via depletion of PDE4A5 results in decreased $p75^{NTR}$ trafficking to lipid rafts.

As cAMP can mediate effects via non-PKA pathways, it would also be of interest to determine if $p75^{NTR}$ expression regulates activity of cyclic nucleotide gated ion channels, or Epac activity. Finally, it would be of great importance to identify additional genes that are regulated by expression of $p75^{NTR}$. Microarray analysis of wt, *$p75^{NTR}$-/-*, and *PDE4A-/-* cells (CGNs or SCs), or heterologous cells overexpressing these proteins may lead to identification of additional genes regulated by $p75^{NTR}$/PDE4A5 interactions.

6.5. Additional roles for the regulation of tPA by p75^{NTR}/PDE4A5

Given the numerous non-fibrinolytic roles of tPA/plasmin, it would be of great interest to determine additional processes that are regulated by the p75^{NTR}/PDE4A5 mediated inhibition of tPA. For example, it would be important to evaluate if expression of p75^{NTR} inhibits processing of pro-neurotrophins to mature neurotrophins. This would provide a mechanism whereby p75^{NTR} could be a master regulator of neurotrophin signaling by controlling the ligand availability to itself and to each of the Trk receptors. It would also be of great interest to determine if expression of p75^{NTR} blocks the activation of latent TGF-β. The regulation of TGF-β by p75^{NTR} would provide a second mechanism whereby p75^{NTR} could play a major role in tissue fibrosis. Both of these possibilities could be examined using ELISA based techniques comparing the effect of p75^{NTR} expression on the relative amounts of pro vs mature, or latent vs active forms of these proteins.

7. General Conclusions

Since its cloning more than twenty years ago, the function of p75^{NTR} has expanded dramatically, from a receptor for NGF important for the survival of neurons, to a protein that binds numerous ligands to regulate everything from cell survival, differentiation and death to myelination and tissue repair both in the presence and absence of ligands. We have identified a novel, neurotrophin-independent role for p75^{NTR} in the inhibition scar resolution and extracellular matrix remodeling after sciatic nerve and acute lung injuries. We have also identified a new signaling pathway downstream of p75^{NTR} mediated by a previously unidentified interaction with the

phosphodiesterase, PDE4A5. The recruitment of PDE4A5 to $p75^{NTR}$ results in the targeted degradation of cAMP and an overall reduction in cAMP signaling. Through the elucidation of the interacting sequences in these two proteins, we have identified a novel drug target for the treatment of diseases associated with the pathogenic regulation of cAMP by $p75^{NTR}$-PDE4A5 interactions. It is my hope that future research will lead to the development of an inhibitor of this interaction that can be evaluated for efficacy in mouse models of human disease. We have also provided some evidence that PDE4A may be the specific phosphodiesterase isoform that contributes to the inhibition of neurite outgrowth in an *in vitro* model of axonal regeneration. I hope that future research confirms these findings using knockout mice with a clean genetic background and identifies a specific target to promote axonal regeneration *in vivo*. The findings my dissertation research are still far too nascent to be directly applied to the treatment of human disease. However, by defining some of the molecular mechanisms underlying disease pathology, we have increased our understanding of the disease process and have hopefully taken a small step towards this lofty goal.

8. Acknowledgments

Chapter 5, in part, has been published and appears in Biochemical Society Transactions 2007. Sachs, Benjamin; Akassoglou, Katerina, Portland Press 2007. The dissertation author was the primary investigator and author of this paper.

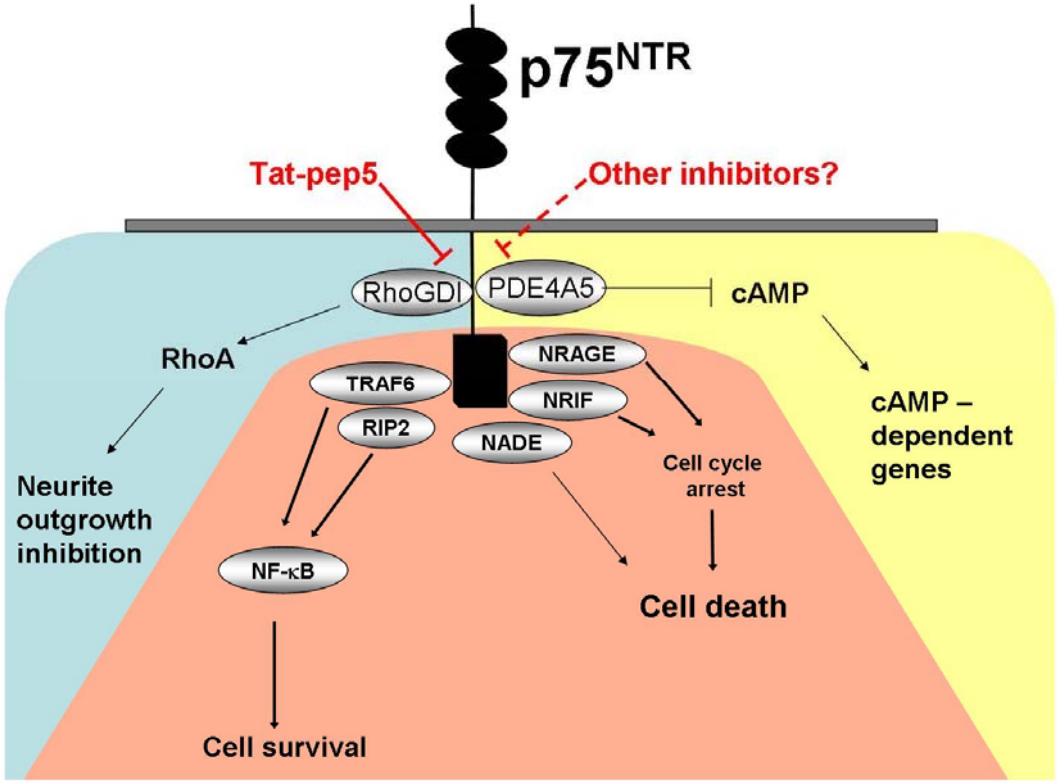

Figure 54. Potential targeting of specific intracellular signaling pathways with cell permeable inhibitors.
Schematic depicting the different signaling pathways activated in response to interactions between $p75^{NTR}$ and its various intracellular signaling mediators. It is possible that by blocking specific interactions between $p75^{NTR}$ and a given intracellular signaling pathway, other $p75^{NTR}$ induced signals would be preserved, thus allowing for selective targeting of $p75^{NTR}$ function.

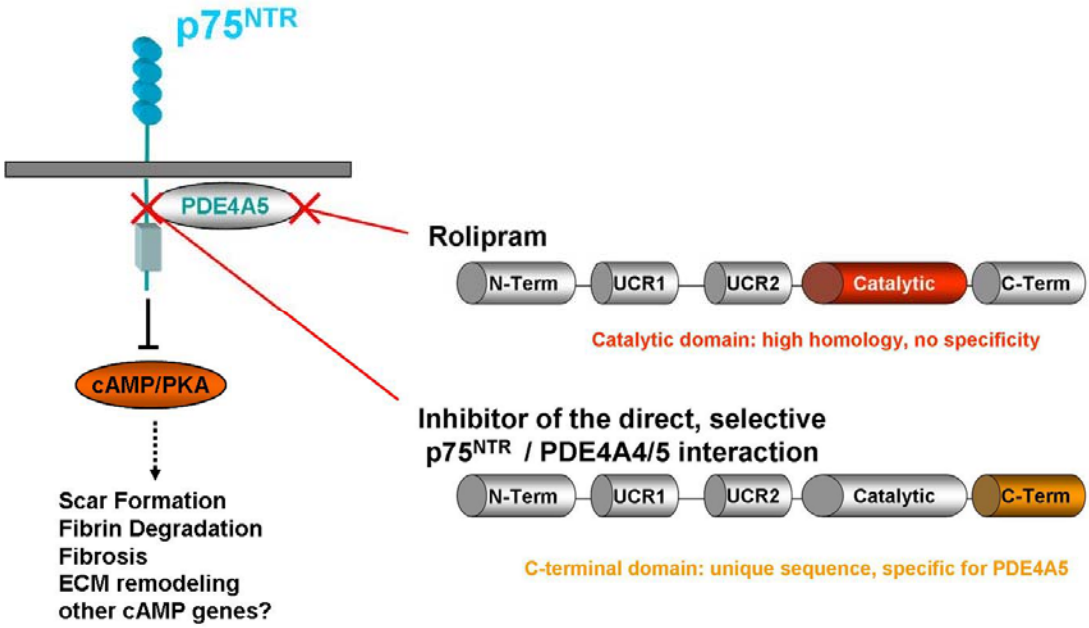

Figure 55. The unique C-terminus of PDE4A4/5 as a potential drug target. Currently available inhibitors of PDE4s, such as rolipram, target the catalytic domain of PDE4. However, the catalytic domain is highly conserved across all PDE4 isoforms, and thus these currently available inhibitors do not specifically target individual isoforms. The lack of specificity leads numerous side effects that preclude their widespread use clinically. In contrast, by targeting unique sequences in specific isoforms, it may be possible to block the effects of individual isoforms. For example, the C-terminus of PDE4A4/5 is not shared by other PDE4 isoforms, and represents a potential specific drug target in diseases characterized by pathogenic PDE4A4/5-$p75^{NTR}$ signaling.

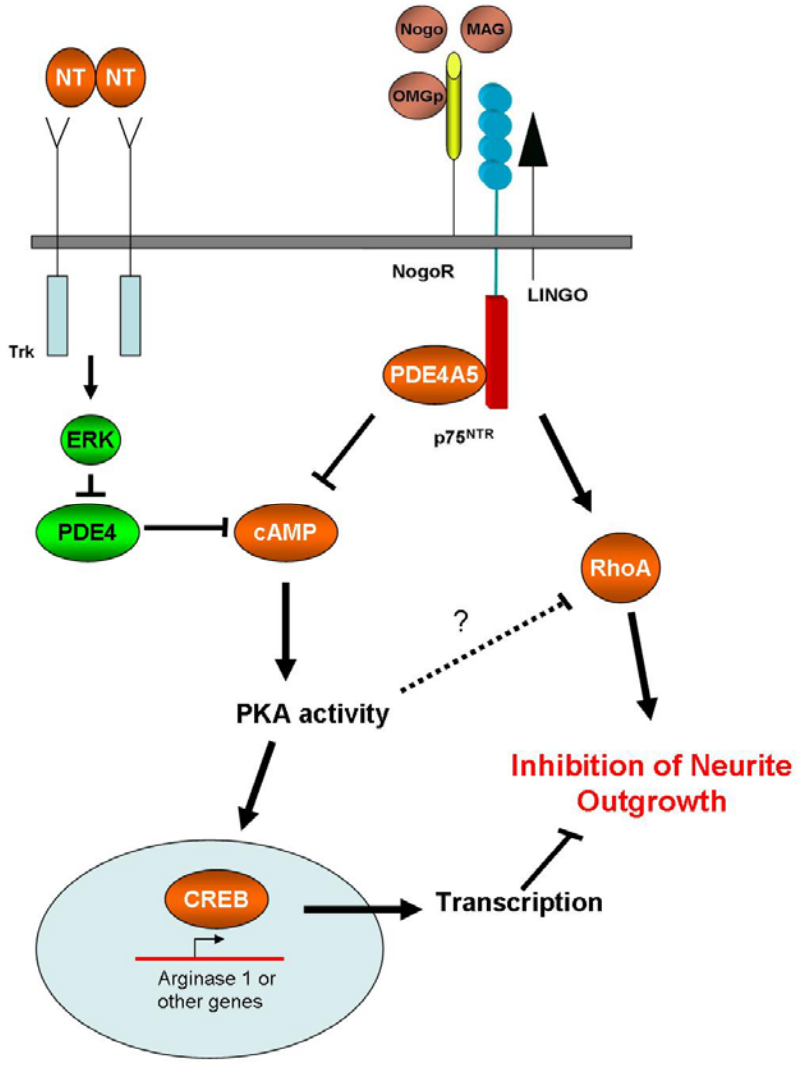

Figure 56. The role of p75^{NTR} and PDE4 in neurite outgrowth inhibition. Previous work from the Filbin group had revealed that neurotrophin signaling through Trk elevates cAMP via the inhibition of a PDE4. This elevated cAMP has been shown to result in increased transcription of a number of cAMP target genes important for the disinhibition of neurite outgrowth, including Arginase 1. In contrast, p75^{NTR} has been implicated in inhibiting neurite outgrowth by promoting RhoA activation through its interactions with RhoA and with Rho-GDI. Our research has identified an additional mechanism whereby p75^{NTR} may regulation of RhoA activity and neurite outgrowth inhibition. Through its interaction with PDE4A5, p75^{NTR} might antagonize the disinhibition of neurite outgrowth that results from BDNF/TrkB/cAMP signaling. By reducing cAMP, p75^{NTR} inhibits neurite outgrowth and may prevent inhibition of RhoA by PKA phosphorylation. We have thus identified a second mechanism whereby p75^{NTR} can contribute to neurite outgrowth inhibition and have provided experimental evidence that cAMP elevation leads to disinhibition of neurite outgrowth at the level of RhoA activation, as well by modulating transcriptional activity.

References

1. Levi-Montalcini, R. and V. Hamburger, *Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo.* J Exp Zool, 1951. **116**(2): p. 321-61.

2. Bueker, E.D., *Implantation of tumors in the hind limb field of the embryonic chick and the developmental response of the lumbosacral nervous system.* Anat Rec, 1948. **102**(3): p. 369-89.

3. Aloe, L., *Rita Levi-Montalcini: the discovery of nerve growth factor and modern neurobiology.* Trends Cell Biol, 2004. **14**(7): p. 395-9.

4. Johnson, D., et al., *Expression and structure of the human NGF receptor.* Cell, 1986. **47**(4): p. 545-54.

5. Hofer, M.M. and Y.A. Barde, *Brain-derived neurotrophic factor prevents neuronal death in vivo.* Nature, 1988. **331**(6153): p. 261-2.

6. Leibrock, J., et al., *Molecular cloning and expression of brain-derived neurotrophic factor.* Nature, 1989. **341**(6238): p. 149-52.

7. Jones, K.R. and L.F. Reichardt, *Molecular cloning of a human gene that is a member of the nerve growth factor family.* Proc Natl Acad Sci U S A, 1990. **87**(20): p. 8060-4.

8. Hallbook, F., C.F. Ibanez, and H. Persson, *Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary.* Neuron, 1991. **6**(5): p. 845-58.

9. Roux, P.P. and P.A. Barker, *Neurotrophin signaling through the p75 neurotrophin receptor.* Prog Neurobiol, 2002. **67**(3): p. 203-33.

10. Chao, M.V., *Neurotrophins and their receptors: a convergence point for many signalling pathways.* Nat Rev Neurosci, 2003. **4**(4): p. 299-309.

11. He, X.L. and K.C. Garcia, *Structure of nerve growth factor complexed with the shared neurotrophin receptor p75.* Science, 2004. **304**(5672): p. 870-5.

12. Dechant, G. and Y.A. Barde, *The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system.* Nat Neurosci, 2002. **5**(11): p. 1131-6.

13. Barker, P.A., *p75NTR is positively promiscuous: novel partners and new insights.* Neuron, 2004. **42**(4): p. 529-33.

14. Yeiser, E.C., et al., *Neurotrophin signaling through the p75 receptor is deficient in traf6-/- mice.* J Neurosci, 2004. **24**(46): p. 10521-9.

15. Mukai, J., et al., *NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated by the common neurotrophin receptor p75NTR.* J Biol Chem, 2000. **275**(23): p. 17566-70.

16. Linggi, M.S., et al., *Neurotrophin receptor interacting factor (NRIF) is an essential mediator of apoptotic signaling by the p75 neurotrophin receptor.* J Biol Chem, 2005. **280**(14): p. 13801-8.

17. Jordan, B.W., et al., *Neurotrophin receptor-interacting mage homologue is an inducible inhibitor of apoptosis protein-interacting protein that augments cell death.* J Biol Chem, 2001. **276**(43): p. 39985-9.

18. Chittka, A. and M.V. Chao, *Identification of a zinc finger protein whose subcellular distribution is regulated by serum and nerve growth factor.* Proc Natl Acad Sci U S A, 1999. **96**(19): p. 10705-10.

19. Chittka, A., et al., *The p75NTR-interacting protein SC1 inhibits cell cycle progression by transcriptional repression of cyclin E.* J Cell Biol, 2004. **164**(7): p. 985-96.

20. Yamashita, T., H. Higuchi, and M. Tohyama, *The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho.* J Cell Biol, 2002. **157**(4): p. 565-70.

21. Yamashita, T. and M. Tohyama, *The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI.* Nat Neurosci, 2003. **6**(5): p. 461-7.

22. Passino, M.A., et al., *Regulation of hepatic stellate cell differentiation by the neurotrophin receptor p75NTR.* Science, 2007. **315**(5820): p. 1853-6.

23. Irie, S., et al., *Functional interaction of Fas-associated phosphatase-1 (FAP-1) with p75(NTR) and their effect on NF-kappaB activation.* FEBS Lett, 1999. **460**(2): p. 191-8.

24. Wooten, M.W., et al., *The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor.* J Biol Chem, 2001. **276**(11): p. 7709-12.

25. Wooten, M.W., et al., *The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination.* J Biol Chem, 2005. **280**(42): p. 35625-9.

26. Gu, C., et al., *Oligodendrocyte apoptosis mediated by caspase activation.* J Neurosci, 1999. **19**(8): p. 3043-9.

27. Wang, X., et al., *Characterization of a p75(NTR) apoptotic signaling pathway using a novel cellular model.* J Biol Chem, 2001. **276**(36): p. 33812-20.

28. Troy, C.M., J.E. Friedman, and W.J. Friedman, *Mechanisms of p75-mediated death of hippocampal neurons. Role of caspases.* J Biol Chem, 2002. **277**(37): p. 34295-302.

29. Casaccia-Bonnefil, P., et al., *Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75.* Nature, 1996. **383**(6602): p. 716-9.

30. Kenchappa, R.S., et al., *Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons.* Neuron, 2006. **50**(2): p. 219-32.

31. Kanning, K.C., et al., *Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability.* J Neurosci, 2003. **23**(13): p. 5425-36.

32. Jung, K.M., et al., *Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor.* J Biol Chem, 2003. **278**(43): p. 42161-9.

33. Domeniconi, M., et al., *MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth.* Neuron, 2005. **46**(6): p. 849-55.

34. Frade, J.M., *Nuclear translocation of the p75 neurotrophin receptor cytoplasmic domain in response to neurotrophin binding.* J Neurosci, 2005. **25**(6): p. 1407-11.

35. Dobrowsky, R.T., et al., *Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor.* Science, 1994. **265**(5178): p. 1596-9.

36. DeFreitas, M.F., P.S. McQuillen, and C.J. Shatz, *A novel p75NTR signaling pathway promotes survival, not death, of immunopurified neocortical subplate neurons.* J Neurosci, 2001. **21**(14): p. 5121-9.

37. Reichardt, L.F., *Neurotrophin-regulated signalling pathways.* Philos Trans R Soc Lond B Biol Sci, 2006. **361**(1473): p. 1545-64.

38. Lee, R., et al., *Regulation of cell survival by secreted proneurotrophins.* Science, 2001. **294**(5548): p. 1945-8.

39. Yaar, M., et al., *Binding of beta-amyloid to the p75 neurotrophin receptor induces apoptosis. A possible mechanism for Alzheimer's disease.* J Clin Invest, 1997. **100**(9): p. 2333-40.

40. Della-Bianca, V., et al., *Neurotrophin p75 receptor is involved in neuronal damage by prion peptide-(106-126).* J Biol Chem, 2001. **276**(42): p. 38929-33.

41. Tuffereau, C., et al., *Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus.* Embo J, 1998. **17**(24): p. 7250-9.

42. Chao, M.V., *Neurotrophin receptors: a window into neuronal differentiation.* Neuron, 1992. **9**(4): p. 583-93.

43. Huang, E.J. and L.F. Reichardt, *Trk receptors: roles in neuronal signal transduction.* Annu Rev Biochem, 2003. **72**: p. 609-42.

44. Lad, S.P., et al., *Individual and combined effects of TrkA and p75NTR nerve growth factor receptors. A role for the high affinity receptor site.* J Biol Chem, 2003. **278**(27): p. 24808-17.

45. Hempstead, B.L., et al., *High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor.* Nature, 1991. **350**(6320): p. 678-83.

46. Barker, P.A. and E.M. Shooter, *Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells.* Neuron, 1994. **13**(1): p. 203-15.

47. Verdi, J.M., et al., *p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells.* Neuron, 1994. **12**(4): p. 733-45.

48. Culmsee, C., et al., *Nerve growth factor survival signaling in cultured hippocampal neurons is mediated through TrkA and requires the common neurotrophin receptor P75.* Neuroscience, 2002. **115**(4): p. 1089-108.

49. Vesa, J., A. Kruttgen, and E.M. Shooter, *p75 reduces TrkB tyrosine autophosphorylation in response to brain-derived neurotrophic factor and neurotrophin 4/5.* J Biol Chem, 2000. **275**(32): p. 24414-20.

50. Hempstead, B.L., *The many faces of p75NTR.* Curr Opin Neurobiol, 2002. **12**(3): p. 260-7.

51. Bibel, M., E. Hoppe, and Y.A. Barde, *Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR.* Embo J, 1999. **18**(3): p. 616-22.

52. Huber, L.J. and M.V. Chao, *A potential interaction of p75 and trkA NGF receptors revealed by affinity crosslinking and immunoprecipitation.* J Neurosci Res, 1995. **40**(4): p. 557-63.

53. Wehrman, T., et al., *Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors.* Neuron, 2007. **53**(1): p. 25-38.

54. Chang, M.S., J.C. Arevalo, and M.V. Chao, *Ternary complex with Trk, p75, and an ankyrin-rich membrane spanning protein.* J Neurosci Res, 2004. **78**(2): p. 186-92.

55. Bilderback, T.R., et al., *Caveolin interacts with Trk A and p75(NTR) and regulates neurotrophin signaling pathways.* J Biol Chem, 1999. **274**(1): p. 257-63.

56. Nykjaer, A., et al., *Sortilin is essential for proNGF-induced neuronal cell death.* Nature, 2004. **427**(6977): p. 843-8.

57. Mazella, J., et al., *The 100-kDa neurotensin receptor is gp95/sortilin, a non-G-protein-coupled receptor.* J Biol Chem, 1998. **273**(41): p. 26273-6.

58. Chen, Z.Y., et al., *Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway.* J Neurosci, 2005. **25**(26): p. 6156-66.

59. Teng, H.K., et al., *ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin.* J Neurosci, 2005. **25**(22): p. 5455-63.

60. Singh, K.K., et al., *Developmental axon pruning mediated by BDNF-p75NTR-dependent axon degeneration.* Nat Neurosci, 2008.

61. Wang, K.C., et al., *P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp.* Nature, 2002. **420**(6911): p. 74-8.

62. Domeniconi, M., et al., *Myelin-associated glycoprotein interacts with the Nogo66 receptor to inhibit neurite outgrowth.* Neuron, 2002. **35**(2): p. 283-90.

63. Fournier, A.E., T. GrandPre, and S.M. Strittmatter, *Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration.* Nature, 2001. **409**(6818): p. 341-6.

64. Wang, K.C., et al., *Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth.* Nature, 2002. **417**(6892): p. 941-4.

65. Mi, S., et al., *LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex.* Nat Neurosci, 2004. **7**(3): p. 221-8.

66. Song, X.Y., et al., *Suppression of p75NTR does not promote regeneration of injured spinal cord in mice.* J Neurosci, 2004. **24**(2): p. 542-6.

67. Zheng, B., et al., *Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo.* Proc Natl Acad Sci U S A, 2005. **102**(4): p. 1205-10.

68. Roux, P.P., et al., *The p75 neurotrophin receptor activates Akt (protein kinase B) through a phosphatidylinositol 3-kinase-dependent pathway.* J Biol Chem, 2001. **276**(25): p. 23097-104.

69. Rabizadeh, S., et al., *Induction of apoptosis by the low-affinity NGF receptor.* Science, 1993. **261**(5119): p. 345-8.

70. Yamashita, T., K.L. Tucker, and Y.A. Barde, *Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth.* Neuron, 1999. **24**(3): p. 585-93.

71. Lee, K.F., et al., *Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system.* Cell, 1992. **69**(5): p. 737-49.

72. von Schack, D., et al., *Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system.* Nat Neurosci, 2001. **4**(10): p. 977-8.

73. Sehgal, A., N. Patil, and M. Chao, *A constitutive promoter directs expression of the nerve growth factor receptor gene.* Mol Cell Biol, 1988. **8**(8): p. 3160-7.

74. Paul, C.E., et al., *A pro-apoptotic fragment of the p75 neurotrophin receptor is expressed in p75NTRExonIV null mice.* J Neurosci, 2004. **24**(8): p. 1917-23.

75. Majdan, M., et al., *Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis.* J Neurosci, 1997. **17**(18): p. 6988-98.

76. Patil, N., E. Lacy, and M.V. Chao, *Specific neuronal expression of human NGF receptors in the basal forebrain and cerebellum of transgenic mice.* Neuron, 1990. **4**(3): p. 437-47.

77. Lomen-Hoerth, C. and E.M. Shooter, *Widespread Neurotrophin Receptor Expression in the Immune System and Other Nonneuronal Rat Tissues.* J Neurochem, 1995. **64**(4): p. 1780-1789.

78. Beattie, M.S., et al., *ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury.* Neuron, 2002. **36**(3): p. 375-86.

79. Scott, A.L., J.F. Borisoff, and M.S. Ramer, *Deafferentation and neurotrophin-mediated intraspinal sprouting: a central role for the p75 neurotrophin receptor.* Eur J Neurosci, 2005. **21**(1): p. 81-92.

80. Ceni, C. and P.A. Barker, *Getting RIP'd Stunts Your Growth.* Neuron, 2005. **46**(6): p. 839-40.

81. Cosgaya, J.M., J.R. Chan, and E.M. Shooter, *The neurotrophin receptor p75NTR as a positive modulator of myelination.* Science, 2002. **298**(5596): p. 1245-8.

82. Horner, P.J. and F.H. Gage, *Regenerating the damaged central nervous system.* Nature, 2000. **407**(6807): p. 963-70.

83. Shao, Z., et al., *TAJ/TROY, an Orphan TNF Receptor Family Member, Binds Nogo-66 Receptor 1 and Regulates Axonal Regeneration.* Neuron, 2005. **45**(3): p. 353-359.

84. Park, J.B., et al., *A TNF Receptor Family Member, TROY, Is a Coreceptor with Nogo Receptor in Mediating the Inhibitory Activity of Myelin Inhibitors.* Neuron, 2005. **45**(3): p. 345-351.

85. Podlesniy, P., et al., *Pro-NGF from Alzheimer's disease and normal human brain displays distinctive abilities to induce processing and nuclear translocation of intracellular domain of p75NTR and apoptosis.* Am J Pathol, 2006. **169**(1): p. 119-31.

86. Harrington, A.W., et al., *Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 6226-30.

87. Copray, S., et al., *Deficient p75 low-affinity neurotrophin receptor expression exacerbates experimental allergic encephalomyelitis in C57/BL6 mice.* J Neuroimmunol, 2004. **148**(1-2): p. 41-53.

88. Kust, B., et al., *Deficient p75 low-affinity neurotrophin receptor expression does alter the composition of cellular infiltrate in experimental autoimmune encephalomyelitis in C57BL/6 mice.* J Neuroimmunol, 2006. **174**(1-2): p. 92-100.

89. Soilu-Hanninen, M., et al., *Treatment of experimental autoimmune encephalomyelitis with antisense oligonucleotides against the low affinity neurotrophin receptor.* J Neurosci Res, 2000. **59**(6): p. 712-21.

90. Ramer, M. and M. Bisby, *Reduced sympathetic sprouting occurs in dorsal root ganglia after axotomy in mice lacking low-affinity neurotrophin receptor.* Neurosci Lett, 1997. **228**(1): p. 9-12.

91. Song, X.Y., et al., *Knockout of p75(NTR) impairs re-myelination of injured sciatic nerve in mice.* J Neurochem, 2006. **96**(3): p. 833-42.

92. Tomita, K., et al., *The neurotrophin receptor p75NTR in Schwann cells is implicated in remyelination and motor recovery after peripheral nerve injury.* Glia, 2007. **55**(11): p. 1199-208.

93. Ferri, C.C. and M.A. Bisby, *Improved survival of injured sciatic nerve Schwann cells in mice lacking the p75 receptor.* Neurosci Lett, 1999. **272**(3): p. 191-4.

94. Trim, N., et al., *Hepatic stellate cells express the low affinity nerve growth factor receptor p75 and undergo apoptosis in response to nerve growth factor stimulation.* Am J Pathol, 2000. **156**(4): p. 1235-43.

95. Cassiman, D., et al., *Human and rat hepatic stellate cells express neurotrophins and neurotrophin receptors.* Hepatology, 2001. **33**(1): p. 148-58.

96. Alpers, C.E., et al., *Nerve growth factor receptor expression in fetal, mature, and diseased human kidneys.* Lab Invest, 1993. **69**(6): p. 703-13.

97. Zhu, Z., et al., *Up-regulation of p75 neurotrophin receptor (p75NTR) is associated with apoptosis in chronic pancreatitis.* Dig Dis Sci, 2003. **48**(4): p. 717-25.

98. Reddypalli, S., et al., *p75NTR-mediated signaling promotes the survival of myoblasts and influences muscle strength.* J Cell Physiol, 2005. **204**(3): p. 819-29.

99. Pezzati, P., et al., *Expression of nerve growth factor receptor immunoreactivity on follicular dendritic cells from human mucosa associated lymphoid tissues.* Immunology, 1992. **76**(3): p. 485-90.

100. Jippo, T., et al., *Involvement of transcription factor encoded by the mouse mi locus (MITF) in expression of p75 receptor of nerve growth factor in cultured mast cells of mice.* Blood, 1997. **90**(7): p. 2601-8.

101. Li, C., et al., *Expression of nerve growth factor (NGF), and its receptors TrkA and p75 in the reproductive organs of the adult male rats.* Zoolog Sci, 2005. **22**(8): p. 933-7.

102. Kraemer, R., *Reduced apoptosis and increased lesion development in the flow-restricted carotid artery of p75(NTR)-null mutant mice.* Circ Res, 2002. **91**(6): p. 494-500.

103. Botchkarev, V.A., et al., *A role for p75 neurotrophin receptor in the control of apoptosis-driven hair follicle regression.* Faseb J, 2000. **14**(13): p. 1931-42.

104. Kerzel, S., et al., *Pan-neurotrophin receptor p75 contributes to neuronal hyperreactivity and airway inflammation in a murine model of experimental asthma.* Am J Respir Cell Mol Biol, 2003. **28**(2): p. 170-8.

105. Renz, H., S. Kerzel, and W.A. Nockher, *The role of neurotrophins in bronchial asthma: contribution of the pan-neurotrophin receptor p75.* Prog Brain Res, 2004. **146**: p. 325-33.

106. Krygier, S. and D. Djakiew, *The neurotrophin receptor p75NTR is a tumor suppressor in human prostate cancer.* Anticancer Res, 2001. **21**(6A): p. 3749-55.

107. Tsunoda, S., et al., *Significance of nerve growth factor overexpression and its autocrine loop in oesophageal squamous cell carcinoma.* Br J Cancer, 2006. **95**(3): p. 322-30.

108. Sinnappah-Kang, N.D., et al., *Heparanase, TrkC and p75NTR: their functional involvement in human medulloblastoma cell invasion.* Int J Oncol, 2005. **27**(3): p. 617-26.

109. Zhao, S.P. and X.F. Zhou, *Co-expression of trkA and p75 neurotrophin receptor in extracranial olfactory neuroblastoma cells.* Neuropathol Appl Neurobiol, 2002. **28**(4): p. 301-7.

110. Iwamoto, S., et al., *The p75 neurotrophin receptor, relative to other Schwann cell and melanoma markers, is abundantly Expressed in spindled melanomas.* Am J Dermatopathol, 2001. **23**(4): p. 288-94.

111. Reis-Filho, J.S., et al., *Distribution and significance of nerve growth factor receptor (NGFR/p75NTR) in normal, benign and malignant breast tissue.* Mod Pathol, 2006. **19**(2): p. 307-19.

112. Beutel, G., et al., *Expression of the p75 neurotrophin receptor in acute leukaemia.* Br J Haematol, 2005. **131**(1): p. 67-70.

113. Marchetti, D., et al., *Brain-metastatic melanoma: a neurotrophic perspective.* Pathol Oncol Res, 2003. **9**(3): p. 147-58.

114. Jin, H., et al., *p75 neurotrophin receptor inhibits invasion and metastasis of gastric cancer.* Mol Cancer Res, 2007. **5**(5): p. 423-33.

115. Jin, H., et al., *p75 neurotrophin receptor suppresses the proliferation of human gastric cancer cells.* Neoplasia, 2007. **9**(6): p. 471-8.

116. Johnston, A.L., et al., *The p75 neurotrophin receptor is a central regulator of glioma invasion.* PLoS Biol, 2007. **5**(8): p. e212.

117. Bugge, T.H., et al., *Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency.* Cell, 1996. **87**(4): p. 709-19.

118. Taub, R., *Liver regeneration: from myth to mechanism.* Nat Rev Mol Cell Biol, 2004. **5**(10): p. 836-47.

119. Akassoglou, K., et al., *Fibrin inhibits peripheral nerve regeneration by arresting Schwann cell differentiation.* Neuron, 2002. **33**: p. 861-875.

120. Imokawa, S., et al., *Tissue factor expression and fibrin deposition in the lungs of patients with idiopathic pulmonary fibrosis and systemic sclerosis.* Am J Respir Crit Care Med, 1997. **156**(2 Pt 1): p. 631-6.

121. Couser, W.G., *Glomerulonephritis.* Lancet, 1999. **353**(9163): p. 1509-15.

122. Valenzuela, R., et al., *Immunoelectrophoretic and immunohistochemical characterizations of fibrinogen derivatives in atherosclerotic aortic intimas and vascular prosthesis pseudo-intimas.* Am J Pathol, 1992. **141**(4): p. 861-80.

123. Rybarczyk, B.J. and P.J. Simpson-Haidaris, *Fibrinogen assembly, secretion, and deposition into extracellular matrix by MCF-7 human breast carcinoma cells.* Cancer Res, 2000. **60**(7): p. 2033-9.

124. Adams, R.A., et al., *The fibrin-derived gamma377-395 peptide inhibits microglia activation and suppresses relapsing paralysis in central nervous system autoimmune disease.* J Exp Med, 2007. **204**(3): p. 571-82.

125. Chang, A., et al., *NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions.* J Neurosci, 2000. **20**(17): p. 6404-12.

126. Idell, S., *Coagulation, fibrinolysis, and fibrin deposition in acute lung injury.* Crit Care Med, 2003. **31**(4 Suppl): p. S213-20.

127. Adams, R.A., et al., *Fibrin mechanisms and functions in nervous system pathology.* Mol Interv, 2004. **4**(3): p. 163-176.

128. Dowling, P., et al., *Up-regulated p75NTR neurotrophin receptor on glial cells in MS plaques.* Neurology, 1999. **53**(8): p. 1676-82.

129. Park, J.A., et al., *Co-induction of p75NTR and p75NTR-associated death executor in neurons after zinc exposure in cortical culture or transient ischemia in the rat.* J Neurosci, 2000. **20**(24): p. 9096-103.

130. Taniuchi, M., H.B. Clark, and E.M. Johnson, Jr., *Induction of nerve growth factor receptor in Schwann cells after axotomy.* Proc Natl Acad Sci U S A, 1986. **83**(11): p. 4094-8.

131. Herrmann, J.L., et al., *Mediation of NGF-stimulated extracellular matrix invasion by the human melanoma low-affinity p75 neurotrophin receptor: melanoma p75 functions independently of trkA.* Mol Biol Cell, 1993. **4**(11): p. 1205-16.

132. Wang, S., et al., *p75(NTR) mediates neurotrophin-induced apoptosis of vascular smooth muscle cells.* Am J Pathol, 2000. **157**(4): p. 1247-58.

133. Schachtrup, C., et al., *Fibrinogen inhibits neurite outgrowth via beta 3 integrin-mediated phosphorylation of the EGF receptor.* Proc Natl Acad Sci U S A, 2007. **104**(28): p. 11814-9.

134. Lijnen, H.R., *Elements of the fibrinolytic system.* Ann N Y Acad Sci, 2001. **936**: p. 226-36.

135. Werb, Z., et al., *Endogenous activiation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator.* N Engl J Med, 1977. **296**(18): p. 1017-23.

136. Saksela, O. and D.B. Rifkin, *Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity.* J Cell Biol, 1990. **110**(3): p. 767-75.

137. Horbach, D.A., et al., *Beta2-glycoprotein I is proteolytically cleaved in vivo upon activation of fibrinolysis.* Thromb Haemost, 1999. **81**(1): p. 87-95.

138. Vassalli, J.D., A.P. Sappino, and D. Belin, *The plasminogen activator/plasmin system.* J Clin Invest, 1991. **88**(4): p. 1067-72.

139. Akassoglou, K., et al., *Tissue plasminogen activator-mediated fibrinolysis protects against axonal degeneration and demyelination after sciatic nerve injury.* J Cell Biol, 2000. **149**(5): p. 1157-66.

140. Siconolfi, L.B. and N.W. Seeds, *Mice lacking tPA, uPA, or plasminogen genes showed delayed functional recovery after sciatic nerve crush.* J Neurosci, 2001. **21**(12): p. 4348-55.

141. Degen, J.L., et al., *Genetic manipulation of fibrinogen and fibrinolysis in mice.* Ann N Y Acad Sci, 2001. **936**: p. 276-90.

142. Gay, F.W., et al., *The application of multifactorial cluster analysis in the staging of plaques in early multiple sclerosis. Identification and characterization of the primary demyelinating lesion.* Brain, 1997. **120 (Pt 8)**: p. 1461-83.

143. Ricci, A., et al., *Neurotrophin and Neurotrophin Receptor Protein Expression in the Human Lung.* Am. J. Respir. Cell Mol. Biol., 2004. **30**(1): p. 12-19.

144. Donovan, M.J., et al., *Identification of the neurotrophin receptors p75 and trk in a series of Wilms' tumors.* Am J Pathol, 1994. **145**(4): p. 792-801.

145. Carmeliet, P., et al., *Physiological consequences of loss of plasminogen activator gene function in mice.* Nature, 1994. **368**(6470): p. 419-24.

146. Sappino, A.P., et al., *Extracellular proteolysis in the adult murine brain.* J Clin Invest, 1993. **92**(2): p. 679-85.

147. Ling, Q., et al., *Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo.* J Clin Invest, 2004. **113**(1): p. 38-48.

148. Khursigara, G., J.R. Orlinick, and M.V. Chao, *Association of the p75 neurotrophin receptor with TRAF6.* J Biol Chem, 1999. **274**(5): p. 2597-600.

149. Yamamoto, K. and D.J. Loskutoff, *Fibrin deposition in tissues from endotoxin-treated mice correlates with decreases in the expression of urokinase-type but not tissue-type plasminogen activator.* J Clin Invest, 1996. **97**(11): p. 2440-51.

150. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

151. Lansink, M., et al., *Effect of steroid hormones and retinoids on the formation of capillary- like tubular structures of human microvascular endothelial cells in fibrin matrices is related to urokinase expression.* Blood, 1998. **92**(3): p. 927-38.

152. Syroid, D.E., et al., *Induction of postnatal schwann cell death by the low-affinity neurotrophin receptor in vitro and after axotomy.* J Neurosci, 2000. **20**(15): p. 5741-7.

153. Michie, A.M., et al., *Rapid regulation of PDE-2 and PDE-4 cyclic AMP phosphodiesterase activity following ligation of the T cell antigen receptor on thymocytes: Analysis using the selective inhibitors erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and rolipram.* Cellular Signalling, 1996. **8**(2): p. 97-110.

154. Beavo, J.A. and D.H. Reifsnyder, *Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors.* Trends in Pharmacological Sciences, 1990. **11**(4): p. 150-155.

155. Zhang, J., et al., *Insulin disrupts beta-adrenergic signalling to protein kinase A in adipocytes.* Nature, 2005. **437**(7058): p. 569-73.

156. Griesbeck, O., et al., *Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications.* J Biol Chem, 2001. **276**(31): p. 29188-94.

157. McPhee, I., et al., *Association with the SRC family tyrosyl kinase LYN triggers a conformational change in the catalytic region of human cAMP-specific phosphodiesterase HSPDE4A4B. Consequences for rolipram inhibition.* J Biol Chem, 1999. **274**(17): p. 11796-810.

158. Allen, M.D. and J. Zhang, *Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters.* Biochem Biophys Res Commun, 2006. **348**(2): p. 716-21.

159. Frank, R., *The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications.* J Immunol Methods, 2002. **267**(1): p. 13-26.

160. Khursigara, G., et al., *A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2.* J Neurosci, 2001. **21**(16): p. 5854-63.

161. Bolger, G.B., et al., *Scanning peptide array analyses identify overlapping binding sites for the signalling scaffold proteins, beta-arrestin and RACK1, in cAMP-specific phosphodiesterase PDE4D5.* Biochem J, 2006. **398**(1): p. 23-36.

162. Bolger, G.B., et al., *The Unique Amino-terminal Region of the PDE4D5 cAMP Phosphodiesterase Isoform Confers Preferential Interaction with {beta}-Arrestins.* J. Biol. Chem., 2003. **278**(49): p. 49230-49238.

163. Chen, D., et al., *Inhibition of intravascular thrombosis in murine endotoxemia by targeted expression of hirudin and tissue factor pathway inhibitor analogs to activated endothelium.* Blood, 2004. **104**(5): p. 1344-9.

164. Seasholtz, T.M., et al., *Rho-mediated cytoskeletal rearrangement in response to LPA is functionally antagonized by Rac1 and PIP2.* J Neurochem, 2004. **91**(2): p. 501-12.

165. Schwab, M.E. and P. Caroni, *Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro.* J Neurosci, 1988. **8**(7): p. 2381-93.

166. Cai, D., et al., *Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism.* Neuron, 1999. **22**(1): p. 89-101.

167. Zheng, B., et al., *Lack of enhanced spinal regeneration in Nogo-deficient mice.* Neuron, 2003. **38**(2): p. 213-24.

168. Niederost, B.P., et al., *Bovine CNS myelin contains neurite growth-inhibitory activity associated with chondroitin sulfate proteoglycans.* J Neurosci, 1999. **19**(20): p. 8979-89.

169. Akassoglou, K., et al., *Fibrin is a regulator of Schwann cell migration after sciatic nerve injury in mice.* Neurosci Lett, 2003. **338**(3): p. 185-8.

170. Yamauchi, J., J.R. Chan, and E.M. Shooter, *Neurotrophins regulate Schwann cell migration by activating divergent signaling pathways dependent on Rho GTPases.* Proc Natl Acad Sci U S A, 2004.

171. Bentley, C.A. and K.F. Lee, *p75 is important for axon growth and schwann cell migration during development.* J Neurosci, 2000. **20**(20): p. 7706-15.

172. Lemke, G. and M. Chao, *Axons regulate Schwann cell expression of the major myelin and NGF receptor genes.* Development, 1988. **102**(3): p. 499-504.

173. Zorick, T.S. and G. Lemke, *Schwann cell differentiation.* Curr Opin Cell Biol, 1996. **8**(6): p. 870-6.

174. Hsu, K.C. and M.V. Chao, *Differential expression and ligand binding properties of tumor necrosis factor receptor chimeric mutants.* J Biol Chem, 1993. **268**(22): p. 16430-6.

175. Qian, Z., et al., *Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation.* Nature, 1993. **361**(6411): p. 453-7.

176. Huber, K., *Plasminogen activator inhibitor type-1 (part one): basic mechanisms, regulation, and role for thromboembolic disease.* J Thromb Thrombolysis, 2001. **11**(3): p. 183-93.

177. Medcalf, R.L., M. Ruegg, and W.D. Schleuning, *A DNA motif related to the cAMP-responsive element and an exon-located activator protein-2 binding site in the human tissue-type plasminogen activator gene promoter cooperate in basal expression and convey activation by phorbol ester and cAMP.* J Biol Chem, 1990. **265**(24): p. 14618-26.

178. Santell, L. and E. Levin, *Cyclic AMP potentiates phorbol ester stimulation of tissue plasminogen activator release and inhibits secretion of plasminogen activator inhibitor-1 from human endothelial cells.* J. Biol. Chem., 1988. **263**(32): p. 16802-16808.

179. Fiumelli, H., et al., *BDNF stimulates expression, activity and release of tissue-type plasminogen activator in mouse cortical neurons.* Eur J Neurosci, 1999. **11**(5): p. 1639-1646.

180. Gao, Y., et al., *Neurotrophins elevate cAMP to reach a threshold required to overcome inhibition by MAG through extracellular signal-regulated kinase-dependent inhibition of phosphodiesterase.* J Neurosci, 2003. **23**(37): p. 11770-7.

181. Bender, A.T. and J.A. Beavo, *Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use.* Pharmacol Rev, 2006. **58**(3): p. 488-520.

182. West, R.E., Jr., et al., *Pertussis toxin-catalyzed ADP-ribosylation of transducin. Cysteine 347 is the ADP-ribose acceptor site.* J Biol Chem, 1985. **260**(27): p. 14428-30.

183. Houslay, M.D., P. Schafer, and K.Y. Zhang, *Keynote review: phosphodiesterase-4 as a therapeutic target.* Drug Discov Today, 2005. **10**(22): p. 1503-19.

184. Brunton, L.L., *PDE4: arrested at the border.* Sci STKE, 2003. **2003**(204): p. PE44.

185. Walikonis, R.S. and J.F. Poduslo, *Activity of cyclic AMP phosphodiesterases and adenylyl cyclase in peripheral nerve after crush and permanent transection injuries.* J Biol Chem, 1998. **273**(15): p. 9070-7.

186. Khursigara, G., J.R. Orlinick, and M.V. Chao, *Association of the p75 Neurotrophin Receptor with TRAF6.* J. Biol. Chem., 1999. **274**(5): p. 2597-2600.

187. Bolger, G.B., et al., *Attenuation of the activity of the cAMP-specific phosphodiesterase PDE4A5 by interaction with the immunophilin XAP2.* J Biol Chem, 2003. **278**(35): p. 33351-63.

188. Bolger, G.B., et al., *The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with beta-arrestins.* J Biol Chem, 2003. **278**(49): p. 49230-8.

189. Barber, R., et al., *Differential expression of PDE4 cAMP phosphodiesterase isoforms in inflammatory cells of smokers with COPD, smokers without COPD, and nonsmokers.* Am J Physiol Lung Cell Mol Physiol, 2004. **287**(2): p. L332-43.

190. Savov, J.D., et al., *Fibrinolysis in LPS-induced chronic airway disease.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(4): p. L940-8.

191. Sklar, P.B., R.R. Anholt, and S.H. Snyder, *The odorant-sensitive adenylate cyclase of olfactory receptor cells. Differential stimulation by distinct classes of odorants.* J Biol Chem, 1986. **261**(33): p. 15538-43.

192. Frey, U., Y.Y. Huang, and E.R. Kandel, *Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons.* Science, 1993. **260**(5114): p. 1661-4.

193. Greengard, P., *The neurobiology of slow synaptic transmission.* Science, 2001. **294**(5544): p. 1024-30.

194. Tanis, K.Q. and R.S. Duman, *Intracellular signaling pathways pave roads to recovery for mood disorders.* Ann Med, 2007. **39**(7): p. 531-44.

195. Nestler, E.J., *Is there a common molecular pathway for addiction?* Nat Neurosci, 2005. **8**(11): p. 1445-9.

196. Spencer, T. and M.T. Filbin, *A role for cAMP in regeneration of the adult mammalian CNS.* J Anatomy, 2004. **204**(1): p. 49-55.

197. Menniti, F.S., W.S. Faraci, and C.J. Schmidt, *Phosphodiesterases in the CNS: targets for drug development.* Nat Rev Drug Discov, 2006. **5**(8): p. 660-70.

198. Montminy, M.R., et al., *Identification of a cyclic-AMP-responsive element within the rat somatostatin gene.* Proc Natl Acad Sci U S A, 1986. **83**(18): p. 6682-6.

199. Cai, D., et al., *Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro.* Neuron, 2002. **35**(4): p. 711-9.

200. Bergamaschi, A., M. Corsi, and M.J. Garnier, *Synergistic effects of cAMP-dependent signalling pathways and IL-1 on IL-6 production by H19-7/IGF-IR neuronal cells.* Cell Signal, 2006. **18**(10): p. 1679-84.

201. Cao, Z., et al., *The cytokine interleukin-6 is sufficient but not necessary to mimic the peripheral conditioning lesion effect on axonal growth.* J Neurosci, 2006. **26**(20): p. 5565-73.

202. Tao, X., et al., *Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism.* Neuron, 1998. **20**(4): p. 709-26.

203. Sachs, B.D., et al., *p75 neurotrophin receptor regulates tissue fibrosis through inhibition of plasminogen activation via a PDE4/cAMP/PKA pathway.* J Cell Biol, 2007. **177**(6): p. 1119-32.

204. Cai, D., et al., *Neuronal Cyclic AMP Controls the Developmental Loss in Ability of Axons to Regenerate.* J. Neurosci., 2001. **21**(13): p. 4731-4739.

205. Nikulina, E., et al., *The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery.* Proc Natl Acad Sci U S A, 2004. **101**(23): p. 8786-90.

206. Pearse, D.D., et al., *cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury.* Nat Med, 2004. **10**(6): p. 610-6.

207. Lu, P., et al., *Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury.* J Neurosci, 2004. **24**(28): p. 6402-9.

208. Teng, F.Y. and B.L. Tang, *Axonal regeneration in adult CNS neurons--signaling molecules and pathways.* J Neurochem, 2006. **96**(6): p. 1501-8.

209. McKerracher, L. and H. Higuchi, *Targeting Rho to stimulate repair after spinal cord injury.* J Neurotrauma, 2006. **23**(3-4): p. 309-17.

210. Laudanna, C., J.J. Campbell, and E.C. Butcher, *Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants.* J Biol Chem, 1997. **272**(39): p. 24141-4.

211. Tsirka, S.E., et al., *Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator.* Nature, 1995. **377**(6547): p. 340-4.

212. Samson, A.L. and R.L. Medcalf, *Tissue-type plasminogen activator: a multifaceted modulator of neurotransmission and synaptic plasticity.* Neuron, 2006. **50**(5): p. 673-8.

213. Baranes, D., et al., *Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway.* Neuron, 1998. **21**(4): p. 813-25.

214. Lyons, R.M., J. Keski-Oja, and H.L. Moses, *Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium.* J Cell Biol, 1988. **106**(5): p. 1659-65.

215. Indyk, J.A., et al., *Laminin chain expression suggests that laminin-10 is a major isoform in the mouse hippocampus and is degraded by the tissue plasminogen activator/plasmin protease cascade during excitotoxic injury.* Neuroscience, 2003. **116**(2): p. 359-71.

216. Wu, Y.P., et al., *The tissue plasminogen activator (tPA)/plasmin extracellular proteolytic system regulates seizure-induced hippocampal mossy fiber outgrowth through a proteoglycan substrate.* J Cell Biol, 2000. **148**(6): p. 1295-304.

217. Pang, P.T., et al., *Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity.* Science, 2004. **306**(5695): p. 487-91.

218. Rogove, A.D., et al., *Activation of microglia reveals a non-proteolytic cytokine function for tissue plasminogen activator in the central nervous system.* J Cell Sci, 1999. **112 (Pt 22)**: p. 4007-16.

219. Teng, K.K. and B.L. Hempstead, *Neurotrophins and their receptors: signaling trios in complex biological systems.* Cell Mol Life Sci, 2004. **61**(1): p. 35-48.

220. Baillie, G.S., J.D. Scott, and M.D. Houslay, *Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract.* FEBS Lett, 2005. **579**(15): p. 3264-70.

221. Perry, S.J., et al., *Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins.* Science, 2002. **298**(5594): p. 834-6.

222. Sands, W.A. and T.M. Palmer, *Regulating gene transcription in response to cyclic AMP elevation.* Cell Signal, 2008. **20**(3): p. 460-6.

223. Arnsten, A.F., et al., *Protein kinase A as a therapeutic target for memory disorders: rationale and challenges.* Trends Mol Med, 2005. **11**(3): p. 121-8.

224. Greengard, P., P.B. Allen, and A.C. Nairn, *Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade.* Neuron, 1999. **23**(3): p. 435-47.

225. Menegon, A., et al., *Protein kinase A-mediated synapsin I phosphorylation is a central modulator of Ca2+-dependent synaptic activity.* J Neurosci, 2006. **26**(45): p. 11670-81.

226. Liu, S.J., et al., *Tau becomes a more favorable substrate for GSK-3 when it is prephosphorylated by PKA in rat brain.* J Biol Chem, 2004. **279**(48): p. 50078-88.

227. Epstein, D.J., et al., *Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway.* Development, 1996. **122**(9): p. 2885-94.

228. Waschek, J.A., et al., *Hedgehog signaling: new targets for GPCRs coupled to cAMP and protein kinase A.* Ann N Y Acad Sci, 2006. **1070**: p. 120-8.

229. Higuchi, H., et al., *PKA phosphorylates the p75 receptor and regulates its localization to lipid rafts.* Embo J, 2003. **22**(8): p. 1790-800.

230. Baron, P., et al., *Immunocytochemical expression of human muscle cell p75 neurotrophin receptor is down-regulated by cyclic adenosine 3',5'-monophosphate.* Neurosci Lett, 1997. **234**(2-3): p. 79-82.

231. Chen, S., J. Wang, and S.A. Siegelbaum, *Properties of hyperpolarization-activated pacemaker current defined by coassembly of HCN1 and HCN2 subunits and basal modulation by cyclic nucleotide.* J Gen Physiol, 2001. **117**(5): p. 491-504.

232. Holz, G.G., et al., *Cell physiology of cAMP sensor Epac.* J Physiol, 2006. **577**(Pt 1): p. 5-15.

233. Petersen, R.K., et al., *cAMP-mediated stimulation of adipocyte differentiation requires the synergistic action of Epac- and PKA-dependent processes.* Mol Cell Biol, 2008.

234. Monaghan, T.K., et al., *PACAP-38 induces neuronal differentiation of human SH-SY5Y neuroblastoma cells via cAMP-mediated activation of ERK and p38 MAP kinases.* J Neurochem, 2008. **104**(1): p. 74-88.

235. Yokoyama, U., et al., *The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals.* Proc Natl Acad Sci U S A, 2008. **105**(17): p. 6386-91.

236. Dwivedi, Y., et al., *Differential and brain region-specific regulation of Rap-1 and Epac in depressed suicide victims.* Arch Gen Psychiatry, 2006. **63**(6): p. 639-48.

237. Kawasaki, T., et al., *Vascular release of plasminogen activator inhibitor-1 impairs fibrinolysis during acute arterial thrombosis in mice.* Blood, 2000. **96**(1): p. 153-60.

238. Lijnen, H.R., et al., *Function of the plasminogen/plasmin and matrix metalloproteinase systems after vascular injury in mice with targeted inactivation of fibrinolytic system genes.* Arterioscler Thromb Vasc Biol, 1998. **18**(7): p. 1035-45.

239. Cherry, J.A. and R.L. Davis, *Cyclic AMP phosphodiesterases are localized in regions of the mouse brain associated with reinforcement, movement, and affect.* The Journal of Comparative Neurology, 1999. **407**(2): p. 287-301.

240. Filbin, M.T., *Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS.* Nat Rev Neurosci, 2003. **4**(9): p. 703-713.

241. M. Xu, C.V.R., B. D. Sachs, A. Makino, O. Platoshyn, W. H. Dillmann, K. Akassoglou, and J. X.-J. Yuan., *p75 Neurotrophin Receptor Regulates Agonist-induced Pulmonary Vasoconstriction.* Am J Physiol Heart Circ Physiol., submitted.

242. Okumura, T., et al., *The biological role of the low-affinity p75 neurotrophin receptor in esophageal squamous cell carcinoma.* Clin Cancer Res, 2006. **12**(17): p. 5096-103.

243. Higuchi, H., et al., *Functional inhibition of the p75 receptor using a small interfering RNA.* Biochem Biophys Res Commun, 2003. **301**(3): p. 804-9.

244. Botchkarev, V.A., et al., *p75 Neurotrophin receptor antagonist retards apoptosis-driven hair follicle involution (catagen).* J Invest Dermatol, 2003. **120**(1): p. 168-9.

245. Weskamp, G. and L.F. Reichardt, *Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors.* Neuron, 1991. **6**(4): p. 649-63.

246. Saez, E.T., et al., *Production of nerve growth factor by beta-amyloid-stimulated astrocytes induces p75NTR-dependent tau hyperphosphorylation in cultured hippocampal neurons.* J Neurosci Res, 2006. **84**(5): p. 1098-106.

247. Massa, S.M., et al., *Small, nonpeptide p75NTR ligands induce survival signaling and inhibit proNGF-induced death.* J Neurosci, 2006. **26**(20): p. 5288-300.

248. Gretarsdottir, S., et al., *The gene encoding phosphodiesterase 4D confers risk of ischemic stroke.* Nat Genet, 2003. **35**(2): p. 131-8.

249. Lehnart, S.E., et al., *Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias.* Cell, 2005. **123**(1): p. 25-35.

250. Millar, J.K., et al., *DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling.* Science, 2005. **310**(5751): p. 1187-91.

251. Dimou, L., et al., *Nogo-A-deficient mice reveal strain-dependent differences in axonal regeneration.* J Neurosci, 2006. **26**(21): p. 5591-603.

252. Rittirsch, D., L.M. Hoesel, and P.A. Ward, *The disconnect between animal models of sepsis and human sepsis.* J Leukoc Biol, 2007. **81**(1): p. 137-43.