DISTINCT ROLES OF P75 REGULATION ON MYELINATION IN THE PERIPHERAL NERVOUS SYSTEM AND CENTRAL NERVOUS SYSTEM

DISSERTATION

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ABSTRACT

Induction of p75 expression after trauma and resulting pathology in the adult nervous system makes this receptor an attractive target for therapeutic intervention. The ability of p75 to induce often opposite phenotypes, such as promoting cell survival and apoptosis, however, renders it a challenge. It thus becomes necessary to obtain a comprehensive picture of p75 signaling mechanisms, so as to prevent activation (or inactivation) of untargeted pathways and their potentially deleterious side effects. Here, we examined the mechanism by which p75 induces apoptosis in myelinating oligodendrocytes of the mature central nervous system after traumatic spinal cord injury. We identified the role of neuronal specific JNK3 stress-activated protein kinase in initiation of the mitochondrial death pathway specifically among oligodendrocytes after injury.

Our next goal was to disrupt the pathways that contribute to oligodendrocyte apoptosis by blocking injury specific proNGF binding to p75. We implemented this goal with a nontoxic and minimally invasive non-peptide small molecule that was able to cross the blood-brain-barrier. We observed functional recovery among specific behavioral parameters that correlate with sparing of ventro-lateral descending tracts, suggesting that this compound is potentially applicable as a therapeutic treatment.
Lastly, we examined the role of p75 in the peripheral nervous system during development, and elucidate the mechanism by which p75 promotes myelination. We identified a novel co-receptor complex between p75 and the ErbB2 receptor that formed in response to BDNF. BDNF has been classically defined as a ligand for p75 and TrkB receptors, but we report that ErbB2 is activated in response to BDNF. Collectively, the diverse roles of p75 in governing myelination in PNS development and apoptosis in CNS adult injury further uncovers the complexity of this receptor in context-dependent situations.
ACKNOWLEDGEMENTS

Out of the numerous traits that I respect in my advisor, it is her sense of persistence and patience that I admire the most. It is the possession of these traits that I believe not only makes her a brilliant scientist, but also a quality mentor. She has been remarkably patient with me during my scientific training while working on improving my weaknesses. At the same time, she has always been able to identify my strengths, even when I did not recognize them myself. I am forever indebted to her for her guidance and advice while fostering my personal scientific growth.

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Major Field: Ohio State Biochemistry Program
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
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<td>BBB</td>
<td>blood brain barrier</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BMS</td>
<td>Basso Mouse Scale</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>CC1</td>
<td>mast cell chymase antibody</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CSPG</td>
<td>chondroitin sulfate proteoglycan</td>
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<tr>
<td>cytC</td>
<td>cytochrome c</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>days post-injury</td>
</tr>
<tr>
<td>EC</td>
<td>eriochrome cyanine</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>Term</td>
<td>Description</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
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<td>JNK</td>
<td>c-jun N-terminal kinase</td>
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<tr>
<td>MAP-K</td>
<td>mitogen activated protein (kinase)</td>
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<tr>
<td>μ-</td>
<td>micro</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>Mcl-1</td>
<td>myeloid leukemia cell differentiation protein</td>
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<tr>
<td>mg/Kg</td>
<td>milligrams per Kilograms</td>
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<tr>
<td>NeuN</td>
<td>neuronal nuclei</td>
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<td>NF-kB</td>
<td>nuclear factor kappa B</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<td>NRG</td>
<td>neuregulin</td>
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<td>NT3/4</td>
<td>neurotrophin 3/4</td>
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<td>OCT</td>
<td>optimal cutting temperature</td>
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<td>PAK</td>
<td>p21-activated kinase</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDZ</td>
<td>post synaptic density protein (PSD-95), drosophila disc large tumor suppressor (DlgA), zonula occludens-1 protein (ZO-1)</td>
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<tr>
<td>Pin1</td>
<td>peptidyl-prolyl cis/trans isomerase-1</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>Pro</td>
<td>proline</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<td>RT-PCR</td>
<td>reverse transcription PCR</td>
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<tr>
<td>SCI</td>
<td>spinal cord injury</td>
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<tr>
<td>Ser</td>
<td>serine</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>TB</td>
<td>tris buffer</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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INTRODUCTION

By interacting with different type of receptors in response to different ligands, p75 is able to promote a variety of cellular functions, including survival, apoptosis and facilitation or inhibition of axonal growth. Because p75 has no intrinsic enzymatic function, p75 signals by recruiting intracellular binding proteins to mediate specific functions (Cragnolini et al., 2008; Hempstead, 2002). Mature neurotrophins bind preferentially to both Tropomyosin-related kinase (Trk) and p75 receptors to facilitate neuronal survival and differentiation (DeFreitas et al., 2001; Roux et al., 2001). Upon binding precursor forms of neurotrophins in the presence of co-receptor, sortilin, p75 regulates pro-apoptotic signaling (Nykjaer et al., 2004; Salehi et al., 2000; Teng et al., 2005). In response to myelin inhibitory factors, p75 functions as a component of the Nogo receptor signaling complex to block axonal growth (Wang et al., 2002; Wong et al., 2002). Although p75 is not widely expressed in adult neuronal tissues, its expression is induced after injuries and under pathologic conditions. p75 is also abundantly expressed in neuronal and glial cells during development (Cragnolini et al., 2008). Because of these expression profiles, p75 function has mostly been described in injury models and during development. Here, we delineate the diversity of p75 function as a positive modulator of myelination during development in the peripheral nervous system (PNS) and as a mediator of apoptosis after spinal cord injury in the adult central nervous system (CNS).
I. *p75 contributes to oligodendrocyte loss in the adult spinal cord after injury*

In addition to functioning as a center for reflex coordination, the spinal cord functions as a channel for motor information, which travels down the spinal cord to muscular effectors. It also conducts sensory information from the peripheral nervous system (PNS), which travels up the spinal cord to the brain (Afifi and Bergman, 45). Injury to the spinal cord impairs both pathways and results in a loss of function caudal to the injury site. The repercussions of spinal cord injury impact the quality of life of the affected individual in many ways. The chief grievances are pain, loss of bowel/bladder function, sexual function, and limitations in hand usage and breathing; the desire to walk is secondary to the desire to regain function in these basic bodily functions (McDonald et al., 2002; Rossignol et al., 2007; Rowland et al., 2008; Siddall et al., 2003). The extent of loss of function in these areas is dependent upon the severity of injury, as well as the level of the spinal cord at which the damage occurs. Although initial trauma activates several molecular and cellular changes that occur within minutes to days, experimental studies have established that much of the post-traumatic tissue damage and correlative neurological defects are the result of secondary reactive processes that can occur within days to months (Genovese et al., 2008; Rowland et al., 2008; Schwab and Bartholdi, 1996). Identifying the mechanisms behind these secondary processes is an attractive target for therapeutic intervention.

Spinal cord injury can be categorized into two phases: primary injury and secondary injury. During the primary phase of injury, the spinal cord undergoes swelling and hemorrhaging due to vascular disruption, which can lead to cord ischemia that can occur both rostral and caudal to the injury. Displaced bone and ligament fragments can
damage the cord by exerting force on the tissue, resulting in damaged blood vessels, disrupted axons and sheared neural membranes (McDonald et al., 2002; Rowland et al., 2008; Schwab and Bartholdi, 1996). Severed axons, necrotic neurons and glia can result in loss of function at and below the site of injury. Axons that are not initially sheared by necrotic trauma occupy a “subpial rim” of spared tissue and can impart neurological function by traversing the lesion site (Rowland et al., 2008). Other pathophysiological processes also commence, including activation of microglial cells, upregulation of proinflammatory cytokines (TNF-α and IL-β) and the release of extracellular glutamate (Rowland et al., 2008).

In the early acute phase of secondary injury mechanisms (up to two days after injury), edematous swelling causes the cord to occupy the entirety of the spinal canal, causing further secondary ischemia (Lee et al., 2005; Rowland et al., 2008; Saadoun et al., 2008). Hemorrhage in the gray matter also spreads both radially and axially and cause spinal neurogenic shock, leading to systemic hypotension and exacerbation of ischemia (McDonald et al., 2002; Rowland et al., 2008). Reactive astrocytes become hypertrophic and proliferative during the subacute phase of secondary injury. These reactive astrocytes then accumulate at the margin of the lesion and build up a dense network of processes to form a gliotic scar between the spared tissue and the wound, forming both a physical and chemical barrier to axon outgrowth (Rowland et al., 2008; Schwab and Bartholdi, 1996). Despite forming a physical and chemical barrier to axon outgrowth, the astrocytes themselves are also able to promote the re-establishment of ionic homeostasis and the integrity of the blood-brain-barrier (Hermann et al., 2008). During the chronic phase of secondary injury, which occurs later than 6 months post
lesion, the lesion is stabilized, as there is continued scar formation and development of
cysts. Wallerian degeneration of injured or severed axons still continues over time,
resulting in further neurological impairment well beyond the initial time of injury
(Rowland et al., 2008).

Many cells die during the primary and secondary injury phases. The cell death
that occurs during the primary phase is mainly necrotic, caused by excitotoxic insults,
lipid peroxidation and free radical damage that occur within the first 24 hours after the
initial trauma. Cell death that occurs during the secondary phase appears to be mainly
apoptotic, occurring in intact axons and the rim of spared white matter distant from the
origin of the initial lesion for an extended period of time (Beattie et al., 2002; Crowe et
al., 1997; Liu et al., 1997). Continued loss of myelin in the preserved outer rim of white
matter can be potentially damaging, because some neurological function is still enabled
with fewer than 10% of long-tract spared connections intact (McDonald et al., 2002;
Rosenberg et al., 1997). Apoptosis among the myelin-producing oligodendrocytes in
distant rostral and caudal tracts of the white matter can induce degeneration of spared
axons days and weeks after the initial injury as a part of the process of Wallerian
degeneration (Beattie et al., 2002; Crowe et al., 1997; Liu et al., 1997; Park et al., 2004;
Schwab et al., 1996). Because there is a relatively slow development of pathology in the
white matter as compared to gray matter, this allows a greater window of opportunity for
treatment (Rosenberg et al., 1997). For instance, myelin formation of intact but naked
connections can be reinitiated by actively promoting remyelination by surviving
oligodendrocytes or directly replacing lost oligodendrocytes. This can be a sensible
approach, since more focused restoration may yield some control for bowel and bladder
movement, hand function, breathing and pain as small anatomical gains can bring about disproportionate functional benefits (McDonald et al., 2002). Obviously, complete rebuilding of the damaged spinal cord is not possible, but targeted restoration of a particular circuit based on remyelination strategy can potentially deliver greater benefit to the patient (Schwab and Bartholdi, 1996).

**Targeting apoptosis of oligodendrocytes**

Upon analyzing the pattern of apoptotic death of oligodendrocytes in rats and monkeys, the Beattie group observed a rostro-caudal distribution of apoptotic cells and degenerating axons (Crowe et al., 1997). In rostral regions of the injury site, they found that axonal degeneration was typically associated with apoptosis of oligodendrocytes in the ascending tracts, rather than descending pathways. In caudal regions, on the other hand, descending tracts were mainly degenerating and apoptotic oligodendrocytes were again associated with these tracts. These results suggest that oligodendrocytes were undergoing apoptosis in response to degeneration of the damaged axons (Crowe et al., 1997). The authors proposed two hypotheses for the cause of oligodendrocyte apoptosis. One is the result of loss of axonal support, where there is withdrawal of trophic factors that normally sustained oligodendrocyte survival. The other is apoptosis being induced as a consequence of cytokines and blood-borne elements released in the inflammatory response (Shuman et al., 1997).

In experimental models of injury, impact is often delivered on the dorsal surface of the cord, leaving a mixture of injured and spared axons in the ventral and lateral funiculus. Because one oligodendrocyte is responsible for myelinating several axons,
oligodendrocyte apoptosis is considered to further augment loss of spared axons in regions with a mixture of dying and surviving axons in this region of the spinal cord. Myelin loss also has serious implication in functional recovery after injury. In most areas of the white matter, the fiber tracts of both ascending and descending pathways are intermixed, so that oligodendrocytes have the capacity to myelinate axons from both pathways (Shuman et al., 1997). If oligodendrocytes are indeed dependent on axons for trophic support, degeneration in one sensorimotor tract may aggravate deleterious effects on its corresponding oligodendrocyte, which in turn fail to provide myelinating support to other tracts.

**Mechanism of oligodendrocyte apoptosis: Immune response and inflammation**

After physical trauma to the spinal cord, the body responds by initiating complex immunological events that are not only implicated in mediating secondary pathogenesis after injury, but may also contribute to tissue repair (Barrette et al., 2008; Genovese et al., 2008; Popovich et al., 1997, 1999; Schwab and Bartholdi, 1996). The first group of cells that respond to CNS trauma is resident microglia of the spinal cord. Upon activation, microglia assume a rounded, phagocytic morphology and secrete pro-inflammatory cytokines (Schwab and Bartholdi, 1996; Popovich and Hickey, 2001; Shuman et al., 1997). These cytokines further facilitate leukocyte recruitment by increasing synthesis of adhesion molecules (Ma et al., 2002). Peripheral inflammatory cells invade the site of injury in two stages: an early invasion by neutrophils and a later recruitment of phagocytic macrophages (with some lymphocytes at even later time points) (Dusart and Schwab, 1994). The neutrophils function to prevent infection by destroying bacteria that
may have been introduced upon lesion. By releasing lysosomal enzymes and producing oxygen radicals in the process, however, they have the potential to further damage tissue and contribute to secondary injury. As soon as three days after injury, hematogenous monocytes from the periphery invade the lesion site and rapidly differentiate into tissue macrophages, and assume a similar morphology to that of activated microglia residing in the spinal cord (Popovich and Hickey, 2001). Similarly to neutrophils, recruited macrophages function to eradicate pathogenic organisms. Macrophages also facilitate custodial duties in tissue repair and homeostasis, and remove apoptotic neutrophils from the lesion site. The rate at which macrophages invade the CNS is much slower than in the PNS, suggesting that degenerating myelin is removed to a much greater extent in the PNS than the CNS. Unfortunately, nitrogen/oxygen metabolites, cytokines and proteases are released by macrophages during repair processes, which may augment the secondary injury response further and damage surviving cells, making it even more difficult for CNS neurons to grow (Noble et al., 2002; Popovich, 2000; Scholz et al., 2007).

The role of inflammation at the injury site is controversial, as it is unclear whether reducing inflammation to limit the degree of bystander damage may conflict with the need to permit inflammation in order to promote tissue repair and regeneration. Reports detailing blocking leukocyte and neutrophil function with antibodies against membrane adhesion glycoproteins are conflicting, in that ischemia and neurologic defects in the spinal cord were either reduced, or unaffected (Clark et al., 1991; Forbes et al., 1994). Published reports have demonstrated that diminishing the accumulation of peripheral macrophages at the lesion site decreased tissue damage and promoted recovery (Popovich et al., 1999). But others have shown impaired peripheral recovery upon macrophage
depletion (Barrette et al., 2008). This duality observed in macrophages is due to upregulation of a pro-inflammatory subset of macrophages induced by lesion-associated factors, which offsets the ratio of subsets of anti-inflammatory macrophages that promote growth (Kigerl et al., 2009). Currently, the only approved pharmacologic therapeutic treatment for spinal cord injury is early administration of high-dose steroids (methylprednisolone) which is utilized to reduce post-traumatic inflammatory response. This therapeutic agent reduces infiltration of monocytes into the lesion site, and limits production of pro-inflammatory cytokines and mediators (Bartholdi and Schwab, 1995; Bracken et al., 1992; Oudega et al., 1999). However, there has been much debate over application of methylprednisolone and adverse side effects (Schwab and Bartholdi, 1996). Complications in patients receiving methylprednisolone were urinary tract infections, pneumonia, and higher incidence of wound infection and gastrointestinal hemorrhage (Schwab and Bartholdi, 1996).

To address whether elements of the immune response were responsible for mediating oligodendrocyte cell death after injury, the expression profiles of microglial activation was analyzed. Microglial activation occurred in a pattern similar to that of apoptotic oligodendrocytes in the spinal cord after injury (Shuman et al., 1997). Morphology of the microglia in the ascending fiber tracts rostral to the lesion exhibited short, thickened processes, indicative of an active state. Microglia was also observed to be active in the descending tracts caudal to the lesion, where degeneration and apoptosis was localized after injury to rats and monkeys (Crowe et al., 1997; Popovich et al., 1997; Shuman et al., 1997). It is unclear whether microglia activation is involved in causing apoptosis directly, although the microglial processes were in contact with apoptotic
oligodendrocytes in these injured tissues (Shuman et al., 1997). Cytokines released from microglia, such as TNF-α, have been reported to induce apoptosis of oligodendrocytes *in vitro* (McLaurin et al., 1995). Indeed, neutralizing antibodies that blocked TNF-α signaling have demonstrated functional recovery after injury (Bethea et al., 1999). However, knockout studies of TNF-α have demonstrated conflicting roles of this cytokine in vitro and after SCI, where TNF-α knockout mice had higher number of cells undergoing apoptosis, larger lesion size and aggravated functional recovery (Kim et al., 2001). These contradictory reports underline the need for further delineation of the benefits and detriments of the immune response after SCI.

**Mechanism of oligodendrocyte apoptosis: Excitotoxicity, phospholipid hydrolysis and formation of free radicals**

Excitatory amino acids, such as glutamate and aspartate, are normally secreted from the end tips of axons to function as neurotransmitters that bind receptors on target neurons, stimulating those cells to fire impulses. However, within minutes of injury to tissues, there is an excess of neurotransmitters released by axons and astrocytes, resulting in toxic over-excitation of neighboring neurons. The over-stimulated cells then depolarize, causing an influx of calcium ions that lead to production of free radicals, activation of proteases, caspases, phospholipases and endonucleases that can attack other cell components and healthy neurons (Genovese et al., 2008; McDonald et al., 2002; Schwab and Bartholdi, 1996). Because oligodendrocytes express the AMPA receptors for glutamate, they are also vulnerable to excitotoxicity, leading to hypoxic cell death and demyelination of spared axons (Liu et al., 2002; Matute et al., 1997; McDonald et al.,
In injury and disease models, white matter degeneration and neurological deficits were attenuated upon blocking AMPA receptors (Agrawal and Fehlings, 1997; Alberdi et al., 2006; Fern and Moller et al., 2000; Pitt et al., 2000; Wrathall et al., 1997), defining yet another therapeutic target to address after pathology occurs. Additionally, increased levels of glutamate can sensitize oligodendrocytes to complement attack in multiple sclerosis models, further compounding the detrimental effects of innate immune response (Alberdi et al., 2006). These complement attacks permeabilize the membrane of oligodendrocytes, allowing toxic levels of Ca++ to flood into the cell and induce apoptosis in a caspase-independent pathway (Alberdi et al., 2006). The mechanisms of glutamate toxicity and over-activation of respective receptors is yet another target that has a therapeutic potential.

Normally, regular metabolism generates reactive oxygen species that are contained by a natural antioxidant defense system, which includes superoxide dismutase, catalase, peroxidases and endogenous anti-oxidants. But under pathology, the production of ROS exceeds endogenous antioxidant capacity, causing oxidative stress and further damage to tissue from uncontained ROS (Genovese et al., 2008). Additionally, activation of calcium-dependent phospholipases can lead to phospholipid hydrolysis and release of free fatty acids (Schwab and Bartholdi, 1996). The accumulation of polyunsaturated fatty acids, including various metabolites of arachidonic acid, can increase levels of platelet-activating factors, which affect blood flow and inflammation and exacerbate the secondary damage response to injury (Schwab and Bartholdi, 1996). Metabolism of arachidonic acid, oxidative invading neutrophils and oxidative catecholamines all contribute to the formation of oxygen radicals after injury, which can damage the
components of biological membranes (Genovese et al., 2008). Therapeutic interventions aim to counteract the production of reactive oxygen species. They include administration of SOD mimetics, caspase inhibitors, and exogenous catalytic antioxidants that scavenge for free radicals (Genovese et al., 2008). But publications reporting the use of several SODs and catalase have produced mixed results, due to the fact that these compounds are very large, which limits cell permeability and circulation, precluding application as a therapeutic drug, at least until smaller compound mimetics are designed (Genovese et al., 2008; Sahawneh et al., 2010; Svensson et al., 1986).

**Mechanism of oligodendrocyte apoptosis: Fas receptor signaling pathways**

Oligodendrocytes have been reported to undergo apoptosis via death receptor mediated pathways. Two well known cell surface receptors that are involved in apoptosis include Fas receptor (CD95 or Apo1) and p75, which are members of the TNF-α (tumor necrosis factor) receptor superfamily (Ashkenazi and Dixit, 1998; Casha et al., 2001). Upon binding of cognate “death ligands” (Fas-L), the Fas receptor interacts with cytoplasmic FADD (Fas-associated death domain protein) adaptor through its intracellular death domain to activate caspase 8 and 10 cascades and initiate apoptosis (Casha et al., 2001). While there is limited expression of Fas in adult CNS tissue, the expression of Fas receptor has been localized to astrocytes, microglia and mostly oligodendrocytes. These are the cells exhibiting apoptotic profiles after injury or pathology, implicating Fas in apoptotic mechanisms (Casha et al., 2001, D’Souza et al., 1996). Fas-L expression in microglia and activated T-cells is upregulated between three and seven days after injury (Nagata and Golstein, 1995; Popovich et al., 1997). Fas
expression progressed and peaked at 7 days after injury, and ultimately decreased by 14 days after injury (Casha et al., 2001). In addition to improvements in axonal survival and myelin preservation, Fas-deficient mutant mice exhibited a decrease in apoptosis among oligodendrocytes selectively, suggesting that targeting Fas signaling is an effective means of decreasing secondary injury mechanisms after trauma (Casha et al., 2005). Because p75 expression still remained elevated in apoptotic oligodendrocytes past 7-14 days, this may be another effective target for limiting degeneration in spinal cord injury.

**Mechanism of oligodendrocyte apoptosis: p75 signaling pathways**

We aim to investigate the molecular signaling events that trigger oligodendrocyte apoptosis during Wallerian degeneration, in hopes of identifying and ultimately manipulating the intracellular and receptor signaling pathways that mediate death of oligodendrocytes. p75 expression in the adult brain is limited, but it is induced in neuronal and glial populations after injury (Fahnestock et al., 2001; Harrington et al., 2004; Lee et al., 2001; Beattie et al., 2002). Indeed, three days after contusion injury, we observed p75 expression localized to the fasciculus gracilis rostral to the lesion and among oligodendrocyte processes in the spared white matter after contusion injury (Figure 2.11). This localization of p75 is similar to the expression of apoptotic oligodendrocytes found in the ascending pathways rostral to the lesion as was observed in Crowe et al., 1997. We also detected p75 expression in motoneurons in the gray matter and among invading sensory fibers in the dorsal horn (Figure 2.11). Localization to areas of apoptotic cells after injury implicates p75 in its critical role in governing cell death.
Oligodendrocytes were initially reported to undergo p75 mediated apoptosis in vitro, mediated by RacGTPase activity, JNK phosphorylation and caspase activation (Cassacia-Bonnefil et al., 1996; Gu et al., 1999; Harrington et al., 2002; Yoon et al., 1998). This was later confirmed in vivo, when p75 was found to be necessary for oligodendrocyte apoptosis after spinal cord injury (Beattie et al., 2002; Casha et al., 2001). After injury, p75 was upregulated in the spinal cord, and most of the p75 positive cells were apoptotic oligodendrocytes (Beattie et al., 2002). In correlation with this, there was a decrease in activated Caspase 3 observed in oligodendrocytes in p75 null mice (Beattie et al., 2002). The precursor form of NGF, proNGF, was reported to bind to p75 and the sorting receptor, sortilin, in high affinity, inducing death of corticospinal neurons and oligodendrocytes (Fahnestock et al., 2001; Harrington et al., 2004; Lee et al., 2001; Beattie et al., 2002; Nykjaer et al., 2004). Because p75 is involved in oligodendrocyte cell death, we employed the use of a non-peptide small molecule in hope of blocking proNGF mediated apoptosis thereby preserving spared white matter that support damaged axons, as discussed in chapter two.

Early investigation into the mechanism of oligodendrocyte cell death after traumatic spinal cord injury implicated the stress-activated protein kinase pathways in transmission of apoptotic signaling among dying cells. These JNK pathways are activated by proinflammatory signals and stress in the nervous system (Shaulian and Karin, 2002). JNK and p38 activation was observed among apoptotic cells (Nakahara et al., 1999). As demonstrated in sympathetic and hippocampal neurons and PC12 cells, p75 mediated apoptosis correlated with increased JNK kinase activity and c-jun phosphorylation in oligodendrocytes (Bamji et al., 1998; Casaccia-Bonnefil et al., 1996;
Friedman, 2000; Roux et al., 2001; Yoon et al., 1998). Similarly, dominant-negative JNK and chemical inhibitors of the JNK pathway inhibited p75-mediated apoptosis (Friedman, 2000; Harrington et al., 2001). We then questioned the mechanism of the JNK family of proteins in apoptosis, and whether it plays a role in mitochondrial death pathways. Indeed, JNK was involved in cytochrome c release, Caspase 9 activation and subsequent apoptosis of sympathetic neurons upon NGF withdrawal or murine embryonic fibroblasts upon stress (Bruckner et al., 2001; Deshmukh et al., 1996; Harding et al., 2001, Tournier et al., 2000). In chapter one, we will address the role of a neuronal specific JNK isoforms, JNK3, in mediating apoptosis of oligodendrocytes via the mitochondrial death pathway after spinal cord injury. We will also demonstrate that targeting this pathway by blocking proNGF binding to p75 results in functional recovery after spinal cord injury.

II. p75 promotes myelination in the peripheral nervous system during development

Similar to p75 expression in the central nervous system, p75 is widely expressed in neurons and glia during PNS development and is upregulated in the myelinating Schwann cells after injury in the adult (Cragnolini et al., 2008). The process of myelination in the peripheral nervous system is a dynamic progression of regulated events consisting of three major phases: proliferation and migration of Schwann cells along the axon in the proliferative stage, elongation and ensheathment along the axon in the premyelinating stage, and ultimately initiation and upregulation of myelin protein in the myelination stage (Chan et al., 2001). Neurotrophins that are released by the axon play an important role in regulating the progression of these processes. The levels of
BDNF and p75 expression were down-regulated at the onset of myelination in the PNS, suggesting that p75 may be playing a role in the events prior to the myelinating stage. This is further supported by the observed impairment of Schwann cell migration and axon ensheathment in p75 null mice (Bentley and Lee, 2000; Cosgaya et al., 2002; Mirsky and Jessen, 1999). Later reports demonstrated the role of BDNF, acting via p75, in inhibiting NT3-mediated Schwann cell migration (Yamauchi et al., 2004). BDNF signaling from the axon was reported to promote myelination in the peripheral nervous system (Chan et al., 2001, Ng et al., 2007). Upon analysis of DRG neuron-Schwann cell co-cultures and sciatic nerves from p75 mice, p75 was identified as the receptor for BDNF mediated signaling in PNS myelination (Cosgaya et al., 2002). In correlation with this, the peripheral nerves from p75 null mice were hypo-myelinated and unresponsive to attempts to promote myelination via BDNF injection (Cosgaya et al., 2002). Inhibiting p75 function with blocking antibodies also resulted in hypo-myelination in sciatic nerves, further underlining the significance of p75 signaling in PNS myelination.

A notable feature of Schwann cell myelination is that there is constant cell to cell contact between the axon and the ensheathing Schwann cell during development. This suggests that there are intricate cell-to-cell communications that govern myelination process at the different stages of myelination. It was reported that DRG neurons secreted BDNF along the surface of the axon in an anterograde fashion to trigger the onset of myelination (Ng et al., 2007). In addition to BDNF, Schwann cells also encounter axonal neuregulin glial growth factors, which have been implicated in Schwann cell survival, proliferation, migration, myelin thickness and de-myelination (Garratt et al., 2000; Huijbregts et al 2003; Meintanis et al., 2001; Zanazzi et al., 2001). Neuregulins receptors,
ErbB2 and ErbB3, are abundantly expressed in premyelinating Schwann cells. In a similar fashion to BDNF and p75 expression, ErbB2 levels decreased significantly after myelination was initiated (Grinspan et al., 1996). In chapter three, we demonstrate a novel interaction between p75 and the ErbB2 receptor that is induced by BDNF. We find that this complex is mediated by the polarity protein, Par3, which is enriched at the axoglial junction. Par3 has been reported to recruit p75 to this junction in response to BDNF in order to initiate myelination (Chan et al., 2006). We demonstrate that Par3 plays a critical role in distinguishing between BDNF and neuregulin axonal signaling for p75-mediated initiation of myelination.
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CHAPTER 1

OPPOSITE REGULATION OF OLIGODENDROCYTE APOPTOSIS

BY JNK3 AND PIN1 AFTER SPINAL CORD INJURY

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INTRODUCTION

Following spinal cord injury, a large portion of proximal oligodendrocytes undergoes apoptotic death during an extended period of time, with up to half the population ultimately lost from the immediate area of injury (Crowe et al., 1997; Beattie et al., 2002). The end result of this process is chronic demyelination (Totoiu and Keirstead, 2005). The mechanism by which oligodendrocytes die is, however, still not clear. Some of the extracellular signals that are known to regulate oligodendrocyte apoptosis in vivo include Fas, proNGF and TNF-α (Kim et al., 2001; Beattie et al., 2002; Demjen et al., 2004). These three ligands all activate the c-jun N-terminal kinase (JNK) pathway as part of their apoptotic signals when they bind their cognate receptors, at least in culture.

The JNK pathways are typically activated by proinflammatory as well as various stress signals in the nervous system (Shaulian and Karin, 2002). Under these conditions, where JNK proteins induce apoptosis, their main action appears to be phosphorylating the API family of key transcription factors, such as c-jun, thereby affecting the expression of their target genes (Kallunki et al., 1996; Behrens et al., 1999; Yoshida et al., 2002). In addition, JNK can also regulate the mitochondrial apoptotic pathway directly by facilitating cytochrome C (cytC) release after stress in culture (Tournier et al., 2000).

The physiological conditions that activate the JNK pathway, however, do not always result in an apoptotic response in vivo, suggesting that JNK proteins play diverse
roles during development as well as in the adult nervous system (Kuan et al., 1999). Although this may be the case for JNK1 and JNK2, a certain degree of specificity has been observed for JNK3. For instance, JNK3 expression is largely restricted to the nervous system (Mohit et al., 1995), and knockout analyses indicate that JNK3 initiates the apoptotic program under a wide range of pathological conditions, such as ischemia (Kuan et al., 2003; Okuno et al., 2004), axotomy (Herdegen et al., 1998; Kenney and Kocsis, 1998), seizure (Yang et al., 1997), and in animal models of neurodegenerative diseases (Hunot et al., 2004; Brecht et al., 2005). Of the three apoptotic receptors that play roles in oligodendrocyte apoptosis after spinal cord injury, p75, in particular, was shown to activate JNK3 in oligodendrocyte cultures (Harrington et al., 2002). Whether JNK3 regulates mitochondrial homeostasis in the nervous system, and which proteins are the physiological targets involved in cytC release, however, have remained unknown. The goal of the present study was therefore to identify the key JNK3 targets and elucidate the mechanisms by which JNK3 regulates these targets to induce apoptosis in vivo.

As a member of the MAP kinase family, JNK3 recognizes and phosphorylates Ser/Thr in SerPro and ThrPro motifs. These consensus sites are also the potential binding sites for Pin1, a phosphorylation-specific propyl-isomerase (Yaffe et al., 1997). Pin1 is known to regulate the stability of many important MAP kinase substrates, including p53, β-catenin, and p65, NF-κB subunit, thereby affecting proliferation of tumor cells (Wulf et al., 2005). In the nervous system, Pin1 action appears to be neuroprotective, blocking tau fibrillization by binding it directly (Lu et al., 1999). The data from Pin1-/- mice also support this view, where the mice develop progressive neuropathy and tau hyperphosphorylation/fibrillization when Pin1 was deleted (Liou et al., 2002). Recently
however, it was shown that Pin1 binds BimEL and JIP3, a neuronal form of JIP, in cultured neurons and promotes apoptosis (Becker and Bonni, 2006). In spinal cord injury paradigm on the other hand, we find that Pin1 plays a mainly anti-apoptotic role, maintaining cytC in the mitochondria in part by binding and stabilizing Mcl-1 in the cytosol. Being activated after injury, JNK3 then perturbs this interaction by phosphorylating Mcl-1, thereby promoting its rapid decay and subsequent cytC release.
MATERIALS AND METHODS

Construction of plasmids

The Ser\textsuperscript{121} and Thr\textsuperscript{163} residues in Mcl-1 were mutated to Ala individually and together, using a hybrid PCR strategy (Zhong and Bajaj, 1993). The PCR products were placed into pCR-II vector (Invitrogen), and sequenced for any errors. The Mcl-1 fragments containing single and double mutations were digested with Kpn I/Not I and used to replace the corresponding fragments in p3XFLAG-CMV-10-Mcl-1. For GST-Mcl-1 constructs with the wild type and mutant Mcl-1, the residues from 31 to 229 in Mcl-1 were excised by PCR with Bam HI and Sal I containing primers, verified by sequencing, and placed into Bam HI/Sal I sites in pGEX4T1 (Amersham). For Pin1 construct, the cDNA was excised from pEGFP-Pin1 by PCR using primers with Bgl II and Sal I sites, sequenced, and placed into Bgl II/Sal I in Bam HI/Sal I in pGEX4T1.

JNK3 and Pin1 mouse breeding

JNK3\textsuperscript{+/+} and JNK3\textsuperscript{−/−} mice were obtained from breeding JNK3\textsuperscript{+/−} mice, originally generated in Dr. Richard Flavell’s laboratory. The genotype was determined by PCR. Pin1\textsuperscript{+/+} and Pin1\textsuperscript{−/−} mice were also obtained from breeding Pin1\textsuperscript{+/−} mice.

Spinal cord injury and processing of the cord

Mice were anesthetized with ketamin/xylazine and subjected to hemisection at the thoracic 8-9 level using mini-Vannas scissors. The control laminectomized mice
underwent the same procedure, except for the hemisection. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee and followed the NIH guidelines for the proper use and care of laboratory animals. For processing the cord for proteins, a 6mm spinal cord tissue block (3mm rostral and 3mm caudal regions of the injury epicenter) was quickly dissected and frozen in liquid N2 at the indicated times. The cords were homogenized in the lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 10 mM Na pyrophosphate, 1% NP40, 10mM Na3P2O7, 10mM NaF, 1μg/ml aprotinin, 10μg/ml leupeptin, 1mM vanadate, 1mM phenylmethylfulfonyl fluoride, and 10% glycerol. The resulting lysates were used in Western analyses as described above. For processing of the cord for immunohistochemistry, mice were perfused with 3% paraformaldehyde in 0.1M phosphate buffer transcardially, and a 6mm segment of the cord that centered about the injury epicenter was removed and cryoprotected in 20% sucrose in 0.1M phosphate buffer. The spinal cord was cut in the horizontal or coronal plane in 20μm thicknesses, and processed for immunostaining.

**TUNEL labeling and quantification**

For TUNEL assays, a 6mm segment of the cord that centered around the injury epicenter were cut in 20μm thicknesses in the coronal plane, mounted onto slides that were precoated with 0.15% gelatin and 0.2μg/ml poly-D-lysine. To access the nuclei for TUNEL labeling, the sections were boiled in Antigen Retrieval Citra solution (BioGenex) for 2min at the full power and 15min at 10% power using a microwave oven. TUNEL reaction was performed using Roche TUNEL kit, and counterstained for
oligodendrocytes and neurons. For quantification, every 4\textsuperscript{th} sections were processed and counted.

\textbf{JNK immunoprecipitation/kinase assays}

At the indicated times, the spinal cord lysates were prepared, subjected to immunoprecipitation using JNK1/2 and JNK3 antibodies. The immune-complexes were used in kinase reactions with \(^{32}\text{P}\)-\(\gamma\)-ATP in the presence of GST-c-jun as an exogenous substrate. The kinase reaction was separated on SDS-PAGE, and autoradiographed.

\textbf{Immunoprecipitation/kinase assays}

The lysates from the spinal cord or 293T cells were subjected to immunoprecipitation with appropriate antibodies and the immune complexes were used in \textit{in vitro} kinase assays as described in the JNK kinase procedure, except that GST-Mcl-1 wild type and mutant proteins were used as substrates.

\textbf{Mitochondrial fractionation}

Mitochondria were prepared by sucrose density gradients (Marchenko et al., 2000; Liu et al., 2004). Briefly, the 6mm spinal cord tissue blocs were homogenized using Dounce homogenizer in 5 volumes of MS buffer (210mM mannitol, 70mM sucrose, 5mM EDTA, 5mM Tris (pH 7.5), 10mM NaF, 1µg/ml aprotinin, 10µg/ml leupeptin, 1mM vanadate, 1mM phenylmethylfulfonyl fluoride). Nuclei were collected after centrifugation for 10min at 2,500rpm at 4°C, and the resulting supernatants were spun at 15,000rpm for 10min at 4°C. The supernatants were subjected to a 1hr spin at 100,000g to obtain the S100 fraction, while the crude mitochondrial pellet was

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resuspended in MS buffer and overlayed onto a step gradient of 1M and 1.5M sucrose. The gradient was centrifuged for 30min at 85,000g. The mitochondrial band at the interface was pulled, diluted in 2 volumes of MS buffer, and spun for 10min at 15,000rpm.

**Immunoprecipitation/Western analyses**

The procedures for Western analyses are as described (Beattie et al., 2002). The antibodies used in the study include JNK1, cytochrome C, mouse Mcl-1 (Pharminogen), JNK2, rabbit Mcl-1, ubiquitin, actin, Bax, and Bid (Santa Cruz Biotechnology), CC1 (Oncogene Sciences), Pin1, p-c-jun (S63), c-jun, Bcl-xL, p-Thr-Pro, p-GSK-3β, and active caspase 3 (Cell Signaling), HA (Covance), NeuN (Chemicon), Cox IV (Molecular Probe), Flag, RhoGDI (Sigma).

**Phospho-S121 specific Mcl-1 antibody production**

The sequence of the phosphopeptide used to generate phospho121-Mcl-1 antibody was EEMAASAAAIV(pS)PEEELDCEPAIG. The peptide was synthesized by Tufts University Core Facility, and coupled to keyhole hemocyanin and injected into Rabbits at Pocono Rabbit Farms. Serum was affinity purified by sulfolink coupling gel (Pierce) to which the phospho-peptide was coupled.

**Two-Dimensional Electrophoresis**

The lysates were either untreated or treated with CIP at 37°C for 1hr and subjected to dialysis in a buffer containing 8M urea, 2% CHAPS, and 2mM tributyl phosphine. The dialyzed lysates were concentrated using Centricon.
Microbe Genomic Facility, 20μg of the protein was loaded onto an immobilized pH gradient strip (BioRad, pH 3-10) on the first dimension and further resolved at the second dimension in a 8-16% gradient gel. Following electrophoresis, the proteins were transferred and probed for Mcl-1 as described (Beattie et al., 2002).

**Immunohistochemistry**

The procedures for immunohistochemistry were as described (Kim et al., 2003).
RESULTS

JNK3 activity is induced after spinal cord injury and plays a critical role in oligodendrocyte apoptosis

To address whether JNK3 activity itself is regulated under pathological conditions, a standard set of conditions was established based on spinal cord injury. Mice were subjected to either laminectomy (control) or lateral hemisection at thoracic level 9-10 as reported (Beattie et al., 2002), and lysates were prepared at suitable intervals. To measure JNK3 activity specifically, we performed immunoprecipitation/kinase reactions using JNK3-specific antibody (JNK3 antibody fails to detect proteins from JNK3−/− mice) (Figure 1.1A). JNK1/2 activities were also measured in parallel using JNK1/2 antibodies in immunoprecipitation/kinase assays. Beginning 1hr after injury, we detected 3 to 3.5-fold increase in JNK3 activity, which was sustained in a prolonged fashion until several days after injury (Figure 1.2A). The JNK1/2 activities showed a transient increase at 1-4hr time points, but their peak activities were 500 fold lower than that of JNK3 (Figure 1.1B, 1.2B), suggesting that JNK3 is the major kinase whose activity is regulated in a prolonged manner after spinal cord injury.

We next asked which types of cells activate JNK in vivo by performing immunohistochemistry with p-c-jun563 antibody and cell type specific antibodies. P-c-jun immunoreactivity was not detected in the control mice, but beginning 1day after injury (dpi), it was detected mainly among NeuN+ neurons in the gray matter and CC1+
oligodendrocytes in the white matter (Figure 1.2C). P-c-jun immunoreactivity was not detected among GFAP+ astrocytes or OX42+ microglia, and the c-jun immunoreactivity was detected at the same extent in both the control and injured mice (data not shown). Quantification demonstrates that the number of p-c-jun+ cells increased over time both among NeuN+ and CC1+ cells (Figure 1.3A). The number of p-c-jun+/CC1+ cells, however, dropped by 3 fold in JNK3−/− compared to that in JNK3+/+ mice (* in Figure 1.4A, p=0.02 and 0.01 at 3 and 5dpi, respectively, student t-test, n=3), suggesting that JNK3 is the major kinase that is activated and phosphorylating c-jun after injury among oligodendrocytes. The number of p-c-jun+/NeuN+ also decreased in JNK3−/−, but the difference between JNK3−/− and JNK3+/+ mice was not statistically significant (Figure 1.3A).

Oligodendrocytes were previously reported to undergo apoptosis after spinal cord injury both in rodents as well as in monkeys (Crowe et al., 1997), typically resulting in 50% loss of oligodendrocytes (Beattie et al., 2002). We therefore asked whether JNK3 plays a role in oligodendrocyte apoptosis by quantifying the number of TUNEL+ apoptotic oligodendrocytes. The number of TUNEL+ cells among CC1+ cells decreased by 2-3 fold in JNK3−/− mice compared to JNK3+/+ littermates (Figure 1.4B), while the number of TUNEL+/NeuN+ cells did not differ greatly between the two genotypes (Figure 1.3B). The total JNK protein levels did not vary significantly between JNK3−/− and JNK3+/+ mice (Figure 1.1A), in agreement with a previous report (Kuan et al., 2003). These results suggest that JNK3 is the key kinase whose activation plays a critical role in the injury-induced apoptosis of oligodendrocytes.
JNK3 is necessary for cytochrome C release

JNK1/2 were shown to induce cytochrome C (cytC) release from the mitochondria in embryonic fibroblast cultures upon UV irradiation (Tournier et al., 2000). We therefore tested whether cytC was released after injury, and whether JNK3 regulates cytC release. For these experiments, the injury lysates were subjected to sucrose gradient to isolate pure mitochondrial fraction (Marchenko et al., 2000; Liu et al., 2004). In parallel, S100 and nuclear fractions were also prepared. The purity of each fraction obtained in a typical fractionation is demonstrated in Figure 1.4C (bottom panel). In JNK3\textsuperscript{+/+} mice, cytC was released into the S100 fraction with a concomitant decrease occurring in the mitochondria beginning 4hr after injury (Figure 1.4C), a time course that is notably similar to what has been reported in various in vitro studies (Nijhawan et al., 2003). In contrast, cytC release was significantly reduced in JNK3\textsuperscript{-/-} mice (Figure 1.4C, D). These results suggest that JNK3 activity is involved in regulating injury-mediated cytC release, the initial step that is necessary for activating the caspase cascade (Liu et al., 1996).

JNK3 is necessary for injury-induced degradation of Mcl-1

Since the Bcl-2 family members are involved in regulating cytC release from the mitochondria (Gross et al., 1999), we screened for the Bcl-2 family member whose expression is regulated by JNK3, and whose expression levels also change in accordance with cytC release kinetics. Of the Bcl-2 family members screened (Figure 1.5), Mcl-1 and Bcl-2 fit both criteria. Of the two anti-apoptotic Bcl-2 family members, Mcl-1 was recently reported as the critical cytosolic inhibitor having a short, 40min half-life, which regulates cytC release from the mitochondria under various apoptotic stimuli (Nijhawan
et al., 2003; Zhong et al., 2005). Increasing Mcl-1 levels experimentally blocked cytC release after UV irradiation (Nijhawan et al., 2003) and inhibiting Mcl-1 ubiquitination by downregulating its ubiquitin ligase, Mule, also resulted in increased cell survival when DNA damaging agents were applied to cells (Zhong et al., 2005). These results suggest that degradation of Mcl-1 is a prerequisite for cytC release and subsequent activation of the caspase cascade (Nijhawan et al., 2003). We therefore focused on Mcl-1 as a potential substrate for JNK3 that can regulate cytC release. In agreement with these culture data, Mcl-1 protein levels began to decrease 4hr after spinal cord injury in JNK3+/+ mice, which coincides in time-lapse period with the initial cytC release (Figure 1.6A, B). In JNK3−/− mice, however, Mcl-1 protein levels remained significantly elevated compared to those in the wild type (Figure 1.6A, B), suggesting that JNK3 regulates Mcl-1 stability.

**JNK3 phosphorylates Mcl-1 at S121 after injury**

Mcl-1 has two MAP kinase phosphorylation sites, S\(^{121}\)P and T\(^{163}\)P. It was shown in culture that ERK phosphorylates Mcl-1 at T\(^{163}\)P in the basal state (Domina et al., 2004) and JNK phosphorylates Mcl-1 at S\(^{121}\) and T\(^{163}\) in response to H\(_2\)O\(_2\) treatment (Inoshita et al., 2002). In agreement with these data, we found that Mcl-1 can be phosphorylated by ERK as well as p38, JNK1 and JNK3 *in vitro* (Figure 1.7A). Of these MAP kinases, JNK and ERK are the only ones whose activities become induced after spinal cord injury; the peak JNK1/2 activity is very low and the activation was transient and p38 activity does not increase with injury until 3dpi (Figure 1.1, 1.8B). It should be pointed out that although both JNK3 and ERK activities are induced by injury, ERK differs from JNK3 by remaining active in the basal state without injury, based on p-ERK/ERK Western
Among JNK proteins, JNK1 and JNK3 appear to phosphorylate Mcl-1 better than JNK2, at least in vitro (Figure 1.6C).

We next determined which of the two sites are phosphorylated by ERK and JNK3 in vitro and in vivo. For in vitro experiments, 293T cells were treated with EGF or transfected with JNK3 cDNA, and the resulting lysates were subjected to immunoprecipitation/kinase reactions using GST-Mcl-1-S121A (AT), T163A (SA), and S121A/T163A (AA) mutants in addition to Mcl-1 wt (WT) as substrates. The results indicate that JNK3 phosphorylates Mcl-1 at both S121P and T163P sites, while ERK phosphorylates T163 mainly (Figure 1.6C). The observation that ERK preferentially phosphorylates Mcl-1 at T163P and ERK remains active in the spinal cord without injury suggests that Mcl-1 should remain phosphorylated at that site in the basal state. Indeed, Mcl-1 was found to be phosphorylated at T163P in the uninjured spinal cord, based on the pT-P-specific immunoreactivity detected from the immunoprecipitated Mcl-1 (Figure 1.8A). This basal phosphorylation was independent of JNK3 genotype. These data together suggest that ERK is most likely to be responsible for phosphorylating Mcl-1 in the basal state, in agreement with the published data from culture studies (Inoshita et al., 2002; Domina et al., 2004). It should also be noted that pT163P was recently discovered to be a part of GSK target site, S159LPST163, where T typically remains constitutively phosphorylated as a priming site for GSK which phosphorylates Mcl-1 at S159 (Cohen and Frame, 2001; Maurer et al., 2006).

Since JNK3 was necessary for Mcl-1 degradation after injury, we believed it was highly likely that JNK3 is responsible for phosphorylating Mcl-1 at S121P after injury in vivo. Two different approaches were taken to address whether JNK3 phosphorylates Mcl-1 at S121P after injury. First, in Western analyses of the injured lysates following two-
dimensional gel electrophoresis, Mcl-1 protein yielded two additional phosphorylated bands in JNK3<sup>+/+</sup>, but not in JNK3<sup>−/−</sup> (Figure 1.8B, two stars), suggesting that Mcl-1 is phosphorylated in a greater degree in JNK3<sup>+/+</sup> than in JNK3<sup>−/−</sup> mice. In agreement with this data, a CIP treatment of the same lysates revealed that the distance between unphosphorylated Mcl-1 (Figure 1.8B, band i) and the partially phosphorylated Mcl-1 (Figure 1.8B, band ii) is greater in JNK3<sup>+/+</sup> compared to that in JNK3<sup>−/−</sup>. These data together suggest that Mcl-1 phosphorylation is attenuated in the absence of JNK3.

As the second approach, we generated a phosphorylation specific antibody against the peptide sequence around pS<sup>121</sup>P in Mcl-1 (peptide sequences are shown in the methods). The antibody detected Mcl-1 only when S121 was phosphorylated <i>in vitro</i> (Figure 1.8C,D), suggesting that the antibody is indeed specific to pS121-Mcl-1. We next subjected the injury lysates to immunoprecipitation with Mcl-1 antibody, and Western with the pS121-Mcl-1 antibody. The intensity of pS121-specific immunoreactivity increased at 1hr after injury in JNK3<sup>+/+</sup>, while it was significantly reduced in JNK3<sup>−/−</sup> mice (Figure 1.8E). A similar increase in pS121-specific signal from the wild type but not in the knockout mice was also observed in a direct Western with pS121-specific antibody (Figure 1.8E). These results together suggest that JNK3 phosphorylates Mcl-1 at S121 upon spinal cord injury <i>in vivo</i>.

Mcl-1 was recently reported to be phosphorylated by GSK upon IL-3 withdrawal in culture (Maurer et al., 2006). We, on the other hand, found that GSK-3 activities decrease with injury independently of JNK3 genotype (Figure 1.9). We interpret these results as suggesting that GSK is not likely to participate in Mcl-1 phosphorylation/ubiquitination in the spinal cord. What is regulated by the injury,
however, is S121 phosphorylation by JNK3 and this phosphorylation is critical for Mcl-1 stability in vivo.

**Mcl-1 recruits Pin1 at its constitutively phosphorylated site T163P**

The phosphorylation sites in Mcl-1, S$^{121}$P and T$^{163}$P, are the potential binding sites for Pin1, a phosphorylation-specific propyl-isomerase (Yaffe et al., 1997). We therefore addressed whether Pin1 is involved in regulating Mcl-1 stability after injury, by first examining whether Pin1 binds Mcl-1 and which of the two putative sites is involved, using two Mcl-1 mutants, S$^{121}$A (AT) and T$^{163}$A (SA). In order to facilitate phosphorylation of S$^{121}$P and T$^{163}$P, 293T cells were transfected with the wild type and mutant Mcl-1s, and treated with 50ng/ml of anisomycin for 30min to activate the endogenous JNK1/2. In subsequent GST-Pin1 pulldown assays, Pin1 bound Mcl-1 at pT$^{163}$P and not pS$^{121}$P (Figure 1.10A). Since Mcl-1 is phosphorylated at T$^{163}$P in the basal state, it could be deduced that Pin1 must bind Mcl-1 at pT$^{163}$P in the absence of injury. Indeed, Pin1 interacted with Mcl-1 in the basal state, and its interaction decreased after injury in the wild type, while the extent of its decrease was reduced in JNK3$^{-/-}$ mice (Figure 1.10B, C). These results suggest that Pin1 may protect Mcl-1 from being degraded in the absence of injury.

**Pin1 action is anti-apoptotic in vivo**

If neuroprotective action of Pin1 includes promoting stability of Mcl-1, a cytosolic regulator of cytC release, one would expect cytC release to increase in the absence of Pin1, opposite of what we observed in JNK3$^{-/-}$ mice. In Pin1$^{-/-}$ mice, cytC was constitutively released, and remained elevated to the levels higher than in the wild type
In addition, overall Mcl-1 levels were reduced at all the time points but its decay kinetics was similar to that in Pin1+/+ mice (Figure 1.11A, C). When the apoptotic profile was examined, however, there were no TUNEL+ cells in the uninjured Pin1−/− mice, despite the fact that cytC release was constitutive (Figure 1.11D). Only with injury, the overall extent of apoptosis increased 3-5-fold in oligodendrocytes in the absence of Pin1. These results suggest that cytC release alone is not sufficient to induce apoptosis, and an injury-induced signal is necessary for oligodendrocytes to die. The opposing phenotypes that are observed between JNK3−/− and Pin1−/− mice suggest that JNK3 is at least one of the critical signals that initiate the apoptotic cascade in spinal cord injury models.

**Pin1 binding inhibits Mcl-1 ubiquitination**

We hypothesized that one of the mechanisms by which Pin1 protects Mcl-1 from degradation is to interfere with Mcl-1 ubiquitination. To test this hypothesis, 293T cells were transfected with increasing amounts of Pin1 plus HA-Ubc and treated with MG132, a proteosome inhibitor, to block Mcl-1 degradation following ubiquitination. In the absence of added Pin1, Mcl-1 underwent ubiquitination, which was considerably attenuated by Pin1 addition in a dose-dependent manner (Figure 1.12A). To test next whether this inhibitory effect was due to Pin1 binding to Mcl-1, we transfected the wild type Mcl-1 or the mutant Mcl-1 whose Pin1 binding site, T163P, was mutated to A163P (SA mutant in Figure 1.12B). While the increasing amount of Pin1 protected the wild type Mcl-1 from being ubiquitinated, it failed to do so with the A163P (SA) mutant (Figure 1.12B). These results together suggest that direct Pin1 binding protects Mcl-1 from degradation by interfering with its ubiquitination under normal condition. As
predicted from this notion, a simple overexpression of Pin1 in 293T cells delayed the McI-1 decay following cycloheximide treatment (Figure 1.12C). Since Pin1 binds McI-1 in vivo via the constitutively phosphorylated p\textsuperscript{T163}P site, we interpret these results as suggesting that JNK3 activity counteracts Pin1 action toward McI-1 as it phosphorylates McI-1 at S121 after injury.

**JNK3 phosphorylation at S121 is necessary for Pin1 to dissociate from McI-1**

What are the mechanisms by which JNK3 counteracts the protective action of Pin1? One of the consequences of S121 phosphorylation may be to facilitate Pin1 to dissociate from McI-1. Unleashed from Pin1, McI-1 would undergo a conformational shift, which could allow subsequent ubiquitination. Our effort to distinguish McI-1 that is phosphorylated at S121 from other forms that are not was difficult in vivo. We therefore turn to 293T cells, in which we can ask directly whether JNK3 phosphorylation of S121 is necessary for Pin1 to dissociate from McI-1. If JNK3-dependent phosphorylation of S121 were indeed necessary for Pin1 to dissociate, one would expect that rendering S121 unavailable for JNK activation would allow Pin to remain bound to McI-1. For these experiments, 293T cells were transfected with Pin1 plus McI-1\textsuperscript{SE} or McI-1\textsuperscript{AE} mutants and subjected to 250ng/ml anisomycin. At this concentration, anisomycin not only inhibits protein synthesis but also activates the endogenous JNK pathway, which leads to phosphorylation of both S121 and T163 in 293T cells (data not shown). We also mutated T163 to a pseudo-phosphorylation site, E163, in order to allow S121 to be the only site being targeted by JNK. After anisomycin treatment, the amount of Pin1 bound to McI-1\textsuperscript{SE} decreased, while the amount of Pin1 bound to McI-1\textsuperscript{AE} did not (Figure 1.13A). Accordingly, the degradation of McI-1\textsuperscript{AE} was delayed compared to that
of Mcl-1SE (Figure 1.13B). It should be added that we obtained similar results with A/S$^{121}$T$^{163}$ as we did with A/S$^{121}$E$^{163}$ (data not shown). These results together suggest that S121 phosphorylation is necessary for Pin1 to dissociate from Mcl-1.
DISCUSSION

We report here that JNK3 activation is critical for apoptosis of oligodendrocytes after spinal cord injury. JNK3 activity itself is induced by the injury, regulating cytC release by phosphorylating Mcl-1 at S121, a pro-survival member of the Bcl2 family. The outcome of this phosphorylation is to facilitate proteosome-mediated degradation of Mcl-1. Our data suggest that Mcl-1 degradation is normally inhibited in part by Pin1, which binds Mcl-1 at PT163P. Since Pin1 fails to dissociate from Mcl-1 mutant (S121A) even after JNK is activated, we conclude that the phosphorylation of Mcl-1 at S121 by JNK3 is necessary for displacing Pin1 from Mcl-1 after injury. Loss of Pin1 leads to Mcl-1 ubiquitination, probably by Mule, and subsequent degradation, which ultimately results in cytC release. In agreement with the protective role of Pin1, cytC release remains constitutive and apoptosis increase dramatically after injury in Pin1−/− mice. A model depicting our data is shown in Fig 1.14.

Activation of the caspase pathways initiated by cytC release is a fundamental aspect of the apoptotic process (Liu et al., 1996), observed in many pathological conditions (Benn and Woolf, 2004). For cytC to be released under pathological stimuli, Mcl-1 must be degraded (Nijhawan et al., 2003; Maurer et al., 2006). Our results are in agreement with the above report; when Mcl-1 levels are elevated in JNK3−/− mice, cytC release was attenuated and oligodendrocyte survival was enhanced. The mechanism by which Mcl-1 stability is regulated, however, has remained unknown, except for the fact that it is ubiquitinated (Zhong et al., 2005). Here, we document that Pin1 is involved in
maintaining Mcl-1 stability by inhibiting its ubiquitination. Without Pin1, Mcl-1 levels were reduced and cytC release remained constitutive in the absence of injury.

As a phosphorylation-specific propyl isomerase, Pin1 regulates the protein turnover of some of the key transcriptional factors that are critical for proliferation as well as cytosolic proteins, such as tau, that are involved in neurodegeneration (Wulf et al., 2005). Indeed, Pin1$^{-/-}$ mice develop tau filament formation over time (Liou et al., 2003), suggesting that Pin1 in the cytosol plays a pro-survival function in the CNS. Our results here provide the evidence for an additional mechanism by which Pin1 acts to promote cell survival in the cytoplasm, via maintaining normal mitochondrial homeostasis by protecting Mcl-1 from being ubiquitinated and degraded unless an apoptotic signal is initiated. As mitochondrial dysfunction is observed in many of the neurodegenerative diseases, it is tempting to speculate that a similar opposite regulation between JNK3 and Pin1 plays a role toward maintaining mitochondrial homeostasis.

It was surprising that although cytC was constitutively released in uninjured Pin1$^{-/-}$ mice at significantly higher levels than that found in the injured wild-type mice, apoptosis was not observed until injury was performed. These data suggest that cytC release alone is not sufficient to induce apoptosis in vivo. This conclusion is in complete agreement with a previous report where injection of cytC was insufficient to induce apoptosis in neuronal cultures that were maintained in NGF (Deshmukh and Johnson, 1998). Clearly, an additional event(s) that is initiated by spinal cord injury is necessary to trigger the apoptotic cascade. Our data indicate that JNK3 activation is one of such events that are necessary to initiate the apoptotic cascade under pathological conditions, disengaging the pro-survival function of Pin1 towards Mcl-1.
Another surprising finding in our study is that although neurons activate the JNK pathway based on \( p \)-cjun staining, the extent of their apoptosis was not affected by JNK3 genotype. A simple explanation may be that neurons activate JNK1/2 predominantly and not JNK3 after spinal cord injury. We consider this scenario unlikely, since JNK3 is expressed in both neurons and oligodendrocytes (data not shown) and the counts of \( p \)-cjun\(^+\) cells among neurons were reduced in the absence of JNK3, albeit it was not statistically significant (Figure 1.3). Instead, it is likely that JNK3 is activated among neurons after injury, but it plays a role other than inducing apoptosis. A potential role for JNK3 in neurons is regulating some aspects of autophagic death. Our preliminary data support this possibility, as beclin-1 protein levels are affected in JNK3\(^{-/-}\) mice (our unpublished results). Alternatively, JNK3 may not regulate cell death, but play roles in relaying the injury signals back to the cell bodies for possible regeneration programs, as suggested by Cavalli et al. (Cavalli et al., 2005).
REFERENCES


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Fig 1.1 (A) The specificity of JNK3 antibody used in immunoprecipitation/kinase assays. (B) Actual dpm counts in GST-c-jun in a JNK1/2 immunoprecipitation/kinase assay.
Figure 1.2 JNK3 is the major kinase activated following spinal cord injury. (A, B) JNK3 and JNK1/2 activity was measured in immunoprecipitation/kinase assays using GST-c-jun as the substrate. For specificity of JNK3 immunoprecipitation, see Figure 1.1. Note that JNK1/2 activities are at least 500-fold lower than JNK3 activity based on the radioactivity counts on GST-c-jun. N=3 and the error bars represent the range of the dpm counts. (C) The p-c-jun immunoreactivity, a measure of JNK activation, was detected in oligodendrocytes and neurons after injury. Arrows point to p-cjun\(^+\) cells. Scale bar, 30\(\mu\)m.
Figure 1.3 (A) Reduction of p-c-jun immunoreactivity among NeuN+ cells in JNK3−/− mice is not statistically significant compared to that in JNK3+/+ (p=0.192, 0.061, 0.180 at 1, 3, and 5dpi, respectively; n=3, student t-test). (B) Similarly, although the number of TUNEL+NeuN+ cells increased with injury, it was not regulated by JNK3.
Figure 1.4 JNK3 is necessary for mitochondria-mediated apoptosis of oligodendrocytes after spinal cord injury. (A) Reduction in the number of \( \text{p-c-jun}^+ \) cells among CC1\(^+\) oligodendrocytes in JNK3\(^+/+\) mice compared to that in JNK3\(^{+/+}\) mice. \( \text{p-c-jun} \) immunoreactivity among CC1\(^+\) cells was quantified in JNK3\(^+/+\) and JNK3\(^{-/-}\) mice. The error bars represent s.e.m. (B) Upper: Oligodendrocyte apoptosis after spinal cord injury is attenuated in JNK3\(^{-/-}\) mice compared to JNK3\(^{+/+}\) mice. Adult JNK3\(^{+/+}\) and JNK3\(^{-/-}\) mice were subjected to T9 hemisection. At the indicated time, every 4\(^{th}\) coronal section was processed for TUNEL and CC1 immunostaining and cells that were positive for both staining were counted (p values, Student t-test). The error bars represent s.e.m. Lower: A representative picture of TUNEL\(^{+}/\)CC1\(^{+}\) cells. Arrowheads point to TUNEL\(^{+}/\)CC1\(^{+}\) cells. Scale bar, 30\( \mu \)m. (C) JNK3 is necessary for cytC release from the mitochondria after spinal cord injury. The spinal cord lysates were processed for mitochondrial fractionation using sucrose gradients. Lower: As controls for fractionation, the mitochondrial, S100, and nuclear fractions were analyzed in Western using a mitochondrial marker, Cox-IV, a cytosolic marker, RhoGDI, and a nuclear marker, cjun. (D) Quantification of the cytC levels in S100 fraction from JNK3\(^{+/+}\) and JNK3\(^{-/-}\) mice. Note that stars represent the time points at which two genotypes showed statistical difference. (Student t-test, \( p < 0.02 \)).
Figure 1.4 continued
Figure 1.5 Changes in cytosolic protein levels of the Bcl-2 family members after spinal cord injury in JNK3\(^{+/+}\) and JNK3\(^{-/-}\) mice.
Figure 1.6 JNK3 regulates Mcl-1 stability after injury. (A) Mcl-1 protein levels changed most dramatically beginning 4hrs after injury in JNK3+/+ mice, coinciding in time with cytC release. In JNK3-/- mice, Mcl-1 levels remain elevated. Note that the numbers underneath each lane represents quantification. Actin control is shown and the control for fractionation is shown in Figure 1.6C. (B) Quantification of the relative Mcl-1 levels at different time points after injury. Note that stars represent the time points at which two genotypes showed statistical difference (Student t-test, P < 0.05). (C) JNK3 phosphorylates Mcl-1 in vitro. JNK1, 2, or 3 was immunoprecipitated from 293T cells, and subjected to kinase assays using GST-Mcl-1 as the substrate. The asterisk in the stained gel represents IgG. As a control, the same lysates were subjected to immunoprecipitation and blotted for JNK Western. The experiments were repeated 3-4 times with similar results. (D) JNK3 phosphorylates Mcl-1 at both S121-P and T163-P, while ERK phosphorylates Mcl-1 at T163-P in vitro. GST-Mcl-1 proteins bearing single and double mutations as indicated were used as substrates in kinase reactions. The lower panels show the amount of GST-Mcl-1 protein in each lane as Coomassie-stained controls. The asterisk in the stained gel represents IgG. The experiments were repeated 3-4 times with similar results.
Figure 1.7 Changes in the activities of ERK and p38 after spinal cord injury. (A) MAP kinases phosphorylate Mcl-1 \textit{in vitro}. 293T cells were treated with 250ng/ml anisomycin for 30min, 10ng/ml TNFα for 10min, or 50ng/ml EGF for 5min, and the lysates were subjected to immunoprecipitation kinase assays using GST-Mcl-1 as the substrate. (B) The p38 pathway is activated significantly beginning 1dpi. The injury lysates were probed with \( p\)-p38 and \( p\)-38 antibodies. (C) ERK remains activated in the basal state, and becomes further activated after injury both in JNK3\(^{+/+}\) and JNK3\(^{-/-}\) mice. An active state of ERK in the control laminectomy (L) samples is evident by \( p\)-ERK immunoreactivity as well as the presence of retarded ERK band in ERK Western. The numbers underneath each lane represents quantification.
Figure 1.8 JNK3 phosphorylates Mcl-1 at S121 in vivo after injury. (A) Mcl-1 is phosphorylated at T163P in the uninjured spinal cord regardless of JNK3 genotype. The uninjured lysates were subjected to immunoprecipitation with the control IgG or Mcl-1 antibody and probed in Western with pThr-Pro antibody. The same blot was stripped and reprobed for Mcl-1 as a control. (B) JNK3 phosphorylates Mcl-1 at an additional site after injury in vivo. Injury lysates collected at 1dpi were subjected to two-dimensional electrophoresis and subsequent Western with Mcl-1 with and without CIP treatment. Note that Mcl-1 has two additional phosphorylated bands in JNK3+/+ but not in JNK3−/− mice without CIP treatment, and the distance between unphosphorylated and partially phosphorylated Mcl-1 is greater in JNK3+/+ than in JNK3−/− mice after CIP treatment. (C, D) Specificity of the pS121P-Mcl-1 antibody. 293T cells were transfected with Mcl-1SA and Mcl-1AT mutants (C) or not (D). To phosphorylate the endogenous Mcl-1 or the mutants, 293T cells were treated with 50ng/ml of anisomycin for 30min or 2hrs. In order to inhibit degradation of phosphorylated Mcl-1, 10μM MG132 was also added to cells prior to anisomycin treatment. Note that pS121P-Mcl-1 antibody detects Mcl-1SA and not Mcl-1AT mutant and only when JNK is activated (C), and similarly, the endogenous Mcl-1 only after anisomycin treatment (D). (E) JNK3 phosphorylates Mcl-1 at S121P after injury. The injury lysates were subjected to immunoprecipitation with the control IgG or Mcl-1 antibody and probed in Western with pS121P-Mcl-1 antibody. As controls, direct Western with pS121P-Mcl-1 and Mcl-1 antibodies are also shown. All the experiments were repeated 2-3 times with similar results.
Figure 1.8 continued
Figure 1.9 GSK is constitutively phosphorylated in the spinal cord and its phosphorylation increases with injury independently of JNK3 genotypes.

Figure 1.10 Pin1 binding to Mcl-1 inhibits ubiquitination and degradation of Mcl-1. (A) Pin1 binds Mcl-1 at T^{163}.P. 293T cells were transfected with Mcl-1 wild type and mutants, and treated with 50ng/ml of anisomycin for 30min to activate the endogenous JNK pathway. Lysates were then subjected to pull-down assays with GST or GST-Pin1. The Mcl-1 input controls are also shown. (B) Pin1 binds Mcl-1 in vivo and its binding to Mcl-1 decreases with injury more rapidly in the wild type compared to JNK3^{-/-} mice. The lysates were immunoprecipitated with Mcl-1 or the control IgG, and the bound Pin1 was probed. The levels of Pin1 and Mcl-1 are shown as controls. (C) Quantification of the amount of Pin1 bound to Mcl-1 (n=3). Note that stars represent the time points at which two genotypes showed statistical difference (Student t-test, p < 0.05). The difference at 1hr post injury was different at P=0.08 (Student t-test).
Figure 1.11 Pin1 is necessary for inhibiting cytC release, at least in part by regulating Mcl-1 stability in vivo. (A) CytC is not only released constitutively in the basal state, but also increases further after injury in Pin1−/− mice. (B) Quantification of cytC release at different time points after injury. (C) Quantification of the relative Mcl-1 levels at different time points after injury. Note that stars represent the time points at which two genotypes showed statistical difference (Student t-test, p < 0.05). (D) Apoptosis of oligodendrocytes increases in Pin1−/− mice after spinal cord injury.
Figure 1.12 Pin1 inhibits ubiquitination of Mcl-1. (A) Pin1 inhibits the extent of Mcl-1 ubiquitination in a dose-dependent manner. A day after transfection, 293T cells were treated with 10μM MG132 to stop Mcl-1 degradation, and 6hrs later, the lysates were subjected to immunoprecipitation with Mcl-1 and probed for ubiquitin using HA antibody. Ub-Mcl-1 indicates ubiquitinated Mcl-1. As controls, Mcl-1 and Pin1 Westerns are also shown. (B) Pin1 binding regulates Mcl-1 ubiquitination. The T^{163}A mutant that no longer binds Pin1 becomes ubiquitinated even in the presence of increasing amount of Pin1. The experiments were carried out similarly as in (A). (C) Mcl-1 degradation after cycloheximide treatment is delayed in the presence of excess Pin1. 293T cells were transfected with the indicated constructs, and treated with 50μg/ml cycloheximide for the indicated amount of time. All the experiments were repeated 3-4 times with similar results.
Figure 1.13 JNK3-dependent phosphorylation of Mcl-1 at $S^{121}P$ is necessary for Pin1 to dissociate from Mcl-1. (A) The Pin1 binding to Mcl-1 fails to decrease with $S^{121}A$ mutant. Mcl-1$^{SE}$ and Mcl-1$^{AE}$ mutants were introduced to 293T cells, which were subsequently subjected to 250ng/ml anisomycin treatment. Anisomycin blocks protein synthesis and activates JNK at that concentration. The amount of bound Pin1 to Mcl-1 mutants was assessed. Controls for JNK activation after anisomycin treatment are shown as $p$-JNK Western along with Mcl-1 and Pin1 input controls. (B) Anisomycin-induced Mcl-1 degradation is delayed in Mcl-1$^{AE}$ mutant compared to Mcl-1$^{SE}$ mutant. 293T cells were transfected with the indicated constructs, and treated with 250$\mu$g/ml of anisomycin for the indicated period of time. All the experiments were repeated 4-5 times with similar results.
Figure 1.14 A Model of JNK3 action in vivo. In the absence of injury, Mcl-1 is maintained in its phosphorylated state at T^{163}P, which recruits Pin1 to bind. Pin1 binding inhibits Mcl-1 ubiquitination and degradation. With the Mcl-1 protein level elevated, cytC remains in the mitochondria. With injury, however, JNK3 is activated and phosphorylates Mcl-1 at S^{121}P, which results in a conformational shift in Mcl-1, thereby facilitating Pin1 to dissociate. With Pin1 removed from Mcl-1, Mcl-1 undergoes ubiquitination and subsequent degradation. Degradation of Mcl-1 leads to changes in the dynamics of Bcl-2 molecule interactions, leading to cytC release into the cytosol.
CHAPTER 2

PROMOTING FUNCTIONAL RECOVERY AFTER SPINAL CORD INJURY BY A SMALL MOLECULE P75 LIGAND THAT DISRUPTS PRONGF BINDING TO P75 AND CROSSES BLOOD-BRAIN-BARRIER

Chhavy Tep, Stephen M. Massa, Frank Longo, Michele Basso, and Sung Ok Yoon
INTRODUCTION

Spinal cord injury induces acute necrosis at the center of the injury site, resulting in loss of cell bodies and axons. This is followed by a secondary reaction lasting up to several weeks, which involves degeneration of axons and loss of myelin at sites distant from the origin of the lesion, eventually leading to the loss of additional axons that had not initially been injured (Schwab and Bartholdi, 1996). One of the major causes for such sustained demyelination is apoptotic death of myelinating oligodendrocytes (Crowe et al., 1997; Warden et al., 2001). Since a single oligodendrocyte myelinates multiple axons, apoptosis of one oligodendrocyte will result in degeneration of multiple axons, causing the lesion to expand in a cyclic manner (Crowe et al., 1997).

We have reported that a neurotrophin receptor, p75, plays a role in apoptosis of oligodendrocytes after spinal cord injury (Beattie et al., 2002). As for the ligand that binds and activates the apoptotic cascade of p75 in vivo, we have identified proNGF, a novel form of the ligand which binds p75 selectively (Lee et al., 2001), and whose expression is upregulated consequent to the injury (Beattie et al., 2002; Harrington et al., 2004). Here, we report that blocking p75 activation from the outset by interfering with the ability of proNGF to initiate p75-mediated death can be a feasible strategy to improve myelin retention after SCI, thereby promoting functional recovery in mice. Our strategy was to develop a non-peptide small molecule, LM11A, which not only blocks proNGF-
mediated oligodendrocyte apoptosis in vitro, but also crosses the blood brain barrier. Daily administration of 100 mg/Kg LM11A beginning 4 hrs after the initial impact has resulted in a significant improvement in myelin retention and overall motor coordination, without exhibiting any toxicity or increased pain. These results therefore suggest that targeting oligodendrocytes, a population that undergoes continued cell death up to a year after the initial injury (Crowe et al., 1997), is an approach that should be explored.

The p75 receptor has been identified to be involved in a broad range of physiological and pathological functions, due to its ability interact with a variety of co-receptors and ligands. We have published that p75 is necessary for apoptosis of oligodendrocytes after spinal cord injury (Beattie et al., 2002). p75 expression is upregulated in oligodendrocytes after insult, and there is an increase in survival in p75 null mice (Beattie et al., 2002). ProNGF, the precursor of mature NGF neurotrophin, was reported to have been induced along with p75 after spinal cord injury (Beattie et al., 2002; Harrington et al., 2004). In vitro studies revealed that a recombinant, cleavage-resistant form of proNGF was shown to bind p75 selectively (Lee et al., 2001) and proNGF present in the injured spinal cord lysates induced oligodendrocyte apoptosis in culture (Beattie et al., 2002), demonstrating that proNGF is the pathological ligand for p75 after injury.

Activation of the c-Jun N-terminal kinase, JNK, pathway has been implicated in a variety of neurodegenerative models and diseases. We have reported that p75 activates JNK in oligodendrocyte cultures, and this is necessary for p75-mediated apoptosis (Harrington et al., 2002). Specifically, we determined that the neuronal specific, JNK3, is the major kinase that is activated among oligodendrocytes, and this activation is
necessary for oligodendrocyte apoptosis after spinal cord injury (Li et al., 2007). We further demonstrated support for p75’s role in JNK3 activation by performing JNK3 activity assays in spinal cord lysates from p75 wt and null mice. We find that JNK3 is robustly activated as soon as one hour after injury, but this activation is attenuated in p75 null lysates (Fig. 2.3A,B). This data demonstrates that p75 is necessary for JNK3 activation after spinal cord injury in vivo. In hopes of minimizing the death of myelinating oligodendrocytes after spinal cord injury, we aim to inhibit JNK3 activation by blocking p75 and the correlative signaling pathways that initiate neurodegeneration.

**Can this knowledge be developed into a therapy by blocking proNGF binding to p75?**

Because proNGF binding to p75 is necessary for apoptosis of oligodendrocytes, we aim to minimize oligodendrocyte cell death by blocking p75 activation with a therapeutic agent that can be administered after injury. The blood-brain-barrier (BBB) exists to maintain the integrity of the nervous system, by restricting entry of solutes and limiting access of microorganisms. BBB is thus a critical defense against molecules that are detrimental to normal functioning of the CNS. Although necessary to maintain normal CNS function, the BBB presents a significant hurdle for the efforts to deliver pharmacological reagents designed to treat injury and encourage repair. Therefore, it has been a long-standing goal to develop a non-peptide, small molecule that is capable of functioning as specific neurotrophin ligands as a therapeutic approach to promote desirable biological outcomes (Longo et al., 2008). Using high-throughput virtual screening of small molecule libraries based on neurotrophin β-loop 1 models, we selected
for potential ligands that fit to pharmacophores designed based on the physical and chemical features of protein ligand domain binding to p75 (Longo et al., 2008). After implementing low throughput, physical screening assays, LM11A compounds with the most drug-like characteristics were selected as a small molecule ligand for p75. In oligodendrocyte cultures, in which proNGF promoted cell death, LM11A treatment blocked proNGF action (Massa et al., 2006). In this chapter, we demonstrate the efficacy of this compound with in vivo spinal cord injury models and find that blocking p75 with a nonpeptide small molecule is an effective, noninvasive means of reducing physiological and functional dysfunction after spinal cord injury.
MATERIALS AND METHODS

Animals and Preparation for Injury:

200 adult female mice were used in these experiments (C57BL/6) aged 2-3 months. All mice were housed in standard barrier housing (humidity maintained at 20-35%, light 0800-2000 daily) for the duration of the experiments. Rodent diet (Harlan Biosciences) and acid water were provided ad libitum (mice were changed over to acid water five days prior to experiments). All surgery and procedures were performed in accordance with the Ohio State University Laboratory Animal Care and Use Committee.

Surgical Procedures:

* Laminectomy:

Mice were anesthetized by intraperitoneal injection of ketamine and xylazine mixture (90mg/kg; 10 mg/kg). Lubricant ointment was applied to prevent drying of eyes during surgery and recovery. The back was prepared by shaving, cleaning and sterilizing with iodine solution and 70% ethanol three times. The mouse is stabilized in the stereotaxic apparatus. Surgical procedures are done under a surgical microscope. Under aseptic conditions, mice received a mid-line incision of the skin and superficial muscles of the back, after which a dorsal laminectomy of T9 vertebrae is performed with fine rongeurs (14cm curved, with 0.5mm cup width). Remnants of the periosteal membrane from the dura surface were removed, and bleeding is controlled with sterile swabs.
Contusion:

After midthoracic T9 dorsal laminectomy was performed, the IH Spinal Cord Impactor (Precision Systems and Instrumentation, Lexington, KY), was used to deliver a contusion injury to the exposed spinal cord by applying a controlled force-defined impact with a stainless steel impactor tip designed for mice. A calibrated load cell is used to directly measure the force between the impactor and the specimen, so that no error from specimen movement is introduced (Scheff et al., 2003). The mouse is placed on a platform and small rat-toothed microdissecting forceps are used to secure the lateral processes of T8 and T10. The forceps are tightened and positioned with locking ball joints. After ensuring that the cord is stabilized, and the spinal column is aligned so that the impact surface was perpendicular to the axis of the impactor, the exposed T9 spinal dura is centered under the impactor tip using the fine X-Y axes adjustments of the IH impactor. The impactor tip is slowly lowered to the spinal surface, and then raised to approximately 0.5mm above the dorsal surface using the measured gears on the device. A computer connected to the IH Spinal Cord Impactor is used to deliver a set amount of force (in our case, 50 kilodynes). When commanded to initiate experiments, the tip moves to the spinal cord at the given force, contacts the spinal cord and retracts to start position after desired dwell time, typically 0 sec. If the actual force value after impact was less than 50kdyne, the mouse was not used for experiments. Similarly, if the actual displacement value of the impactor was not in the range of 400-600um, then the mouse was excluded from experiments. The mouse is moved from under the tip and the spinal cord is visually inspected for bruising for the signs of impact. Afterwards, the muscle is
sutured with 6-0 polypropylene suture and the skin stapled with surgical clamps. After recovery, the behavior of the mouse was assessed with BMS rating system, and if the mouse had a score greater than 2 (more than 50% ankle movement), then the mouse was not used for experiments.

**Post Operative Care:**

Immediately after spinal cord injury, the mice were injected with 0.5ml of buprenorphine (0.1mg/kg) subcutaneously. Mice also received subcutaneous fluids (sterile saline at 2.5ml/50g weight) every day for a week. They were placed on a certified heating pad for recovery for 18-24 hrs post-injury. Additionally, contused mice were injected with 0.5ml dose of Baytril (2.5mg/Kg) daily for a week to prevent bladder infection. Mice were maintained with acidified water throughout the duration of the study to maintain urine acidity, as a way to reduce the chances of urinary infection. The mice underwent gentle bladder expression two-three times daily until it was voiding on its own. The mice were checked frequently for skin lesions, infections and bladder problems (cloudy or bloody urine). Weight was monitored daily until 14dpi, and then weekly for the duration of the experiments (42 days post injury). A 10% weight loss was typically observed after injury, and a high caloric nutrient paste (Nutrical, Evsco, Inc.) was provided ad libitum as a dietary supplement. If there was greater than 20% weight loss, or other signs of illness was observed (poor appetite, low activity or infection which is non-responsive to treatment) the animals were euthanized by >70% CO2.
Small Molecule Administration:

The determined drug dosages (0mg/kg, 10mg/kg, 25mg/kg, 100mg/kg and 320mg/kg) were aliquoted into a color-coded system, so as to maintain double-blind measures. Four hours after injury, the drugs were administered to the mice via oral gavage, twice daily. For gavage, the animal was held straight with one hand, and the other hand placed the round-tipped gavage needle into the esophagus, at which point the solution was quickly dispensed and needle withdrawn.

BMS Test for Open field locomotion:

Open field locomotion was assessed with the BMS rating system, which was developed as a more sensitive and reliable measuring of hindlimb recovery in mice following SCI (Basso et al., 2006). The open field was a round table that was 8” high and 5 feet in diameter situated in a quiet testing room with normal lighting. The mice were acclimated to the behavior testing table for 20 min daily for a week before surgery. Mice were observed to move freely about the open field without signs of fear, including crouching. The day before surgery, the mice were tested to obtain base line, pre-op values. After injury, each mouse was observed at 1, 3, 5, 7, 10, 14, 21, 28, 35, and 42 days to evaluate differences in functional recovery among different drug treatments. The extent of force applied in the contusion injuries were moderate-severe thoracic contusions (50 kdyne) so as to allow some degree of weight-supported stepping in recovery. This also ensured that there was some extent of impairment in hindlimb function during over-ground walking.
Two raters that were blind to the treatment groups scored hindlimb locomotion simultaneously with the score sheet from Basso et al., (2006) for 4 min observations per mouse. If the scores differed between the individuals, the lower score was taken. To summarize the steps involved in BMS testing, it was first decided whether the mouse was plantar stepping when it was placed in the open field and the timer was initiated. If not, then ankle movement of dorsal stepping was evaluated and appropriately scored. If there was plantar stepping, then the frequency of stepping and coordination was evaluated:

A) **Plantar Placement:** To classify plantar placement, we observed that the first and last digit of the paw contacted the ground during the motion of stepping, and most importantly, that there was weight support throughout the process of stepping. In order to correctly categorize stepping, we ensured that the hindend and the knees of the mouse did not touch the ground during the process of stepping.

B) **Stepping Frequency:** The scale categorized stepping frequency as none, occasional (<50%), frequent (>50%) or consistent (all of the time of fewer than five missed steps) in injured mice.

C) **Coordination:** For every forelimb step, that there is a hindlimb step that is taken, and that there is alternation of the hindlimbs during an assessable pass (a pass being a distance of at least 3 body lengths). Frequency of occurrence was also quantified in subscore assessment, for if less than 3 assessable passes occurred, there was no coordination. And if there were greater than 3 assessable passes, and if most were not coordinated, then this was defined as some coordination. If most of the assessable passes were coordinated, then this was defined as most coordination.
D) *Paw rotation*: If there was some coordination, paw placement was assessed as to whether the paw was parallel to the body, or rotated on initial contact and lift off from the surface. The observer looks for position of the middle digit of the hindpaw in relation the long axis of the body, and whether the paw is parallel or rotated, both upon initial contact and upon lift off. Uninjured mice position the paw parallel to the body at initial contact and lift off, whereas in injured mice, the paw is often positioned internally or externally to the body axis.

E) *Trunk Stability*: Trunk instability is classified as either severe or mild trunk stability, with the distinguishing factor being that in severe trunk stability, postural deficits or events impair stepping motion in greater than half the time during locomotor movement. Mild trunk stability was defined as five or greater instances of instability, including dipping, rocking, tilting or side tail, with fewer than five events that stop stepping. Severe trunk stability was then assessed for scoliosis, haunch hit, collapse, spasm.

F) *Tail Position*: In scoring observations, the position of tail was classified as either up (always held up), up and down (held up at least once during locomotion) or down (never held up off the surface).

Overall BMS Score consists of a 0-9 point scale, with extensive ankle movement at 2, frequent plantar stepping at 4, and coordination at scores of 7-9, with varying degrees of paw position. Individual parameters including plantar stepping, coordination, trunk stability and paw and tail position were assessed with subscore values (score of 0-11). For analysis, the scores from both hindlimbs were averaged at each time point.
Grid Walking:

To measure deficits in descending sensorimotor control and voluntary movement control, we evaluated the ability of mice to locomote over a wire mesh grid (1”x1” spaced grid that is 8”x21” in size). The grid was mounted on four poles, 15” above the base board. On one end of the mesh grid, a black box was placed with food and a housing unit inside, so as to entice the mouse to enter from crossing over the grid. Mice were acclimated to the wire grid daily for a week before experiments, and observed to move freely on the grid without signs of fear, including non-movement. The day before surgery, mice were videotaped to evaluate normal performance. 42 days after injury, mice were videotaped for at least three minutes on the grid, to obtain at least 30 seconds of uninterrupted movement and two crosses from one end of the grid to the other (a set distance of 45cm). Analysis consisted of observing the number of footfalls, which consisted of the hindlimb paw falling through the grid so that the toes and heels protruded through the wire surface, and the number of steps, in which the hindlimb paw gripped the wire without slipping.
Swim Test:

![Swim tank diagram](image)

**Figure 2.1** Swim tank diagram (figure adapted from Gullo et al., 2003)

To measure locomotor performance in the absence of the weight support, we observed the limb movement of the mice while they swam across a fish tank, (85cm long x 30 cm high x 30 cm deep) according to a protocol established by Schnell et al., and modified for mice (Gullo et al., 2003). The water was kept at 32°C, and a black acrylic divider was placed inside the tank to create a 5” wide swimming track that ran along the length of the aquarium so as to discourage the mice from swimming in circles. At the final destination of the swimming track, the mice are able to climb onto a platform with a rough surface to provide traction that was exposed 1” above the water. A housing unit was also placed on the platform to encourage the mice to climb onto the platform. In order to facilitate the videotaping of the paw positions during swimming, a mirror was placed against the divider to provide a 45° angle from the underside of the mice during videotaping (Figure 2.1). Mice were acclimated to the swim tank for several minutes
daily, a week before experiments, and observed to swim freely from one side of the tank to the exposed platform on the other end of the tank. 42 days after injury, mice were videotaped to obtain at least two passes of 44 cm long distance. The 44cm distance was designated with tape markers at 0, 22 and 44cm (Figure 2.1, marked in yellow). Some mice (n=7) swam past the 22cm mark, but turned around and swam back, so one pass included swimming to the halfway point (22cm) and turning back around to the starting point (total distance 44cm). Mice that had extreme difficulty in swimming after injury and began to sink were promptly removed and were not included in analysis. Analysis included observations of several of the following parameters, each of which was given a score that was ultimately summed to give a total cumulative swimming score:

a) Tail Movement: Impairment of tail movement among injured mice was observed during each swimming pass in the videotaped recordings. The tail of uninjured mice moves in a regular sinusoidal pattern during swimming, and assigned a score of 4. Impaired tail movement was given a subsequent lower score with the lowest score being 1, given to a completely paretic tail. No tail movement was given a score of 0.

b) Hindpaw Position: The hindpaws of uninjured mice are positioned relatively tight to the body axis, but after injury, the hindpaws are positioned more laterally. A score of 3 was given to the normal hindpaw position of uninjured mice, while the most lateral hindpaw position was given a score of 1.

c) Forelimb Analysis: Uninjured mice employed forelimb usage only for steering of the body during swimming, while injured mice used forelimb strokes to propel themselves as compensation for the lack of hindlimb motion. Forelimb usage was given a score of 2 for mice that mostly used the forelimb for steering. A score of 1 was given to
mice that used one forelimb at a time, while a score of 0 was given to mice that mostly employed both forelimbs for propelling the body through the water.

d) *Hindlimb Coordination:* Uninjured mice utilized even left and right hindlimb strokes during swimming as a way to propel themselves, while hindlimb usage was uneven in a severely injured mouse. We gave a score of 2 for even criss-crossing of left/right strokes as seen in injured mice, to designated *most coordination* while swimming; specifically we observed that there were greater than 50% even strokes between the left and right hindlimb. We gave a score of 1 for *some coordination*, where there were less than 50% even strokes between the left and right hindlimb. A score of 0 was given to mice exhibiting no coordination, which included no movement at all in the hindlimb, or uneven usage of only one hindlimb.

e) *Velocity:* Velocity was calculated as the distance that each mouse swam (44cm) divided by the amount of time that it took to traverse each pass (seconds).

**Mechanical Alldynia:**

Mechanical sensitivity studies were conducted 42 days after injury. The dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy) is an automated Von Frey system that was used to assess the withdrawal threshold to graded mechanical pressure. For two days prior to testing, mice were individually acclimated for 20 min to 2 hours in the transparent plexiglass caging that was situated on top of a 1/16” wire mesh platform. Measurements were only assessed when there was minimal grooming or exploratory behavior, and there was plantar placement of the hindpaw. Sometimes, 2 hrs were necessary for the mouse to reach a resting position and cease movements in order to
permit analysis. Mouse hindpaw thresholds were measured by eliciting a paw-flick response upon applying an increasing force (in grams) using a metal filament (0.5mm) on the middle of the plantar surface of the hindpaw (viewed through an angled mirror), at which point the actuator was started. Paw withdrawal thresholds were measured with an applied force that was initially below detection threshold, and increased from 0 to 5 grams. When a withdrawal response occurred, the stimulus was terminated and the response threshold was recorded electronically. The force applied in order to elicit a reflex removal was recorded, and five measurements were taken, with at least 10-20 min intervals in between. Paw withdrawal threshold was calculated as the mean of the threshold of five trials.

**Hyperalgesia:**

Heat Sensitivity was tested 42 days after injury, using a modified Hargreaves method that emits infrared as a heat source upon contact (7370, Ugo Basile). Mice were individually housed in plastic cubicles mounted on a smooth, thin surface. A heat source was positioned under the plantar surface of the hindpaw, and measurement of latency to withdrawal of the stimulated paw was recorded. If the mouse did not withdraw a foot within 20 sec, the heat stimulus was automatically shut off. For two days prior to testing, mice were allowed to acclimate for 20 min to 2 hours. Measurements were only assessed when there was minimal grooming or exploratory behavior, so that there was plantar placement of the hindpaw. Sometimes, 2 hrs were necessary for the mouse to reach a resting position and cease movement in order to permit analysis. Five measurements of latency were obtained, testing each hindpaw alternately, with at least 10-20 min intervals.
in between. The response time was recorded electronically, and paw withdrawal threshold was calculated as a mean of five trials.

**Tissue Processing and Histological Analysis:**

The mice were transcardially perfused with saline, followed by 75ml of 3% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The spinal cords were subsequently dissected and postfixed for two hours in the same fixative. For paraffin sectioning, the cords were maintained in the fixative until embedded. 10 µm sections were collected at every 10th throughout the length of the cord. For cryosectioning, the cords were transferred after the postfix to 20% sucrose in 0.1M PB overnight at 4°C. The frozen cords (and the cords used for paraffin sectioning) were divided into 3mm segments, and the rostral, caudal, and caudal-most end of the epicenter were marked with marker. The tissue blocs were then mounted marker-side down in TissueTek (OCT Compound), and stored at -80°C until sectioning (Figure 2.2). The cords were sectioned at 20um and mounted on SuperFrost PLUS microscope slides. Each slide contained serial 20um transverse sections, spaced 100um apart, cut through on a cryostat.

![Figure 2.2 Tissue mount for cryo-section and paraffin section](image-url)
Histology:

A modified eriochrome cyanine (EC) staining protocol:

Each slide contained 10um coronal sections of the cord spaced 100 um apart. Every slide was stained with eriochrome cyanine to identify myelinated white matter and residual tissue sparing. Digital images were obtained with a Zeiss Axio Observer.Z1. The sections containing the epicenter lesion were the ones that contained the largest extent of lesion and least amount of visible white matter. The lesion was identified by loss of staining and severe disruption of tissue (where the defined borders of the gray matter were lost and there was loss of tissue and degeneration). The white matter was determined to be spared if the eriochrome cyanine staining was normal in appearance and density (lacking cysts, degeneration).

Air-dried sections were cleared of paraffin in xylene, and subsequently hydrated in a decreasing ethanol series before being placed in 10% FeCl3 and 0.2% EC (Sigma) in 0.5% aqueous H2SO4 for 18 minutes. The slides were then differentiated in 10% FeCL3 for 7 min, which was followed by thorough washes in distilled water. Slides were subsequently dehydrated through ethanol and xylene and mounted with PerMount and coverslipped.

The total cross-sectional area of the spinal cord and lesion boundary was measured on digitized images of the epicenter using a computer-assisted image analysis program (AxioVision 4.7, Zeiss). The lesion volume was calculated by multiplying the sum of the digitalized lesion area with the distance between sections (100um). The amount of spared white matter was reported as the percentage of the total cross-sectional
area of the cord at the epicenter. Lesion length was determined to the nearest 100um by examining EC stained sections rostrally and caudally from the epicenter until sections with normal distribution and density of EC staining were observed.

**Immunohistochemistry:**

Sections were pretreated with blocking solution, composed of 5% bovine serum albumin (BSA) in TBS, with 0.3% Triton X-100 with 5% goat and horse serum for 2 hours at room temperature. Primary antibodies were applied with 0.1% Triton X-100 and 5% BSA, and 1% goat and horse serum overnight at room temperature. Antibodies used to detect cellular components in the lesion site include CC1 (Calbiochem; #OP80, 1:200) as a marker for oligodendrocytes. A rat anti-mouse monoclonal antibody was used to label the CD11b antigen (αm integrin chain; Mac-1) present on polymorphonuclear cells, monocytes, and microglia (Serotec, Inc.; #MCA74, 1:200). Slides were then mounted with Vectashield (Vector Labs, Burlingame, California).

**Statistical Analysis:**

Statistical tests were performed using Graphpad Prism2 Software (Instat, Inc.). Differences in biomechanical parameters of injury (including BMS scores, subscores) between C31 treated spinal cord tissues were evaluated using Student's t test or one way ANOVA analysis. Comparison between treatments was evaluated using the Tukey’s multiple comparison test. The probability value to determine statistical significance was set at p<0.05. Swimming scores and open-field locomotor scores were analyzed with
repeated measures analysis of variance (ANOVA), with Tukey’s multiple comparison test at each dosage.
RESULTS

Strategy to inhibit oligodendrocyte apoptosis by blocking proNGF binding to p75 after spinal cord injury.

Small molecule, non-peptide p75 ligands (LM11A) that were designed to mimic β-loop 1 site of NGF domain interacting with the p75 receptor (Figure 2.4A) were selected after virtual in silico screening of 3D conformer library scans (Massa et al., 2006). In previous studies, we demonstrated that LM11A can disrupt proNGF binding to p75 (Massa et al., 2006). Mature oligodendrocyte culture systems were used to analyze LM11A function in vitro. These cell types express p75 but not TrkA, and it was found that treatment with recombinant, cleavage-resistant proNGF promoted cell death. Treatment with LM11A compound inhibited this proNGF induced cell death over a concentrations range of 1-10nM (Figure 2.4B). LM11A compounds also displaced proNGF binding to p75 by nearly 30% in the same culture system (Figure 2.4C). These in vitro studies demonstrate that LM11A can function as a competitor to proNGF binding to p75 and can protect oligodendrocytes from proNGF mediated apoptosis in culture.

LM11A crosses the blood-brain-barrier.

Among the small molecules, LM11A was predicted to have the best BBB penetration and outstanding medicinal properties based on Lipinski rules (Massa et al., 2006). Using standard pharmaceutical industry protocols, assays to determine the ability of this compound to reach non-intravascular brain tissues in mice after oral (PO),
intraperitoneal (IP) and intravascular (IV) administration were conducted by Absorption Systems Inc (Exton, PA). To determine the level of LM11A in plasma, blood was collected at one and three hours post administration and plasma was separated. To determine the level in brain tissues, mice were first perfused with saline to remove intravascular LM11A, and the brain tissue was collected from which extracts were prepared. The level of LM11A in the extract was determined via LC-MS/MS. A single administration of LM11A at 10mg/kg resulted in tissue concentration of 210 and 63nM at one and three hours (Figure 2.4D). The brain to plasma ratio for this compound is greater than 5 (Figure 2.4E), demonstrating that this compound is an efficient means of penetrating CNS tissues for therapeutic treatment.

There is no weight loss after LM11A treatment after spinal cord injury.

Although LM11A exhibited no apparent toxicity in uninjured mice, it was possible that the molecule could exacerbate the normal weight loss that is associated with SCI. We therefore followed the changes in weight after SCI for the duration of the study. Uninjured laminectomy animals gained weight over the period of 42 days, and LM11A administration at every dosage exhibited no deleterious effect on this weight gain (Figure 2.5A, n=8, p>0.05). Injured mice lost less than 10% of their weight by 10 dpi, but began gaining weight reaching the initial weight before the operation. Again, the change in weight in LM11A treated mice was identical to the vehicle treated control group at every dosage tested (Figure 2.5B; n=19-21, p>0.05). The death rate after injury also did not correlate with the treatment (n=1 out of 200). Collectively, these results suggest that administration of LM11A exhibited no serious toxic effects for the mice, even after SCI.
LM11A does not exacerbate pain sensitivity after SCI

NGF is a well-known factor that induces neuropathic pain, mainly acting on TrkA (Lewin et al., 1993; Ro et al., 1999). Although the role of p75 is yet to be determined, it is possible that p75 could augment TrkA signaling in pain signaling as well. If that is the case, LM11A could either exacerbate or attenuate the pain that follows SCI. We therefore measured the effect of LM11A administration on thermal hyperalgesia and allodynia from the hindpaw at 42 dpi, when mice regained plantar stepping.

In both thermal hyperalgesia and mechanical allodynia tests, we found that there was no significant difference amongst drug dosages in both laminectomy control and injured mice, suggesting that this drug does not cause greater sensitivity. When the injured was compared to laminectomized controls, latency withdrawal time after heat stimulus was lower in the injured mice, suggesting increased sensitivity after injury (Figure 2.6A, n=8, p<0.05 one-way ANOVA). Similarly for mechanical Von Frey tests, there was no significant difference in either time or force among the dosage treatments in the control and injured mice, suggesting that administration of this compound does not confer or exacerbate sensitivity (Fig 2.6B, n=8 or 19-21 respectively, p >0.05). When the control group was compared to the injured group, the injured mice exhibited increase in force and time, suggesting that the injured mice exhibited reduced sensitivity to mechanical pain (Figure 2.6B, p<0.05, one-way ANOVA). These results together suggest that LM11A does not exacerbate both thermal and mechanical pain sensitivity after spinal cord injury.
LM11A promotes recovery in trunk stability and coordination in stepping after spinal cord injury at 100mg/Kg

We hypothesized that if treatment with LM11A inhibits apoptosis of oligodendrocytes by blocking proNGF binding to p75, there will be more myelin spared and a greater improvement in behavioral recovery. We first assessed locomotor behavior with BMS open-field test. Among the laminectomy control groups, there were no significant differences in BMS scores. The BMS scores of all dosage groups throughout the extent of experiments remained at normal scores of 9, similar to baseline prior to sham injury (data not shown). This demonstrates that LM11A is not causing impairment of normal stepping function in control mice.

At 50 Kdyne force, all injured animals developed complete paraplegia the day after the contusion, corresponding to a BMS score of 0-1. The vehicle treated control mice began demonstrating improvements in motor function by 2-3 days after injury, and gained plantar stepping by 10dpi with BMS scores observed to be 3.9-4.6 on average (Figure 2.7A, n=19-21). A BMS score of 4-5 signifies frequent plantar stepping without much coordination (Basso et al., 2006). The mice treated with LM11A at 10 and 25mg/Kg behaved similarly to vehicle treated animals, without demonstrating any dose-dependent increase. At 100mg/Kg however, the improved behavioral recovery over the others was evident, reaching a BMS score of 6.1 and 7.1 at 21 and 35dpi respectively. This is a score that is at least 1 point higher than the rest of the group at both time points (p<0.01, student’s t-test). Once reaching a BMS score of 7.0, these scores persist throughout the whole term of study until the end point at 42dpi. A BMS score of 7 signifies at least most coordination while stepping, though trunk stability and normal paw
position placement are still compromised. At 320mg/Kg, the scores fell to the value of 10 and 25mg/Kg treatment. The potential reason for failure of dose-dependency is reviewed in the Discussion. These results demonstrate that LM11A may have an impact on gaining overall coordination after spinal cord injury.

Another parameter that was analyzed from BMS testing was the time at which plantar placement of the hindpaw and frequent stepping was initiated. For stepping to occur, a functioning reticulospinal system is required (Metz et al., 2006; Patestas and Gartner, 2006), and uninjured mice do not miss a step during stepping (Basso et al., 2006). Therefore, improvement with LM11A will suggest that the molecule has an effect on the reticulospinal circuit. A majority of the mice treated with LM11A at 100mg/Kg initiated plantar stepping 2 days earlier than vehicle control (Figure 2.7B, \( p<0.01 \)). In addition, the initial day of stepping ranged from 7 to 28dpi with the vehicle control, while it ranged from 5 to 14dpi with the 100mg/Kg group (Figure 2.7C). These data may be interpreted as suggesting that LM11A is exerting an effect on the reticulospinal system that allows the recovery in one of the more crucial phases in weight-bearing stepping.

The fine details of functional locomotor recovery, such as plantar stepping, coordination, paw position, trunk stability and tail placement, were further analyzed using cumulative subscore values for these parameters. Subscoring allows evaluation of whether there was an improvement in any specific aspect of locomotion after frequent plantar stepping that the overall BMS score did not reflect (Basso et al., 2006). For the vehicle groups, a cumulative subscore increased up to a value of 6.4-6.8 by 35 and 42dpi (Figure 2.7D) The 100mg/Kg group, however, demonstrated statistically significant improvement beginning with a subscore of 5.0 at 21dpi, reaching a subscore of 8.7 at
42dpi ($p<0.01$). When each locomotor parameter among plantar stepping, coordination, paw position, trunk stability and tail placement was analyzed individually, it was trunk stability that contributed to the significant improvement in cumulative subscores at 100mg/Kg (Figure 2.7E).

**Subscore: Trunk Stability**

Mice normally exhibit a high trunk stability score of 2.0 with no observable lateral movements of the trunk in the haunch of the tail during movement. After injury, mice lose stability, losing posture and balance in walking (Basso et al., 2006). We found that after SCI, the trunk stability values vary widely over time, ranging from 0.8 to 1.9, and most regained some trunk stability at values 1.1 to 1.4 at 42dpi (Figure 2.7E). Although the value for the 100mg/Kg was not statistically different from the rest of the groups at 42dpi, their value for the 100mg/Kg group differed from the rest, demonstrating a gradual, steady increase over time (Figure 2.7E, $p<0.01$). These data suggest that LM11A promotes the circuits that are responsible for maintaining trunk stability in open-field locomotion after SCI.

**LM11A improves swimming behavior at 100mg/kg, especially in coordination**

A limitation in the BMS scoring is that it focuses on stereotyped movements controlled by reflex pathways in the spinal cord that rely heavily on the ability to bear weight (Smith et al., 2006). It is possible that LM11A may influence locomotion that is independent of weight-bearing ability. In order to address these behavioral recovery shortcomings, we chose to evaluate motor behavior during swimming at 42dpi, which obviates the need to bear weight, but allows assessment of a phasic relationship between
right and left alternation of limb flexion and extension, as in walking (Smith et al., 2006). This motor behavior relies on the integrity of the descending motor tract in the cord (Smith et al., 2006). Since swimming is not yet well-established in motor function assessment after spinal cord injury, we modified a published protocol for swimming in rats for use in mice (Gullo et al., 2003), and analyzed 5 independent parameters, including forelimb usage, hindpaw position, tail movement and coordination, in addition to velocity.

**Velocity:**

Velocity was calculated by measuring the amount of time it took for a mouse to swim 44cm in distance during swimming (Figure 2.1 in Materials and Methods). The velocity among the vehicle group with laminectomy was 6.5 cm/sec (Figure 2.8A). LM11A administration did not affect the velocity among laminectomized mice at all the doses tested (Figure 2.8A) suggesting that the small molecule did not impair the circuits involved in normal swimming ability. After SCI however, the velocity was reduced by three-fold to an average of 2.5 cm/sec ($p<0.001$), showing that contusion injury therefore impaired the average speed at which the mice could navigate from one end of the tank to the other. LM11A therefore did not improve the velocity at all the doses tested (Figure 2.8A). This suggests that LM11A is not involved in the recovery of the neural pathways that govern speed in locomotion.

**Hindlimb Coordination:**

Overall improvement in swimming behavior was analyzed via cumulative scores of hindlimb coordination, forelimb usage, hindpaw placement and tail movement (Gullo
et al., 2003). As with BMS testing, the 100mg/Kg group alone exhibited statistically significant improvement in functional recovery in swimming ($p<0.001$); the mean was 5.7 at 100 mg/Kg with the values ranging from 2.5 to 9.0, while the mean for the remaining treatments was 4.2 with values ranging from 0.0 to 7.5 (Figure 2.8 B,C). This improvement at 100 mg/Kg was mainly attributable to improved hindlimb coordination (Figure 2.8 B,C). The average score for hindlimb coordination for the 100 mg/Kg group was 1.6, with 66.7% exhibiting a perfect coordination score at 2.0. Some of the mice in other groups also reached the perfect coordination score at 2.0, but the proportion of the mice with 2.0 scores were 38%, 37%, 24% and 29% for the vehicle, 10, 25, and 320 mg/Kg, respectively (Figure 2.8B,C). Since the ventrolateral tracts are mainly responsible for forelimb-hindlimb coordination (Bolton et al., 2006; Metz et al., 2000), these results suggest that LM11A action impinges positively on the sensorimotor pathway.

**Forelimb usage:**

Normal mice use the forelimbs for steering and directional change, while relying on the hindlimb for forward propulsion during swimming. Injured mice, however, rely on the forelimb instead of the hindlimb in order to propel themselves in water (Smith et al., 2006). Our analysis revealed that there was no significant difference among the drug treatments in the injured mice (Figure 2.8F). These data suggest that LM11A is not playing a role in decreasing the dependency on forelimb usage after injury.
LM11A does not improve sensorimotor recovery in gridwalk.

Voluntary sensory or sensorimotor deficits that are not revealed by open-field locomotion and swimming analyses can be analyzed with gridwalk behavioral analysis (Behrmann et al., 1992; Bolton et al., 2006; Smith et al., 2006). In this behavior test, we are able to assess forelimb-hindlimb coordination and deficits in descending motor control while observing foot placement over a wire mesh grid. If LM11A is involved in maintaining the integrity of these tracts, then treated contused mice should display improvement in gridwalk navigation similar to that of untreated mice.

After scoring the total number of completed steps and foot faults for each limb, we calculated for percentage of foot faults out of the total number of placements (steps plus foot faults) for uninjured and contused mice. There were nearly zero foot faults among the laminectomy mice, as they were voluntarily able to navigate the grid successfully. Treatment with LM11A did not appear to affect grid walking by the uninjured mice (n=8, p>0.05). After injury, however, there were an average of 7.1 foot faults along the extent of the 21” gridwalk, amounting to nearly 60% missed steps (Figure 2.9A-B, n=19-21, p<0.0001). LM11A administration did not improve the incidence of foot faults at every dosage (Figure 2.9B, p>0.05). This was also the case when we analyzed the rate of successful gripping and paw placement on the grid: the average was 4.8 steps among the injured, regardless of the dosage of LM11A, while it was 9.4 steps among the laminectomy controls, amounting to nearly 50% impairment in paw placement (Figure 2.9C, p<0.001). These data demonstrate that LM11A is not playing a role in maintaining the integrity of various descending sensorimotor tracts after contusion injury.
LM11A protects against myelin loss after spinal cord injury.

Each neural system within the tissue has a functional specificity and is topographically organized in the spinal cord (Collazos-Castro et al., 2006). Contusion typically causes damage to the gray matter, corticospinal tract and loss of ascending and descending axons in the white matter (Collazos-Castro et al., 2006; Gensel et al., 2006; Nishi et al., 2007). Models of thoracic contusive injury are used to isolate and study white matter deficits. However, gray matter loss in the thoracic area causes less identifiable functional impairments (Gensel et al., 2006). Because we observe functional recovery in mice treated with LM11A, we next asked whether treatment with LM11A played a role in maintaining anatomical structures in the cord and prevention of neural damage after injury. Since LM11A exhibited improvement at 100mg/Kg, histological analyses were performed between the vehicle control and the 100mg/Kg treated injured groups.

Myelin loss and spared white matter were assessed with eriochrome cyanine staining of serial cross-sections of the spinal cord. When all the stained sections through the rostral and caudal axis of 9mm distance were compared, it was apparent that LM11A treatment improved overall myelin sparing compared to vehicle control (Figure 2.10C-F, n=4)). Additionally, damage to the gray matter, corticospinal tract and most of the dorsal column axons were also significantly attenuated by LM11A treatment (Figure 2.10D, F, n=4) with distinct boundaries of the gray matter, and the intact discernable corticospinal tract.

Quantification of the section for myelinated area supported the visual differences. LM11A attenuated the myelin at the rostral and epicenter segments (Figure 2.10G). It
was published that myelin loss is most noticeable in the rostral and epicenter regions in rats as well as monkeys (Crowe et al., 1997). Upon assessing white matter sparing between the 0mg/Kg and 100mg/Kg groups with the AxioVision digital image analysis program, we found that that there was a significant increase in spared tissue at the epicenter with 100mg/Kg LM11A treatment. For the untreated lesion sample, 18.3% of tissue was spared at the epicenter, but in the treated mice, approximately 34.2% of white matter was spared ($p<0.05$). There was no significant difference in the rostral and caudal areas with treatment. This data suggests that LM11A may play a role in preventing oligodendrocyte cell death, therefore accounting for the increase in spared white matter upon 100mg/Kg treatment after spinal cord injury.
p75’s role in apoptosis of oligodendrocytes after spinal cord injury has been previously published by our lab and proNGF has been identified as the ligand that binds p75 selectively (Beattie et al., 2002). We have also demonstrated that small molecule, non-peptide p75 ligands that mimic the β-loop1 domain of NGF block proNGF binding to p75 in cultured oligodendrocytes (Massa et al., 2006). In this report, we present data that demonstrate the therapeutic potential of the prototype p75 ligand, LM11A, as a specific monomeric, synthetic ligand to p75 that can disrupt the ability of proNGF to initiate p75-mediated death during Wallerian degeneration after spinal cord injury. We believe that this non-peptide small molecule has the potential to be effective in translational applications due to its ability to cross the blood-brain-barrier, is minimally invasive, and does not appear to cause weight loss or toxicity in mice. Administration of this compound was also found not to exacerbate sensitivity to hyperalgesia or allodynia with or without spinal cord injury. This demonstrates the efficacy and benefits of this compound as a treatment after spinal cord injury.

Upon histological analysis with eriochrome cyanine staining of injured tissue, we find that myelin loss was significantly reduced in mice treated at 100mg/kg compared to control. In the tissues from LM11A treated mice, we find that the dorsal funiculus and the ventrolateral tracts are intact. These data suggest that reduction in myelin loss accounts, at least partly, for the behavioral improvement that we observed at 100 mg/Kg.
Our data demonstrate that administration of LM11A can be a potentially valuable strategy in improving behavioral and histological recovery after spinal cord injury. If LM11A is indeed blocking proNGF binding to p75, then in correlation with \textit{in vitro} data (Massa et al., 2006), we hypothesize that there should be reduced death of oligodendrocyte after injury. This would account for the increase in spared white matter with 100mg/Kg treatment. One mechanism that could account for functional recovery in the treated mice is the presence of spared white matter in maintaining myelination of surviving neurons, and consequently, prevention of spinal cord dysfunction.

\textbf{LM11A treatment improves behavioral activities governed by spared ventrolateral descending pathways}

Demyelination and axonal damage both contribute to functional deficits after spinal cord injury (Cao et al., 2005). We would have to investigate the extent of white matter sparing further to determine if there is correlation to our behavioral recovery data; variability in improved locomotion may be attributed to topographical organization of each descending, ascending or intrinsic neural system. The injuries specific to this experiment may have only damaged certain pathways and spared others in the cord, and this would only impair certain types of locomotion (Collazos-Castro et al., 2006). The IH spinal cord impactor device used in these experiments impacts directly dorsal to the exposed cord, so the corticospinal tract as well as the ascending sensory tract should be damaged. However, severity of white matter loss in the ventral and lateral funiculi is variable, depending on the extent of injury. Many important descending pathways that govern hindlimb locomotor function reside here, including the reticulospinal tract and the
rubrospinal tract in the dorsolateral funiculi (Brosamle et al., 1997; Cao et al., 2005; Patestas and Gartner, 2006). It has been reported that demyelination injuries in the ventrolateral and dorsolateral regions produce more significant locomotor deficits than injury alone (Cao et al., 2005; Loy et al., 2002). p75 expression (mRNA and immunoreactivity) is found to be localized to the medullary reticulospinal, raphespinal and vestibulospinal tract with limited expression in the corticospinal tract (Barrette et al., 2007). If LM11A treatment targets p75 and attenuates oligodendrocyte loss in these ventral and lateral regions of the spinal cord, then the amount of spared functional white matter will prevent degeneration of these descending pathways. Further analyses of surviving oligodendrocytes (quantified with surviving CC1+ cells) will be completed to address this point.

Upon evaluating functional recovery after compound treatment in injured mice, we found that the aspects of open-field stepping that improved significantly were frequency of hindlimb-forelimb coordination and paw rotation in mice dosed with 100mg/Kg LM11A, as compared to vehicle treatment. Further analysis of the finer parameters of behavior locomotion demonstrates modest improvements in paw position and trunk stability. As a means of furthering analysis of hindlimb recovery upon LM11A treatment, we subjected the mice to swimming behavioral tests. With this approach, we were able to take advantage of the rodent’s natural buoyancy in water and to bypass limitations with above-ground stepping, including difficulty with weight-bearing limb movements and support. Upon quantifying distinct parameters of swimming, we found that improvement in coordination was again demonstrated in the swim tests. The mice treated with LM11A at 100mg/Kg criss-crossed the hindlimbs in a coordinated manner
more frequently compared to the untreated mice, confirming that LM11A is playing a role in functional recovery of coordination in both above-ground stepping and phasic hindlimb swimming.

Hindlimb-forelimb coordination is mediated by the ventrolateral tracts (Bolton et al., 2006; Metz et al., 2000), suggesting that treatment with LM11A may be sparing white matter in these regions of the spinal cord. p75 is expressed in the medullary reticulospinal tracts, and this is topographically located in the ventral region of the spinal cord (Barrette et al., 2007). The medullary reticulospinal tract governs motor control of trunk and limb musculature, which is involved in posture maintenance and orientation of limbs (Patestas and Gartner, 2006). Interestingly, the parameters that were specifically improved in open-field analyses were *trunk stability* and correct *paw position* upon LM11A treatment. This would suggest that p75 in the reticulospinal tracts may be distinctively targeted. It has also been reported that the reticulospinal tract plays a critical role in initiation of locomotor function (Cao et al., 2005; Jordan, 1998). Frequency analyses in our experiments have demonstrated that mice dosed at 100mg/Kg, initiated plantar stepping as soon as 5dpi, which was two days sooner than the mice given the other dosages. Collectively, LM11A may block proNGF mediated oligodendrocyte apoptosis in the spared white matter surrounding the reticulospinal tracts, thus preventing degeneration of these descending pathways and result in recovery in motor control of trunk and limbs.
LM11A may impinge on p75/NgR mediated inhibition of regeneration

The lack of regeneration attributed to injuries to the central nervous system is due to a combination of factors, including death of injured neurons, reduced capacity of adult neurons to grow, lack of trophic support, and the presence of a hostile environment to growth (Woolf et al., 2003). There is the possibility that LM11A treatment functions in perpetuating survival of existing axons, and does not block proNGF mediated oligodendrocyte cell death exclusively. In this case, axonal regeneration would have to be investigated. p75 is known to play a role in enabling the non-permissive action of myelin inhibitors (MAG, OmGP, and Nogo) by interacting with Nogo receptor (NgR) (Hunt et al., 2002). It is possible that LM11A binds to p75 in the raphe-, reticulo- and vestibulospinal tract directly, and this would impair the association of p75 to NgR. The inability of NgR to interact with p75 would prevent downstream signaling of Rho and consequential growth cone collapse (Hunt et al., 2002), thus allowing surviving or regenerating axons to grow without inhibition from myelin. Both anterograde and retrograde neuronal tracing would be necessary to determine if there is indeed axon regeneration in the descending axonal pathways. For anterograde tracing, it would be possible to trace specific descending tracts by directly injecting into the brain region in which the tract originates. For instance, to follow the reticulospinal tract, it would be necessary to inject the tracer into reticular nuclei; to tract the raphespinal tract, it would be necessary to inject tracer into the raphe- nuclei (Barrette et al., 2007).

Walking is the result of coordinated activity of many muscles of the trunk and limbs, and is regulated by integrated activity of descending and peripheral pathways acting upon the neural spinal cord circuitry (Majczynski et al., 2006). According to their
location in the spinal cord, motor activity through the descending pathways can be separated into two principal systems: the medial system and the lateral system. The medial system includes reticulo- and vestibulospinal pathways which govern diffuse action on flexor and extensor muscle activity, while the lateral system consists of the cortico- and rubrospinal pathways which govern fine control and voluntary modification of locomotion (Majczynski et al., 2006). If LM11A is blocking p75-mediated inhibition of axon growth in the medial system, then this would account for the partial recovery in above-ground stepping. Allowing for axon growth in these specific reticulo- and vestibulospinal pathways may play a significant role in the maintaining muscle tonus necessary to support body weight, trunk stability, body posture and step-by-step regulation of locomotor muscle activity. Because these parameters were improved in open-field locomotion, LM11A treatment may block p75/NgR mediated inhibition of regeneration in the reticulo- and vestibulospinal pathways. This is in addition to the rubrospinal pathways that govern excitatory and inhibitory effects on flexor and extensor muscles (Majczynski et al., 2006). Partial improvement in swimming behavior upon LM11A treatment can also be attributed to the compound impinging on the p75 inhibitory pathway, because both swimming and walking involve phasic right and left alternation of limb flexion and extension, with repetitive movements that mimic stepping. Therefore, if LM11A is preventing p75 and NgR mediated inhibition of axon growth, it would be targeted to the reticulo- and vestibulospinal pathways, thus accounting for improvement in trunk stability and paw placement while stepping.
LM11A treatment does not improve voluntary sensorimotor activities governed by the corticospinal tract

Voluntary sensory or sensorimotor deficits that are not made readily apparent by open-field locomotion and swimming analyses can be analyzed with gridwalk behavioral analysis (Behrmann et al., 1992; Bolton et al., 2006; Smith et al., 2006). Gridwalk depends on hindlimb paw placement (mediated by corticospinal tract integrity), forelimb-hindlimb coordination (mediated by the ventrolateral tracts), initiation of stepping (mediated by reticulospinal tracts) and voluntary movement (mediated cortico- and rubrospinal tracts) (Bolton et al., 2006; Metz et al., 2000; Smith et al., 2006). In order to assess improvement in forelimb-hindlimb coordination and descending motor control, we quantified the number of foot faults and completed steps while navigating across a grid. We found no difference among dosage groups for total number of foot faults, suggesting that LM11A treatment is not improving voluntary sensorimotor activity in the gridwalk. Similarly, we found that mice treated with LM11A at 100mg/Kg did not improve in the number of successfully completed steps when traversing the gridwalk, demonstrating that there was still impairment in hindlimb paw placement in the contused mice. Contact placing response and voluntary aspects of limb movement and balance are indicators of cortico- and rubrospinal tract integrity (Bolton et al., 2006; Metz et al 2000). Since there is limited expression of p75 in the cortico- and rubrospinal tract, then LM11A may not be targeted to these descending pathways. Indeed, it has been reported that there is no enhanced regeneration of corticospinal tract axons after dorsal hemisection injury in p75 null mice (Zheng et al., 2005). Because there is no regeneration in the cortico- and rubrospinal tracts, this may explain the sustained impairment of voluntary sensorimotor
function upon navigating the gridwalk with LM11A treatment in our experiments. Further studies observing the behavior of p75 null mice in locomotor recovery tests would determine if there is also impairment in gridwalk stepping, but improvement in coordination and trunk stability in open-field locomotion as we have distinguished with LM11A administration.

**There is no dose response with LM11A treatment**

Based on the behavioral data, it appears as though 100mg/Kg is the optimal dosage at which it acts as an antagonist and recovery occurs, but treatment at the next logarithmic dose (320mg/Kg) does not seem to perpetuate the same behavioral recovery. In fact, the rate of recovery returns to the same level as untreated and lower dosages of 10mg/Kg and 25mg/Kg. If binding of this compound saturates available p75 receptors after injury, the saturation level should plateau and have the same effect at a higher dose, represented as a dose-response curve, but this is not the case. Because LM11A appears to improve behavioral recovery at 100mg/Kg, then the dose response would be represented by a bell-shaped curve. There are many factors that may account for absorption of compound: variation in availability of endogenous proNGF, and variation in receptor (p75), in which case continued stimulation or inhibition of living systems cause the subject to become tolerant or the body has initiated compensatory processes (Dobson and Kell, 2008).

Initially, in design of the compound, active dimeric cyclic peptides mimicking the β-hairpin loop1 domain that interacts with p75 were isolated. However, upon specificity testing of the compound in neurotrophic systems, the authors found that despite the
chemically diverse nature of the pharmacore derivatives, there were similar neurotrophic activities and potencies (Massa et al., 2006). Upon comparison of the peptide sequences of loop1 from both NGF and NT-3, we find that there is only a difference of three amino acids (Massa et al., 2006). There is about 50% sequence identity among the neurotrophins, and similarity among secondary structure estimations of NT3 and NGF from circular dichroism and Fourier transform infrared spectroscopy has been reported (Robinson et al., 1995). The neurotrophins exist as dimeric molecules, and the dimer interface in the NGF homodimer consists of hydrophobic residues that are highly conserved throughout the neurotrophin family (Robinson et al., 1995). A possibility as to why there is no similar dose response at 320mg/Kg could be that at such a high concentration, LM11A compound impinges on other neurotrophic pathways. The structural similarity between NGF and NT-3 could mean that LM11A binds TrkC receptors, and perpetuates the corresponding signaling pathways. It has been found that NT-3 enhances sprouting of transected corticospinal tract neurons after spinal cord injury, and NT-3 is a survival factor for axotomized corticospinal neurons (Schnell et al., 1994; Giehl et al., 2006; Grill et al., 2007). NT-3 was also found to play a role in normal function of the adult spinal cord, in that it is expressed in the medial and lateral α-motoneurons of basil lamina IX, and there is some restricted lower level expression in the dorsal and ventral horns of the gray matter (Scarisbrick et al., 1993). Such high concentrations of LM11A acting upon NT-3 pathways suggest that there may be some regeneration in the corticospinal tract that is acting independently of the p75 signaling pathways, which may also account for some of the functional recovery we observe in the walking, swimming and gripping during gridwalk.
Our functional data demonstrate that LM11A treatment improved recovery in behavioral activities that rely on the integrity of vestibulo- and reticulospinal tracts in the ventrolateral region of the spinal cord. These are the descending tracts that govern hindlimb-forelimb coordination and initiation of stepping/trunk stability respectively, which are the specific parameters that we found to be improved upon LM11A treatment in both open-field stepping and swimming. This correlation suggests that LM11A may be targeted specifically to these pathways, since p75 expression is localized to these regions of the spinal cord. Limited expression of p75 in the cortico- and rubrospinal tracts accounts for impairment of voluntary sensorimotor function in fine paw placement while navigating the gridwalk. Collectively, LM11A is either blocking proNGF mediated apoptosis of oligodendrocytes, thus sparing the myelinating white matter surrounding these pathways, or it is impairing p75/NgR mediated inhibition of regeneration after injury. Targeting these specific descending pathways that are involved in initiation and maintenance of locomotion is an effective means of enhancing functional recovery of locomotion by promoting growth and regeneration of damaged descending axons.
REFERENCES


Figure 2.3 JNK3 kinase activity in spinal cord injury lysates from p75<sup>+/+</sup> and p75<sup>−/−</sup> mice. (A) p75 is necessary for JNK3 activation after spinal cord injury. Lysates prepared from p75<sup>+/+</sup> and p75<sup>−/−</sup> spinal cord tissue injury time course were subjected to immunoprecipitation/kinase assays using JNK3 specific antibody (Upstate/Millipore #05-893). Kinase reactions with <sup>32</sup>P-γ-ATP were performed using GST c-jun as a substrate, and autoradiography values were quantified (n=5, p<0.05). The level of JNK3 activation was reduced to control levels in p75 knockout lysates. For statistical analyses, student t-test was used. The error bars represent s.e.m. Bottom panel shows p75 western blot control, confirming genotype of tissue. (B) Quantification from (A). Student’s t-test was used for statistical analyses.
Figure 2.4 The nonpeptide small molecule, LM11A is an effective, noninvasive compound that selectively blocks proNGF binding to p75. (A) Strategy in selection of small molecule, non-peptide p75 ligands (LM11A): High-throughput virtual screening of small molecule libraries based on neurotrophin β-loop1 models were screened to select for ligands (Massa et al., 2006). (B) LM11A treatment blocks proNGF mediated apoptosis of oligodendrocytes. Cortical oligodendrocyte cultures were treated with 0.05nM recombinant, cleavage-resistant proNGF and/or LM11A compounds at a range of 1-10nM. After fixation and processing for MBP and TUNEL, proNGF mediated cell death was found to be reduced by 30-50% upon LM11A treatment (Massa et al., 2006). (C) LM11A competes with proNGF for p75-mediated signaling. Using ELISA assays, 96-well plates were incubated with p75-Fc, followed by treatment with 1.8nM proNGF and/or LM11A at a concentration range of 1,500-10,000nM. Anti-NGF antibody was used to detect bound proNGF, as determined by optical density of bound horseradish peroxidase conjugate. LM11A treatment displaced proNGF binding to p75 up to 30% at the highest concentration of 10,000nM (Massa et al., 2006). (D) LM11A crosses the blood-brain barrier. After a single administration of 10 mg/Kg LM11A via PO, IP and IV administration, LC-MS/MS determined brain tissue extract to be 210 and 63nM at one and three hours. (E) LM11A penetrates CNS tissue efficiently: The brain to plasma ratio of administered LM11A is greater than 5.
Figure 2.4 continued
Figure 2.5 LM11A administration exhibited no apparent toxicity in mice. (A) There were no significant difference in weight change among various dosages of LM11A in laminectomized mice (n=8, p>0.05). After laminectomy, mice lost <10% of their weight, but regained it within 5dpi. For statistical analyses, student t-test was used. The error bars represent s.e.m. (B) There was no significant difference in weight change among LM11A dosages in injured mice (n=19-21, p >0.05). Mice lost <10% of their weight after injury, and post-operative care provided high calorie nutrient paste (Nutrical, Evesco Inc) ad libitum to aid in recovery of weight loss.
Figure 2.6 LM11A administration did not exacerbate pain sensitivity. (A) Modified Hargreaves infrared test for thermal hyperalgesia found no significant change in latency withdrawal time of the hindpaw among LM11A dosages. Five measurements of latency were obtained, testing each hindpaw alternately, with at least 10-20 min intervals in between, using the Ugo Basile Model 7370 Plantar Test apparatus. The response time was recorded electronically, and paw withdrawal threshold was calculated as a mean of five trials (laminectomy mice n=8, injured mice n=19-21). Compared to laminectomized controls, latency withdrawal time was lower in injured mice, suggesting increased sensitivity after injury (p<0.05, one-way ANOVA). The error bars represent s.e.m. (B) Von Frey testing for mechanical allodynia found no significant difference in latency withdrawal time among LM11A dosages in either time or force. The paw-flick response to an increasing force from a metal filament was measured with the dynamic plantar aesthesiometer apparatus (37450, Ugo Basile, Comerio, Italy). Paw withdrawal threshold was calculated as the mean of the threshold of five trials (laminectomy mice n=8, injured mice n=19-21). The injured mice exhibited an increase in paw withdrawal threshold in both force and time when compared to laminectomized control, suggesting that the injured mice exhibited reduced sensitivity to mechanical pain (p<0.05, one-way ANOVA).
There is improvement in coordination and trunk stability in open-field locomotion at 100mg/Kg LM11A treatment in contused mice. (A) BMS Score assessment among injured mice demonstrates improved behavioral recovery among mice treated with 100mg/Kg LM11A. Recovery in hindlimb locomotion was assessed with four minute observations of each mouse in open-field behavior testing table at 1, 3, 5, 7, 10, 14, 21, 28, 35 and 42 dpi with testing worksheet from Basso et al., (2006). Injured mice began stepping at 10dpi, with scores of 3.9-4.6 on average, while the injured mice dosed with 100mg/Kg reached a BMS score of 6.1 and 7.1 at 21 and 35dpi respectively, which is statistically significant (n=19-21, p <0.01). For statistical analyses, student t-test was used. The error bars represent s.e.m. (B) Mice treated with 100mg/Kg initiated frequent plantar stepping sooner than vehicle control (p<0.01). For statistical analyses, one-way ANOVA and Dunnett’s multiple comparison test were used. (C) Histogram of day of initial stepping among LM11A dosages demonstrates that the majority of mice treated with 100mg/Kg LM11A initiate plantar stepping sooner than untreated mice. The general pattern for 0mg/Kg is outlined in purple and 100mg/Kg is outlined in green. (D) Cumulative BMS subscores was significantly improved upon 100mg/Kg treatment of LM11A (p<0.01). Subscores were calculated from testing worksheet from Basso et al., (2006). (E) Specifically, the trunk stability parameter in subscoring contributed to higher cumulative subscore in mice treated with 100mg/Kg dose of LM11A.
Figure 2.7 continued
Figure 2.8 LM11A improves hindlimb coordination in swimming tests. (A) Velocity: LM11A administration did not affect swimming speed among control and injured mice at all doses tested. Velocity was calculated from an average of two runs from videotaped recordings of mice swimming across a 44cm in the behavioral swimming tank. The error bars represent s.e.m. (B) Histogram for distribution of cumulative swimming scores among dosages demonstrates that majority of mice treated at 100mg/Kg exhibit higher cumulative swimming scores, peaking at 5-6.5 on average. The general pattern for 0mg/Kg treatment is outlined in purple, and 100mg/Kg is outlined in green. (C) Cumulative Swimming Score: The sum of quantified swimming parameters (forelimb usage, hindlimb placement, hindlimb coordination and tail movement) is higher for mice administered LM11A at 100mg/Kg (n=19-21, p<0.001). For statistical analyses, one-way ANOVA and Dunnett’s multiple comparison tests were used. The error bars represent s.e.m. (D) Histogram for distribution of swimming coordination scores among dosages demonstrates that the majority of mice treated with 100mg/Kg LM11A exhibit higher coordination scores. The general pattern for 0mg/Kg treatment is outlined in purple and 100mg/Kg is outlined in green. (E) Treatment with LM11A at 100mg/Kg improves coordination in swimming (p<0.01, one-way ANOVA). Using an average of two videotaped runs, hindlimb coordination score was determined as follows: most coordination is given a score of 2.0, some coordination is given a score of 1.0, and no coordination is given a score of 0.0. (F) Forelimb usage score was determined as follows: mice that employed minimal use of forelimb was given a score of 2.0, some use of forelimb (one forelimb) was given a score of 1.0 and complete dependence on forelimb was given a score of 0.0.
Figure 2.8 continued
Figure 2.9 Mice treated with LM11A do not improve in gridwalk navigation (A). Administration of LM11A did not appear to affect gridwalking in laminectomy control mice (n=8, p>0.05). There is also no significant improvement in foot faults among LM11A dosage treatment after injury (n=19-21, p>0.05). Values were calculated from an average of two videotaped recordings of mice locomotion over a wire mesh grid (1”x1” spaced grid that is 8”x21” in size). Foot faults were quantified by the hindlimb paw falling through the grid so that the toes and heels protruded through the wire surface. For statistical analyses, Dunnett’s multiple comparison test was used. The error bars represent s.e.m. (B) Percentage of foot faults out of total steps (C) The number of successful steps did not improve with LM11A treatments at all dosages in both laminectomized and injured mice. Laminectomized mice averaged 9.4 steps, and injured mice averaged 4.8 steps while navigating the grid, amounting to nearly 50% impairment in voluntary paw placement. The number of successful steps was quantified by correct placement of the hindpaw where the forepaw gripped the wire while navigating the grid without slipping.
Figure 2.10 There is increased white matter sparing upon LM11A treatment (A) Myelin stained with eriochrome cyanine is intact in laminectomized vehicle-treated tissue (n=2). 10um coronal sections were sampled 100 um apart from rostral to caudal axis. (B) The myelin in laminectomized tissue from mice treated with 100mg/Kg LM11A is also intact. (C, E) There is myelin loss and tissue disruption at the epicenter after injury in vehicle treated mice (n=15). (D, F) LM11A treatment at 100mg/Kg spared myelin and attenuated damage to gray matter, corticospinal tract and dorsal column axons (n=16) (G) Quantification of white matter sparing in rostral, center and caudal regions of spinal cord demonstrated significant increase in myelination at the epicenter upon 100mg/Kg LM11A treatment (n=15-16, p<0.05). For statistical analyses, student t-test was used. The error bars represent s.e.m.
Figure 2.10 continued
Figure 2.10 continued
Figure 2.11 p75 expression in the mouse spinal cord three days after injury (A) Differential staining for p75 immunohistochemistry in coronal sections. The bottom left and upper left quadrants are stained with MLR2 antibody, which is specific for the extracellular domain of p75. We find greater immunoreactivity among fibers in the ventral region of the cord, and motoneurons in the ventral horn of the gray matter. The bottom right and upper right quadrants are stained with p75 antibody given to us by Dr. Bruce Carter, which is specific to the intracellular domain of p75. We find greater immunoreactivity among the cell bodies of oligodendrocytes in the white matter, in the sensory fibers in the dorsal horn of the gray matter, and the ascending sensory tract. (B) Representation of coronal section of mouse spinal cord and main descending motor tracts (shaded in blue) and ascending sensory tracts (shaded in purple) (taken from Barrette et al., 2007).
CHAPTER 3

BDNF INDUCES POLARIZED RAC1 SIGNALING AT THE ONSET OF SCHWANN CELL MYELINATION BY ACTIVATING A COMPLEX OF P75 AND ERBB2.

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INTRODUCTION

During development, cell to cell contact plays a critical role in facilitating the exchange of signals between neighboring cells. Myelination by Schwann cells is perhaps one of the best examples, where myelinating glia are in intimate, continuous contact with the axons that they myelinate, forming a reciprocal signaling network between the two cell types. The process of wrapping axons is particularly polarized, being initiated at the axon-glial interface and continuing until mature myelin is formed.

Par3, a member of the polarization complex that includes Par6 and aPKC (1), was recently shown to play a critical role in this process: Par3 was localized at the axon-glial interface, recruiting p75 in response to BDNF (2), which is secreted by the axons (3). In particular, Par3 knockdown markedly inhibited myelination in vitro (2). Par3 contains three PDZ domains, rendering it capable of interacting with a large number of proteins, aside from Par6 and aPKC in a polarization complex. They include Tiam1 (4), KIF3A (5), Inscrutable (6), Nectins (7), 14-3-3 (8), JAM (9), Ku70 (10), and dynein (11).

Considering the ability of Par3 to interact with a variety of proteins, it is possible that the previously reported effect of Par3 knockdown in myelination cultures (2) could be due to disruption of its association with a protein critical for myelination.

Here, we report that Par3 binds ErbB2 and facilitates the association between p75 and ErbB2 in response to BDNF in Schwann cells. The consequence of this interaction is
to activate Rac1 in a spatially restricted manner at the axon-glial interface of the Schwann cell, thereby promoting myelination.
MATERIALS AND METHODS

Reagents

The Fc recombinant proteins were purchased from R&D Systems and BDNF, from Promega. The antibodies used in the study include p-ErbB2 and p-PAK (Cell Signaling), and ErbB2, ErbB3, actin, fyn, and Cdc42 (Santa Cruz Biotechnology), Rac1, Par3, phosphotyrosine, and neurofilament (Millipore), p75 (Promega), myc (Covance). PKI-166 was a gift from Novartis.

Constructs

ErbB2 construct was a gift from Dr. Nancy Hynes and Par3, Dr. Ian Macara. The PDZ 1, 2, and 3 constructs were described in (Chan et al., 2006).

RNAi sequences

The Par3 and control RNAi sequences were published (Chan et al., 2006). The p75 and its control RNAi sequences are published (Vilar et al., 2009). The sequences for ErbB2 RNAi and ErbB3 RNAi are shown below. The oligonucleotides containing the shRNA of the individual RNAi sequences were placed into pSIREN-RetroQ-ZsGreen1 vector (Clontech) using Bam HI and Xba I sites as directed by the vendor. Retroviruses were generated following transient transfection of the shRNA constructs in PlatE cells (Cell Biolabs) and the viral supernatants were concentrated by centrifugation at 20,000
rpm for 4 hrs at 15°C using SW28 rotor (Beckman). The viral pellet was resuspended in small volume of media and frozen at 80°C until use. For infection of Schwann cells in vitro and in vivo, a combined mixture of three different RNAi viruses were used for efficient knockdown.

**ErbB2i-a:** AAGTCTCACAGAGATCCTG  
**ErbB2i-b:** ACCACGTCAAGATTACAGA  
**ErbB2i-c:** ATCATAGGTATCTGTGA  
**ErbB3i-a:** TCTGGACTTCTCATCACC  
**ErbB3i-b:** GAACCTGAATGTCACATCC  
**ErbB3i-c:** CCTGACAAGATGGAAGTAGAT

**Morpholinos**

pard3 antisense MO1:  
(MORPH1404, sequence 5’-TCCAACACTCCTTTCCCGAATCCAAG-3’) was obtained from Open Biosystems. MO2: (5’-TCAAAGGCTCCCGTGCTCTGGTGTC-3’) and a random sequence control MO were obtained from Gene Tool LLC. Both MOs were resuspended in H₂O at a concentration of 1.0 mM. Each MO was diluted to a working concentration of 0.25 mM in H₂O and phenyl red and injected (1-2 nl) into

*Tg(sox10(7.2):mRFP);Tg(olig2:EGFP)* zebrafish embryos (Flanagan-Steet et al., 2005; Kucenas et al., 2008) at one cell stage. The embryos were raised at 28.5°C until analysis at 4 dpf (days post fertilization).

**In situ RNA hybridization in zebrafish**

Zebrafish larvae were fixed in 4% paraformaldehyde for 24 h and stored in 100% methanol at –20°C. The *mbp* RNA probe was synthesized using a digoxigenin labeling kit (Roche) and T7 RNA polymerase (NEB). After in situ RNA hybridization and
staining, embryos were dissected from the yolk and mounted in 75% glycerol on bridged
microscope slides. Images were obtained using Volocity software (PerkinElmer) and a
Zeiss AxioObserver inverted microscope equipped with DIC optics and a Retiga Exi
color digital camera. Images were exported to Photoshop (Adobe). Image manipulations
were limited to cropping and contrast, levels and color matching settings.

*In vivo imaging of zebrafish*

For imaging, larvae were lightly anesthetized using ethyl 3-aminobenzoate
methanesulfonic acid (Tricaine), immersed in 0.8% low-melting temperature agarose and
mounted on their sides in glass-bottomed 35 mm dishes (World Precision Instruments).
Images were captured using a 40X oil-immersion objective mounted on a motorized
Zeiss AxioObserver microscope equipped with a PerkinElmer UltraVIEW VoX spinning
disk confocal system. Z image stacks were collected and compiled using Volocity
software and exported to Photoshop. Image manipulations were limited to levels settings
and cropping.

**Primary Schwann Cell Culture**

Primary rat Schwann cells were isolated from P0-P1 rats and maintained
according to (Chan et al., 2004). For RNAi knockdown experiments, Schwann cells were
infected with the retroviruses that express the shRNA sequences, and the lysates were
harvested 3 days later.
RacGTP assays and Western blotting

RacGTP assays were performed as described (Harrington et al., 2002). For quantification, RacGTP signals were adjusted to total Rac1 to obtain ‘relative RacGTP levels’. The lysates were prepared and processed as described (Harrington et al., 2002).

Preparation of neuregulin 1 type III

Neuregulin 1 type III cDNA was transfected into 293T cells and the membranes were prepared according to (Taveggia et al., 2005), and the membranes were added onto Schwann cell monolayers as described (Maurel and Salzer, 2000; Taveggia et al., 2005). As a control, the membranes from untransfected 293T cells were prepared in parallel. To add the membranes to Schwann cells, 8 μg of membrane preparations was placed onto the cells, and spun for 3 min at 3000 rpm. The cDNA for NRG 1-Type III was a generous gift from Dr. Doug Falls.

Affinity Crosslinking

Affinity cross-linking was performed as described (Yoon et al., 1996), following BDNF and NRG1-Type III addition into 293T cells. Briefly, cells on plates were washed three times with ice-cold PBS which was supplemented with 1% BSA plus 1 mg/ml of glucose. Following 1 hr incubation at 4°C with BDNF and NRG1-Type III on a shaker, BS3 crosslinker (Pierce) was added at 2 mM final concentration. After an additional 30 min of incubation, the reaction was stopped by adding ice-cold PBS with 50 mM lysine. The cells were washed in the same solution two more times, and the lysates were prepared in RIPA buffer for immunoprecipitation.
**Optiprep density gradient centrifugation**

The lysates (800 μg) from P0-P2 sciatic nerves were prepared using Dounce homogenizer in 25 mM Tris (pH 7.5) and 150mM NaCl plus 10mM NaF, 1μg/ml aprotinin, 10μg/ml leupeptin, 1mM vanadate, and 1mM phenylmethylfulfonyl fluoride. The lysates were then mixed with Optiprep to obtain 40% in final Optiprep concentration. The resulting samples were loaded on the bottom of discontinuous Optiprep gradients, which was overlaid sequentially with 30% and 10% Optiprep. The gradients were centrifuged for 16 hrs at 4°C at 40,000 rpm using SW55 rotor (Beckman). Fractions were collected from the top of the tube and subjected to Western analyses.

**Injection of Fc fusion proteins and retroviruses into neonate mice**

Injection of Fc proteins or retroviruses into P0 mouse sciatic nerves were performed as described (Chan et al., 2006). Briefly, 2 μl of Fc solutions (1 μg/μl) or 5 μl of concentrated retroviruses was injected using a glass-pulled pipet over the sciatic nerve midway between the knee and hip by penetrating through the outer skin. For every injection, one side was injected with experimental reagents, while the other side was injected with appropriate controls in a single mouse. Four days after the injection, the 1 cm sciatic nerve segment surrounding the injection site was isolated for immunohistochemistry and biochemical analyses. For statistical analyses, student t-test was used.
**P75 knockout and wild type mice**

The p75 knockout mice that carried the mutation in exon 3 of the p75 gene (Lee et al., 1992) and the wild type mice were obtained from heterozygote mating as littermates. The mice were back-crossed to C57/BL6 for 10 generations to make them congenic. Their genotype was determined by PCR analyses of tail DNA according to Bentley and Lee (Bentley and Lee, 2000).

**Myelination cultures with p75 knockdown**

Rat Schwann cells were transfected with the control-shRNA or p75-shRNA as described (Yoon et al., 2008). Cells were plated in Ultraculture media (BioWhittiker) supplemented with 10% FBS, 2 mM L-glutamine, and 50 ng/ml NGF at a density of 80,000 cells/2.2 cm² per collagen coated coverslip. Myelination was induced 5 days later by adding 50 μg/ml of ascorbic acid in the growth media. Growth media and ascorbic acid were replaced every 2 days. Following 10 days of treatment, cells were fixed and immunostained for myelin basic protein (MBP). For quantification, MBP⁺ internodes were quantified in a blind manner. For statistical analysis, a student t-test was used.

**EM analyses and quantification**

For TEM of the tissues, the sciatic nerves encompassing the segment from the hip to the knee were dissected (≈ 0.5 cm) four days after retrovirus injections, and divided further into three equal parts using a sharp razor blade. The tissues were fixed for 2 hrs at room temperature with 2% paraformaldehyde/2% glutaldehyde in 0.1M Na cacodylate buffer (pH 7.2), and rinsed in 0.1M Na cacodylate buffer, and placed in 1%
osmium/0.1M Na cacodylate for 1 to 1 1/2 hr at room temperature. The tissues were
stained en bloc for 1 hr in 2% uranyl acetate, embedded in Spurr resin following
dehydration procedures. Sections were cut on a coronal plane at 80 nm using a Reichert
Ultracut E ultramicrotome, and collected on 300 mesh grids. Sections were stained in 2%
uranyl acetate and Reynolds lead citrate before observation in FEI Technai G2 Spirit
TEM at 60kV.

For quantification of myelinated axons, three random images were obtained from
each cross section of the sciatic nerve, and three cross sections per mouse (the number of
images = 36 from 4 mice). The electron photomicrographs were prepared at 2550X
magnification, and the number of myelinated axons were counted using Metaview
software. For statistical analyses, student t-test was used.
RESULTS

BDNF activates Rac1 in Par3-dependent manner.

During development in rat sciatic nerves, RacGTP levels peaked before the onset of myelination at embryonic age 19.5 (E19.5) and then gradually declined, reaching the adult level (Figure 3.1A, B). Since Schwann cells are in constant contact with axons in addition to forming a signaling junction with the extracellular matrix, we localized Rac1 activation by phospho-PAK (p-PAK) immunostaining. Although PAK is known to undergo auto-phosphorylation when bound to Rac1- and Cdc42-GTP (Manser et al., 1997), p-PAK staining represents mostly Rac1 activation at the premyelinating stage, since Cdc42 is not activated until myelination begins (Figure 3.2). In dorsal root ganglion neuron (DRG)-Schwann cell cocultures in which myelination was not initiated, p-PAK immunoreactivity was detected within the Schwann cells on the side that was in contact with DRG fibers (Figure 3.1C, left). Also in postnatal day 3 (P3) sciatic nerves, p-PAK immunoreactivity was asymmetrically localized in relation to the axon (Figure 3.1C, right and inset). These results suggest that at least at the premyelinating stage, signals from the axon induce Rac1 activation in Schwann cells.

We hypothesized that BDNF and/or neuregulin (NRG) 1, the two well-known axonal signals (Michailov et al., 2004; Taveggia et al., 2005; Ng et al., 2007), are responsible for activating Rac1 in vivo. To test the hypothesis, TrkB-Fc or ErbB3-Fc was
injected into sciatic nerves of P0 mice, while the control-Fc was injected into sciatic nerves on the contra lateral side. While the RacGTP levels were readily detected from the control-Fc injected sciatic nerves at P4, they were reduced by 80% with TrkB-Fc injections (Figure 3.1D). ErbB3-Fc injection caused a 70% reduction in RacGTP levels (Figure 3.3A). These results suggest that both BDNF and NRG1 are involved in activating Rac1 at P0-P4. It should be noted that myelin protein, P0, levels were also reduced with TrkB-Fc and ErbB3-Fc, suggesting that the mechanisms by which NRG1 and BDNF promote myelination are likely to include Rac1 activation. In line with the in vivo data, BDNF increased RacGTP levels in Schwann cell cultures (Figure 3.1E). NRG1-Type III also induced Rac1 activation in Schwann cells (Figure 3.3B).

Par3, partitioning-defective protein 3, was shown to be asymmetrically localized on the axon-glial interface during the premyelination stage, recruiting p75 upon BDNF stimulation (Chan et al., 2006). Par3 can also bind RacGEF, Tiam1, and thereby regulate Rac1 activation in epithelial cells (Chen and Macara, 2005). Since the p-PAK expression pattern mimics that of Par3 (Chan et al., 2006), we tested whether Par3 plays a role in BDNF- and/or NRG1-Type III-dependent Rac1 activation in Schwann cells. Par3 was knocked down in Schwann cells using retroviruses carrying Par3 functional RNAi or control, nonfunctional Par3 RNAi (Chan et al., 2006), and the changes in RacGTP levels were measured at various times after treatment with BDNF or NRG1-Type III. Par3 knockdown reduced RacGTP levels to the basal level with BDNF (Figure 3.1F, G), but not with NRG1-Type III (Figure 3.3C, D). The reason for the difference was not clear, since ErbB2 can bind Par3: ErbB2 bound Par3 via PDZ1 and/or 3 domains of Par3 in 293T cells (Figure 3.3E, F). These results therefore suggest that the mechanism by which
BDNF activates Rac1 is distinct from that of NRG1-Type III in Schwann cells. More importantly, these data indicate that Par3 plays a role in distinguishing the two different axonal signals.

**Par3 facilitates the interaction between p75 and ErbB2.**

In epithelial cells, ErbB2 was shown to bind directly to Par6-aPKC complex, rather than Par3 (Aranda et al., 2006). Although ErbB2 interacted with Par3 in 293T cells, it is possible that it does not in Schwann cells or sciatic nerves, thereby obviating the need for Par3 in Rac1 activation by NRG1-Type III. In E19.5 rat sciatic nerves, however, ErbB2 readily co-immunoprecipitated with Par3 (Figure 3.4A). Surprisingly, p75 was present in the same immune complex of ErbB2 and Par3: in immunoprecipitation reactions with ErbB2 and Par3 antibodies, the bottom parts of the membrane were blotted with anti-p75 antibody. In a reciprocal immunoprecipitation with anti-p75 antibody, Par3 and ErbB2 were also present (Figure 3.4A). In further support, we found that ErbB2 was asymmetrically localized to the axon-glial interface in P3 sciatic nerves, as with p75 (Figure 3.4B). In addition, a significant fraction of ErbB2 immunoreactivity colocalized with p75 immunoreactivity, suggesting that the two receptors are likely to interact at the axon-glial interface (Figure 3.4B). These results together indicate that p75 forms a complex with ErbB2 and Par3 in vivo. We hypothesized that Par3 functions as a scaffold, facilitating the complex formation between ErbB2 and p75 by binding each receptor via their PDZ binding motifs at their respective C-termini.
If Par3 is indeed responsible for facilitating the interaction between p75 and ErbB2, the interaction should also be inducible with BDNF, since the interaction between Par3 and p75 was also BDNF-inducible (Chan et al., 2006). Indeed, both Par3 and p75 were present in ErbB2 immune complexes after BDNF treatment in Schwann cells (Figure 3.4C), and Par3 knockdown inhibited the BDNF-dependent interaction of p75 and ErbB2 (Figure 3.4D, E). These results indicate that Par3 is necessary for facilitating the interaction between ErbB2 and p75 in response to BDNF in Schwann cells.

**BDNF activates ErbB2 independently of ErbB3 in Schwann cells.**

Since BDNF facilitates the complex formation between ErbB2 and p75 via Par3, we asked whether BDNF can activate ErbB2 itself, and whether ErbB3 is also involved. ErbB2 can be directly activated without heterodimerization with ErbB3 (Tapinos et al., 2006). Indeed, BDNF activated ErbB2 in Schwann cells, as it increased tyrosine phosphorylation of ErbB2 in time and dose-dependent manners (Figure 3.5A, B). The ability of BDNF to activate ErbB2 appears to be specific to BDNF at least in Schwann cells, as NGF and NT3 failed to induce tyrosine phosphorylation of ErbB2 (Figure 3.6). It should also be noted that the full-length TrkB is not present in Schwann cells (Frisen et al., 1993; Funakoshi et al., 1993; Ng et al., 2007), suggesting that TrkB is not involved in tyrosine phosphorylation of ErbB2 in Schwann cells. In further support, K252a treatment failed to affect the extent of ErbB2 tyrosine phosphorylation by BDNF (Figure 3.5C).

It should be noted that ErbB3 was not recruited to the activated ErbB2, when Schwann cells were treated with BDNF, although it was the case with NRG1-Type III (Figure 3.5A). These results suggest that BDNF-dependent ErbB2 activation is not likely
to involve ErbB3. To test this idea more rigorously, we asked whether ErbB3 becomes activated at all by BDNF in Schwann cells. While ErbB3 underwent rapid and robust tyrosine phosphorylation with NRG1-Type III, it did not with BDNF treatment (Figure 3.5D). In addition, knockdown of ErbB3 inhibited tyrosine phosphorylation of ErbB2 by NRG1-Type III, but not by BDNF (Figure 3.5E). Similarly, Rac1 activation by NRG1-Type III was inhibited with ErbB3 knockdown, but not by BDNF (Figure 3.5F). These results indicate that BDNF activates ErbB2 in a novel way, which does not involve ErbB3 in Schwann cells.

The lack of effect with K252a suggests that BDNF activates ErbB2 via p75 (Figure 3.5C). To directly test whether p75 is necessary for BDNF-induced ErbB2 activation, we knocked down p75 in Schwann cell cultures, and measured BDNF-mediated ErbB2 activation. With p75 knockdown, ErbB2 tyrosine phosphorylation was significantly reduced after BDNF treatment, compared to that in the control (Figure 3.5G), suggesting it is p75 that is responsible for activating ErbB2 in response to BDNF. To test whether p75 activates ErbB2 in vivo, we next measured the extent of ErbB2 tyrosine phosphorylation in P0-P2 sciatic nerves from p75+/+ and p75−/− mice. Indeed, the extent of ErbB2 tyrosine phosphorylation was reduced in p75−/−, compared to that in p75+/+ mice, but the amount of ErbB2 that was immunoprecipitated was also lower in p75−/−, compared to that in p75+/+ mice (Figure 3.5H). This is likely due to a loss of DRG neurons and a coordinate reduction in Schwann cell number in p75−/− mice, since DRG neurons also express p75 in addition to Schwann cells. In order to achieve specific knockdown of p75 in Schwann cells and not in DRG neurons, we delivered the p75-RNAi and control RNAi as retroviruses into P0 sciatic nerves in mice. The p75-RNAi are
specific to p75 (Vilar et al., 2009). In the P0 sciatic nerve, it will be Schwann cells and not neurons that will be infected with retroviruses. The infected Schwann cells were readily identified by their GFP signal, since the virus carries the cDNA for GFP in addition to the RNAi duplexes (Figure 3.5I). Upon knocking down p75 in Schwann cells \textit{in vivo}, the extent of ErbB2 tyrosine phosphorylation was attenuated compared to that in the control RNAi injected sciatic nerve, while total ErbB2 levels did not differ between the two (Figure 3.5J). With these results, we conclude that it is p75 in Schwann cells that is activating ErbB2 in response to BDNF \textit{in vivo}.

\textbf{ErbB2 kinase activity is necessary for BDNF to activate Rac1 in Schwann cells.}

We next asked whether ErbB2 itself and its tyrosine kinase activity are necessary for Rac1 activation by BDNF. With knockdown of ErbB2 using its specific RNAi, RacGTP levels were reduced in response to BDNF (Figure 3.7A, B). In addition, inhibiting ErbB2 kinase activity with 5 \textmu M PKI-166 also blocked Rac1 activation by BDNF (Figure 3.7C, D). Although PKI-166 is an ErbB2/EGF receptor-specific inhibitor, (Mellinghoff et al., 2004; Tapinos et al., 2006), the major target of PKI-166 is ErbB2 in these cells, since the EGF receptor is not expressed in Schwann cells (DeClue et al., 2000): PKI-166 also inhibited NRG1-Type III-dependent Rac1 activation (Figure 3.8).

Since BDNF activates ErbB2, we reasoned that BDNF should also activate the canonical signaling pathway that is typically activated by ErbB2. Indeed, the ERK pathway was activated by BDNF, as well as the PI-3 kinase pathway, as evidenced by phosphorylation of AKT, albeit its extent is much smaller than that by NRG1-Type III (Figure 3.7E). ErbB2 activity was necessary for activation of both the ERK and PI-3
kinase pathways, since inhibiting its kinase activity with PKI-166 attenuated the extent of ERK and AKT phosphorylation by BDNF (Figure 3.7F). These results together indicate that BDNF indeed activates ErbB2 and its downstream signaling pathway in Schwann cells.

**P75 forms a heterodimeric complex with active ErbB2.**

What is the mechanism by which p75 activates ErbB2? We hypothesized that BDNF induces functional, tyrosine phosphorylation competent oligomerization of ErbB2 in the presence of p75 and Par3. To test this possibility, we performed crosslinking experiments in intact 293T cells that were transfected with p75, ErbB2, and Par3 in the presence and absence of BDNF. As controls, parallel cultures were transfected with ErbB2 and ErbB3 and treated with NRG1-Type III. Upon immunoprecipitation with an ErbB2 antibody, a new tyrosine phosphorylated complex appeared when p75 and ErbB2 were introduced together either with or without Par3 (an arrow in Figure 3.9A, lanes 5-8; Note that 293T cells have endogenous Par3). The size of the complex was bigger than that of the monomer but smaller than that of the homo-oligomers of ErbB2 on top of the gel. Reprobing the membrane with a HA antibody revealed that the complex contained p75 and its protein level increased with BDNF treatment, although it was observed only when Par3 levels increased by introducing exogenous Par3 (Figure 3.9A, lanes 7-8). We interpret these results as suggesting that Par3 can facilitate a functional coreceptor complex formation between p75 and ErbB2 in response to BDNF, although the fraction of ErbB2 that associates with p75 is small in 293T cells. Alternatively, the normal endogenous Par3 level may not be sufficient to allow easy detection of p75-ErbB2
interaction in 293T cells.

What would be the mechanism by which Par3 fosters a new type of heterodimer formation? Since Par3 is localized at the axon-glial interface, we reasoned that Par3 might define a subdomain in the Schwann cell membrane where p75 and ErbB2 form a functional coreceptor unit to activate Rac1. In that subdomain, Par3 is likely to be enriched. To address the question, we subjected the lysates from P2-P3 sciatic nerves to Optiprep step density gradients. Optiprep density ultracentrifugation allows isolation of different subdomains in the plasma membrane depending on their lipid contents and lipid types (Shah and Sehgal, 2007; Li and Donowitz, 2008). While p75 and ErbB2 existed in two discrete peaks, fractions 1-2 and 7-8, Par3 was present only in one of the two peaks, fractions 7-8 and not in 1-2, which coincided with p-PAK signals in fractions 7-8 (Figure 3.9B). We interpret these data as suggesting that there exists a subdomain in Schwann cell membranes, which can be demarcated by Par3. Within this unique subdomain, Par3 facilitates the heteromeric complex formation between p75 and ErbB2 independently of NRG1-Type III signal.

To test the hypothesis that ErbB2 exists in two different compartments in the membrane, we treated Schwann cells with both BDNF and NRG1-Type III at the same time, and asked whether ErbB2 still associates with p75 even under simultaneous BDNF and NRG1-Type III stimulation. Indeed, when Schwann cells were treated with both BDNF and NRG1-Type III, ErbB2 still interacted with p75 to the extent similar to that with BDNF treatment alone (Figure 3.9C). These results suggest that ErbB2 can be activated by more than one signal at a given moment. A most likely scenario in which such a phenomenon occurs is that ErbB2 resides in more than one compartment in the
membrane. We suggest that Par3 helps isolate ErbB2 so that it can associate with p75 in the absence of ErbB3 in response to BDNF. As a further support of this notion, Par3 knockdown in Schwann cells led to a reduction in the extent of ErbB2 tyrosine phosphorylation by BDNF treatment, but not by NRG1-Type III (Figure 3.9D). These results together suggest that BDNF activates ErbB2 independently of ErbB3 by forming a heteromeric complex with p75, which is facilitated by Par3 in a unique compartment in the Schwann cell membrane.

Selective knockdown of Par3 in Schwann cells in vivo inhibits polarized Rac1 activation and myelination.

Our biochemical data strongly suggest that it is the p75 in Schwann cells through which BDNF acts to promote myelination by engaging the potent ErbB2 receptor. p75 is, however, expressed both in DRG neurons and in Schwann cells, tending to obscure whether p75 plays a positive role in myelination from the neuronal side or the Schwann cell side. Our discovery of Par3-mediated selectivity for BDNF over NRG1-Type III signaling presents an opportunity for testing whether it is the p75 in Schwann cells that is responsible for its effect in myelination. Accordingly, we knocked down Par3 only in Schwann cells by injecting the retrovirus for Par3-RNAi and its control RNAi to P0 sciatic nerve, and examined whether polarized activation of Rac1 as well as myelination are affected. The effect of Par3 knockdown on Rac1 activation was assessed via p-PAK immunoreactivity.

In the control virus infected Schwann cells, red p-PAK immunoreactivity was polarized to one side of GFP+ Schwann cells (Figure 3.10A inset). Par3 knockdown
resulted in a significant reduction in Rac1 activation (Figure 3.10A), as it did with p75 and ErbB2 knockdown (Figure 3.11, Figure 3.12). These results provide additional evidence that p75, ErbB2 and Par3 regulate Rac1 activation in Schwann cells. When we activated Rac1 constitutively using RacV12 retrovirus, however, Rac1 activity was no longer polarized, but distributed throughout Schwann cells (Figure 3.10A, inset). We found that loss of polarized Rac1 activation by Par3 knockdown impacts myelination; P0 protein levels were reduced by 30% in Par3 knockdown nerves, compared to that in the control nerves (Figure 3.10B, C). Attenuation in myelination by Par3 knockdown is also supported by EM analyses of the infected sciatic nerves; the proportion of the axons that were myelinated was reduced by 15% by Par3 knockdown compared to the control (Figure 3.10D, E). It should be pointed out that the extent of reduction that we observed is likely to be an underestimate, since not all Schwann cells were infected with the virus. We therefore interpret these data as suggesting that the p75-ErbB2-Par3 complex regulates polarized activation of Rac1, thereby regulating certain aspects of myelination in vivo.

**Knockdown of pard3 in zebrafish larvae inhibits mbp expression by disrupting normal wrapping of axons.**

Although the EM data suggest that there are less number of axons myelinated with Par3 knockdown in mouse sciatic nerves, it is not clear at which step Par3 regulates myelination. In order to determine the precise step at which Par3 plays a role during myelination, we utilized fluorescently marked transgenic zebrafish, in which Par3 expression was knocked down by injecting into single cell embryos either of two
different antisense morpholino oligonucleotides (MO1 and MO2) designed to block
translation of *pard3* mRNA. Embryos and larvae injected with MO1 and MO2 generally
had few obvious morphological defects except for a reduction in the size of the eyes and
brain and slight curving of the body axis relative to controls (Figure 3.11A, B). At 4 days
post fertilization (dpf), larvae injected with *pard3* MO had a significant reduction in
endogenous RacGTP levels as well as Pard3 protein (Figure 3.11C). This result indicates
that Par3 regulates Rac1 activation in zebrafish as it does in rodent Schwann cells. We
next investigated whether *pard3* knockdown also attenuates the extent of myelination by
performing in situ RNA hybridization using *mbp* as a marker. In all the larvae that
received no MO (n=31) or a control MO (n=19), *mbp* RNA was readily detected at motor
nerves (Figure 3.11D) and the posterior lateral line nerve (Figure 3.11E). In larvae that
were injected with either MO1 (n=20, Figure 3.11F,G) or MO2 (n=65, Figure 3.11H, I),
*mbp* RNA levels were significantly reduced along both motor nerves and the posterior
lateral line nerves. These results suggest that Par3 regulates Schwann cell myelination
both in zebrafish and rodents.

To investigate the steps at which *pard3* regulates Schwann cell myelination, we
injected MO into one cell stage *Tg(sox10(7.2):mRFP);Tg(olig2:EGFP)* embryos, grew
them until 4 dpf and performed live cell imaging. This transgenic combination marks
motor axons with EGFP driven by *olig2* regulatory DNA (Shin et al., 2003) and Schwann
cells with membrane-tethered RFP driven by *sox10* regulatory DNA (Kucenas et al.,
2008). In control larvae, Schwann cell wrapping of motor axons is initiated by 4 dpf
(Figure 3.11J, arrows). In comparably staged *pard3* injected larvae, Schwann cells
migrated to the motor roots but did not appear to wrap axons tightly (Figure 3.11K,
arrows). These results are consistent with the possibility that in the absence of Par3 signaling, Schwann cells lose polarity and fail to form a tight interaction with the axon that they should myelinate. We therefore conclude that Par3-mediated signaling in Schwann cells is critical for proper myelination by helping to establish and maintain the axonal contact at the time of initial wrapping.

**P75 signaling from Schwann cells to activate Rac1 and promote myelination.**

Although our Par3 knockdown data suggest that BDNF acts on Schwann cells most likely through p75, we can not rule out the possibility that Par3 has an unknown target that plays a role in myelination. We therefore tested whether knocking down p75 itself in Schwann cells affects myelination in vitro and in vivo. First in an in vitro study, we utilized DRG-Schwann cell cocultures, where p75 expression was knocked down by transfecting Schwann cells with the p75-RNAi, prior to seeding them onto pure DRG neurons, as described (Yoon et al., 2008). When the extent of myelination was assessed by staining for myelin basic protein (MBP) on the 10-14th day following ascorbic acid treatment, the number of MBP⁺ fibers was reduced by 62% in the p75 knockdown Schwann cells compared to the control cultures (Figure 3.13A, B). These results suggest that it is the p75 in Schwann cells that plays a role in myelination in vitro.

To study the effect of p75 knockdown in vivo, we opted to inject a retrovirus that carries the same p75-shRNA into mouse sciatic nerves. As with Par3, knockdown of p75 resulted in a significant reduction in p-PAK immunoreactivity as well as myelin basic protein (MBP) staining (Figure 3.13C). In correlation with p-PAK results, RacGTP levels in p75/⁺ mice were reduced by 40% compared to that of the wild type mice at
E18.5 (Figure 3.13D,E). The RacGTP levels at P3 and adult stages were not different between the genotypes. We therefore conclude that p75 promotes myelination from the Schwann cell side, in part by regulating Rac1 activation.
CHAPTER 3

DISCUSSION

Of the receptors in the ErbB receptor family, ErbB2 is unique in being able to interact with a different class of receptors, such as CD44 (Sherman et al., 2000) and plexin B1 (Swiercz et al., 2004). Association with these receptors leads to activation of ErbB2 in response to non-classical ligands, such as hyaluronic acid and semaphorin 4D. These results suggest that although ErbB2 belongs to the ErbB receptor family, it is more promiscuous than others in its ability to respond to a variety of different types of ligands. Indeed, ErbB2 can be directly activated within 5 min upon binding to *Mycobacterium leprae*, which does not involve dimerization with ErbB3 (Tapinos et al., 2006). In the case with *M. leprae*, ErbB2 is hypothesized to form a homodimeric receptor cluster. Here, we present an additional route by which ErbB2 is activated; that is, by forming a receptor complex with a receptor from the TNF-receptor family, p75, through a PDZ-domain containing protein, Par3. This interaction allows ErbB2 to be activated by a distinct class of ligand, BDNF. Why is ErbB2 eliciting BDNF signal in addition to NRG1-Type III in Schwann cells? We believe that by recruiting other receptors, such as p75, ErbB2 gains specificity in how it signals to promote proliferation and/or migration of Schwann cells, and/or wrapping of the axon. Our data suggest that by forming a
coreceptor complex with p75, ErbB2 plays a role in identifying the point along the axon at which axon wrapping should be initiated.

A recent study by Xiao et al. (Xiao et al., 2009) demonstrated that BDNF promoted myelination through p75 from the neuron side, and that knocking down the receptor in Schwann cells had no effect. This is in direct contrast to our results presented here. The reason for the difference is not clear, but it should be noted that the age of the DRG neurons used in the two studies differ. In our study, DRG neurons were isolated at E15 and cultured for a week in the presence of NGF before Schwann cells were added. This procedure approximates the neuronal stage when myelination occurs during normal development. On the other hand, Xiao et al. isolated DRG neurons at P2, and cultured them for 2-3 weeks until they become independent of neurotrophins, before adding Schwann cells. The DRG neurons would essentially be adult neurons. Hence, it is possible that p75 plays a critical role in Schwann cells during myelin formation in development, but in the adult when myelin forms, such as after an injury, the receptor has a more critical role in the neurons. Future studies aimed at conditionally deleting p75 in Schwann cells or neurons will be important to better clarify the role of this receptor.

Here, we identified two signals that can activate Rac1, BDNF and NRG1-Type III, in addition to β1 integrin (Nodari et al., 2007). Of these three different inputs to activate Rac1 in Schwann cells, it is the BDNF-dependent Rac-GTP signal that is located to the axon-glial interface. It is our contention that where Rac1 is activated inside the cell is likely to affect which downstream signaling pathways are to be activated, thereby impacting on a particular step that is involved in myelination. Based on Par3 knockdown results from zebrafish, we propose that Par3-dependent Rac1 activation regulates where
on the axon surface that axon wrapping should commence, while it has little effect on
Schwann cell migration along the axon. These data are in agreement with a report, in
which BDNF was shown to inhibit Schwann cell migration in rat Schwann cells
(Yamauchi et al., 2004). Although it needs to be tested whether Par3 in zebrafish is also
linked to BDNF and p75 signaling as in rodents, we surmise that BDNF signaling in
rodents is likely to play a role in establishing the initial wrapping points along the axon.

Although BDNF has been shown to promote myelination by Schwann cells, its
impact on the overall extent of myelination was perhaps shadowed by the potent NRG1
signaling via the ErbB2/ErbB3 signaling pair. Our data demonstrating that BDNF signals
via ErbB2 suggest that ErbB2 plays a critical role by integrating various signals, thereby
regulating multiple processes that result in compact myelin sheath formation by Schwann
cells. It is plausible that integrin signaling also culminates in ErbB2 activation in
Schwann cells; integrin α6β4 was shown to form a complex with ErbB2 in mammary
carcinoma (Guo et al., 2006). It may then be hypothesized that ErbB2 functions as the
key signaling receptor, which switches its receptor partner depending on the role it needs
to play in the course of Schwann cell myelination, such as proliferation and migration of
Schwann cells, sorting of the axons by Schwann cells, initial contact formation with the
axons, and wrapping of the axons.
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Figure 3.1 BDNF and NRG1-Type III activate Rac1 in sciatic nerves. (A) RacGTP levels are highest at E19.5 in rats and gradually decline during development. (B) Quantification of RacGTP levels in (A). n=6-8. (C) Asymmetric localization of phospho-PAK immunoreactivity in premyelinating Schwann cells in culture and in vivo. Note that phospho-PAK staining is along the side of Schwann cells that were in contact with DRG axons in culture (NF, neurofilament; left two panels, scale bar, 5 μm). Similarly, p-PAK immunoreactivity is found predominantly adjacent to axons in P3 sciatic nerves (right panel, scale bar, 12 μm). The inset shows a high magnification view. (D) Injection of TrkB-Fc, and not control Fc, into the sciatic nerves of P0 mice resulted in inhibition of Rac1 activation and P0 protein levels. Quantification is shown next to the figure. (E) BDNF activates Rac1 in primary Schwann cells. BDNF was added at 50 ng/ml for 1 hr. Quantification is shown below the figures. (F) Par3 is necessary for BDNF-mediated Rac1 activation in Schwann cell cultures. Following infection with the retrovirus carrying Par3 RNAi, Schwann cells were treated with 50ng/ml BDNF for the indicated period of time. Control Par3 Western demonstrates the extent of Par3 knockdown. (G) Quantification of (F). Relative RacGTP levels represent the RacGTP levels adjusted to total Rac1 level in each sample. For statistical analyses, student t-test was used.
Figure 3.1 continued
Figure 3.2 (A, B) A time course of CDC42 activation in developing sciatic nerves in the rat.
Figure 3.3. Although NRG1-Type III activates Rac1 in Schwann cells, Par3 is not involved. (A) Injection of ErbB3-Fc, and not control Fc, into the sciatic nerves of P0 mice resulted in inhibition of Rac1 activation and P0 protein levels. Quantification is shown next to the figure. (B) NRG1-Type III activates Rac1 in primary Schwann cells. Membrane fractions from vector or NRG1-Type III transfected 293T cells were added for 1 hr. Membrane fractions were prepared as described (Taveggia et al., 2005). Quantification is shown below the figures. (C) Knocking down Par3 in Schwann cells failed to inhibit Rac1 activation by 8μg/ml NRG1-Type III. (D) Quantification of (C). For statistical analyses, student t-test was used. (E) Par3 binds ErbB2 in 293T cells. Full-length Par3 and ErbB2 were transfected to 293T cells, and the resulting lysates were subjected to immunoprecipitation with ErbB2 and Western with Par3. Control inputs are shown (5% of the lysates used for immunoprecipitation). (F) ErbB2 binds Par3 via PDZ domains 1 and 3. The individual PDZ domain in Par3 was expressed in 293T along with ErbB2. Note that ErbB2 binds PDZ 1 and 3 but not 2. Control inputs are shown (5% of the lysates used for immunoprecipitation).
Figure 3.3 continued
Figure 3.4 Par3 facilitates the interaction between p75 and ErbB2. (A) Par3, ErbB2 and p75 exist in a complex in sciatic nerves. The lysates from E19.5 rat embryos were processed for immunoprecipitation with Par3, ErbB2, p75, and control IgG. For Par3 and ErbB2 immunoprecipitation, the blots were divided in two, and probed for ErbB2/p75 and Par3/p75, respectively. For p75 immunoprecipitation, the blot was stripped after Par3 Western, and reprobed for ErbB2. Control inputs are shown (5% of the lysates used for immunoprecipitation). (B) Asymmetric colocalization of ErbB2 and p75 in the P3 sciatic nerves. White * denotes the asymmetric staining in NF/ErbB2, NF/p75, and p75/ErbB2 double immunocytochemistry. Scale bar, 10-20μm. The inset illustrates colocalization of p75 and ErbB2 in a higher magnification; the white arrows indicate where the two signals overlapped. Scale bar, 4μm. (C) BDNF induces interaction between ErbB2 and p75 and Par3 in Schwann cells. Control inputs are shown (10% of the lysates used for immunoprecipitation). (D) Par3 is necessary for the interaction between p75 and ErbB2 in response to BDNF. The ErbB2 blot was stripped, cut in half and reprobed for p75. Note that Par3 knockdown reduces the amount of ErbB2 that associates with p75. (E) Quantification of p75-ErbB2 interaction shown in D. For quantification, the values for p75 in BDNF treated samples were divided by the values for untreated samples.
Figure 3.4 continued
Figure 3.5 BDNF activates ErbB2 independently of ErbB3 in Schwann cells. (A) BDNF induces tyrosine phosphorylation of ErbB2 at 50 ng/ml in Schwann cells. ErbB2 was immunoprecipitated and blotted with phosphotyrosine antibodies. For the positive control, Schwann cells were treated with NRG1-Type III. The blot was reprobed for ErbB2 and ErbB3. (B) BDNF-induced activation of ErbB2 is dose-dependent in Schwann cells. BDNF was added at the indicated concentrations for 1 hr. (C) Inhibition of the full-length TrkB with K252a has no effect on BDNF-dependent ErbB2 activation in Schwann cells. K252a was added at 100 nM, 30 min prior to BDNF addition for 1 hr. B, BDNF. ErbB3 is not involved in BDNF-dependent activation of ErbB2. (D) BDNF does not activate ErbB3, while NRG1-Type III does in Schwann cells. (E) Knockdown of ErbB3 inhibits ErbB2 activation by NRG1-Type III, but not by BDNF. (F) Knockdown of ErbB3 inhibits Rac1 activation by NRG1-Type III ($p<0.05$), but not by BDNF. Schwann cells were treated for 1 hr. For statistical analyses, student t-test was used. (G) Knockdown of p75 in Schwann cell cultures inhibited ErbB2 activation by BDNF. (H) ErbB2 activation is attenuated in sciatic nerves of p75 knockout mice compared to that in the wild type. Lysates from P0-P2 sciatic nerves were subjected immunoprecipitation with an ErbB2 antibody and Western blotting for phospho-tyrosine or ErbB2 antibodies. Input controls are also shown. (I) Representative images of the retrovirus infected sciatic nerves. GFP signals indicate circular myelin sheath formed by the Schwann cells that were infected with the retroviruses. Scale bar, 15 μm. (J) Specific knockdown of p75 in Schwann cells also resulted in attenuation of ErbB2 tyrosine phosphorylation at P4 sciatic nerves. The extent of p75 knockdown is shown along with control Western results on ErbB2 and tau.
Figure 3.5 continued
Figure 3.6 (A, B) NGF and NT3 failed to induce ErbB2 tyrosine phosphorylation in Schwann cells.
Figure 3.7 ErbB2 kinase activity is necessary for BDNF-mediated signaling in Schwann cells. (A) Knockdown of ErbB2 inhibits Rac1 activation by BDNF in Schwann cells ($p<0.05$). Schwann cells were treated with BDNF for 1 hr. Also shown are controls, which demonstrate that shRNA of ErbB2 is specific to ErbB2 and not to ErbB3. (B) Quantification of the data in (A). For statistical analyses, student t-test was used. (C) Inhibiting ErbB2 kinase activity results in inhibition of Rac1 activation by BDNF in Schwann cells. Schwann cells were treated with 5µM PKI-166 or control DMSO for 30 min prior to BDNF treatments and RacGTP levels were determined. As a control for PKI-166 effect, the lysates were blotted for p-ErbB2. (D) Quantification of (D). For statistical analyses, student t-test was used. (E) BDNF activates both ERK and AKT in Schwann cells. (F) BDNF-mediated activation of ERK and AKT requires ErbB2 kinase activity. Schwann cells were treated with 5µM PKI-166 or control DMSO for 30 min prior to BDNF treatments.
Figure 3.7 continued
Figure 3.8 PKI-166 inhibits ErbB2 activation by NRG1-Type III. Note that the lack of p-ErbB2 signals correlates with a downward shift in the ErbB2-immunoreactive band.
Figure 3.9 p75 forms a heterodimeric complex with active ErbB2 receptors in intact 293T cells. (A) Formation of a crosslinked, heterodimeric p75-ErbB2 complex on the cell surface. Note that a new heterodimeric complex (arrow) that contains active ErbB2 is formed when p75 is introduced with ErbB2 (lanes 5 to 8). Reprobing the membrane revealed that the complex contained p75, whose levels increased with BDNF treatment in the presence of Par3 (lane 8). B, BDNF; III, NRG1-Type III. (B) In Optiprep density gradients, Par3 is present predominantly in one of the two protein peaks (fractions 7/8), while p75, ErbB2, and ErbB3 segregate into two peaks (fractions 1/2 and 6-8). (C) ErbB2 associates with p75 and ErB3 simultaneously in Schwann cells. (D) Par3 knockdown inhibits tyrosine phosphorylation of ErbB2 by BDNF, but not by NRG1-Type III.
Figure 3.10 Selective knockdown of Par3 among Schwann cells in vivo results in disruption of polarized Rac1 activation and attenuation in myelination. (A) Knockdown of Par3 in vivo inhibits polarized Rac1 activation in Schwann cells. Note that p-PAK immunoreactivity is polarized within Schwann cells (Inset). While p-PAK immunoreactivity is detected throughout the cross section of the sciatic nerve from the control virus infected mice, it is significantly reduced in the nerves that were infected with shRNA carrying retroviruses of Par3. In contrast, RacV12 infection increased p-PAK immunoreactivity throughout the sciatic nerve and disrupted its polarization. Scale bars, 45-47 µm. Inset shows a high magnification view. (B) Knockdown of Par3 in vivo resulted in reduction in P0 protein levels. Also shown is the control Western, Par3 and tau. (C) Quantification of P0 proteins from (B). For statistical analyses, student t-test was used. (D) Representative EM images of the retrovirus infected sciatic nerves at P4. Scale bar, 10µm. (E) Quantification of the proportion of the axons that were myelinated. For statistical analyses, student t-test was used.
Figure 3.10 continued
Knockdown of *pard3* in zebrafish larvae resulted in abnormal Schwann cell wrapping and a significant reduction in *mbp* expression. (A, B) Images of living 2 days postfertilization (dpf) control (A) and *pard3* MO1 (B) injected embryos. The eyes, brain and spinal cord of MO injected embryos are reduced in size. (C) *pard3* MO1 reduces Pard3 protein levels and inhibits RacGTP levels in zebrafish larvae. Embryos at 4 dpf were deyolked, and proteins were extracted for RacGTP assays and control Westerns. (D-I) Lateral views at the level of the trunk of 4 dpf larvae hybridized for *mbp*. In control larvae (D), *mbp* is expressed in stripes associated with ventral motor roots (D, asterisks) and along the posterior lateral line nerve (E, arrow). In *pard3* MO1 (F, G) and MO2 (H, I) injected larvae, *mbp* expression is lost at the ventral roots (asterisks) and the posterior lateral line nerve (arrows), whereas it is still evident in the spinal cord (sc, brackets). (J, K) Lateral views at the level of the trunk of 4 dpf *Tg(olig2:EGFP);Tg(sox10:mRFP)* larvae. Motor axons are green and Schwann cells are red. In a control larva (J), Schwann cells wrap motor axons (arrows) whereas in a MO1 injected larva, Schwann cells failed to align along the axon, forming a loose association with the axon (K). MO-injected larva also has a deficit of axon wrapping by oligodendrocytes, which are marked by RFP expression, in the spinal cord (sc).
Figure 3.11 continued
Figure 3.12 Knockdown of ErbB2 among Schwann cells \textit{in vivo} results in reduction in Rac1 activation as measured by p-PAK immunoreactivity.
Figure 3.13 P75 signals from Schwann cells to activate Rac1 and promote myelination. (A) Knockdown of p75 in rat Schwann cells inhibited the extent of myelination in culture. Also shown are the control Westerns for the p75 knockdown with RNAi. For statistical analyses, student t-test was used. (B) Representative images of MBP staining of the cocultures. Scale bar, 10μm. (C) Knockdown of p75 among Schwann cells reduced the extent of p-PAK immunoreactivity. Note that we are using the same image from Figure 3.10A for the control shRNA infected nerves. Scale bars, 45-47 μm. (D) P75 is necessary for Rac1 activation in vivo. RacGTP levels are reduced in p75−/− mice compared to those in p75+/+ at E18.5. As controls, the protein levels of p75, tau and P0 are also shown. Note that there is a reduction in tau protein levels, reflecting a reduction in DRG fibers. (E) Quantification of RacGTP from (D).
Figure 3.13 continued
GENERAL CONCLUSION

Induction of p75 expression upon pathology and trauma in the adult nervous system makes this receptor an attractive target for therapeutic intervention. However, the versatility of p75 function in mediating cell survival, apoptosis and other various pathways renders it a challenge as well. As a result, it is necessary to identify global details of p75 signaling mechanisms extensively, so as to prevent activation (or inactivation) of non-specific pathways and potentially deleterious side effects. Here, we examined the mechanism by which p75 mediates apoptosis in myelinating oligodendrocytes of the central nervous system after traumatic spinal cord injury. We identified the role of neuronal specific JNK3 stress-activated protein kinase in initiation of the mitochondrial death pathway specifically among oligodendrocytes after injury. Our next step was to disrupt these signaling mechanisms with a nontoxic and minimally invasive non-peptide small molecule by blocking injury specific proNGF binding to p75. We observed functional recovery among specific behavioral parameters that correlate with sparing of ventro-lateral descending tracts, suggesting that this compound is potentially applicable as a therapeutic treatment. Lastly, we examined the role of p75 in the peripheral nervous system during development, and elucidate the mechanism by which p75 initiates myelination. We identified a novel co-receptor complex between p75
and the ErbB2 receptor that formed in response to BDNF. BDNF has been classically defined as a ligand for p75 and TrkB receptors (Chao, 2003; Huang and Reichardt, 2001), but we report that ErbB2 is activated in response to BDNF. Collectively, the diverse roles of p75 in governing myelination in PNS development and apoptosis in CNS adult injury further uncovers the complexity of this receptor in context-dependent situations.

We have revealed contradictory roles for p75 in myelinating Schwann cells and oligodendrocytes. In the PNS, p75 serves as a positive modulator of cell survival, in that this receptor promotes myelination by Schwann cells so as to ensure proper axon development. In the CNS, however, p75 plays a critical role in modulating oligodendrocyte cell death, which contributes to the degenerative pathways that encompass axonal vulnerability. The role of p75 in adult PNS injury has been examined as well. BDNF and P75 expression has been reported to be upregulated in Schwann cells after axonal transection (Hempstead and Salzer, 2002; Zhang et al., 2000). Similarly to developmental myelination, NT3 is down-regulated. Because BDNF inhibits NT3-mediated signaling in order to promote developmental myelination, this suggests that BDNF and p75 may be involved in re-myelinating pathways after injury in the adult PNS. Inhibiting endogenous BDNF signaling after sciatic nerve lesion in mice and rats with BDNF blocking antibody resulted in a dramatic reduction in myelinated distal axons and a reduction in the elongation of regenerative axons (Zhang et al., 2000). This suggests that dichotomous p75 signaling may also be involved in promoting myelination after PNS injury, which may be one mechanism as to why the PNS environment is more conducive to regenerative axon outgrowth than the CNS environment. In p75 null mice, however, there is a decrease in sciatic nerve diameter as compared to wild type control.
(Bentley and Lee, 2000; Von Schack et al., 2001). Consistent with this, there is a reduction in the number of Schwann cells collected from postnatal day 3 mutant sciatic nerves (Von Schack et al., 2001) suggesting that p75 is involved in pathways regulating axon size during developmental pathways. Bentley and Lee, 2000, examined peripheral neurons in embryonic p75 null mice and reported that the absence of p75 resulted in neurite outgrowth defects and Schwann cell migration. It would be interesting to investigate the role of p75 in mature PNS systems with conditional p75 knockout lines, and examine the physiology of peripheral nerves upon down-regulated p75 expression in mature mice.

The aim of our work is to identify molecular therapeutic interventions after spinal cord injury that can prevent extended secondary cell death and aid in survival of spared axons. By down-regulating p75 mediated apoptosis of oligodendrocytes, we hope to preserve the white matter that normally is lost in secondary injury mechanisms distal to the lesion site and prevent the chronic demyelination of axons that persist long after the lesion (Crowe et al., 1997; Liu et al., 1997; Rowland et al., 2008; Shuman et al., 1997). Upon treatment with the small molecule non-peptide NGF mimetic, we observed functional recovery in very specific parameters of behavior. In open-field locomotor tests with the BMS rating scale, mice dosed with 100mg/Kg of LM11A exhibited improvement in coordination and trunk stability (Figure 2.7). Coordination was similarly improved in swimming locomotor tests (Figure 2.8), suggesting that the tracts that govern these tasks were at least partly functional after a 50 kdyne contusion. The ventrolateral tracts that govern these behavioral functions include the medullary reticulospinal and rubrospinal tract in the dorsolateral funiculi (Figure 2.11) (Brosamle et al., 1997; Cao et
al., 2005; Patestas and Gartner, 2006). Immunoreactivity and in situ hybridization expression profiles have localized p75 expression to these tracts after spinal cord injury, suggesting that treatment with LM11A may have blocked p75 mediated apoptosis in these regions and maintained the sparing of white matter (Barrette et al., 2007). Because many contusive injury models impact the spinal cord tissue on the dorsal surface, there is a mixture of dying and surviving axons in the ventral and lateral funiculus, which is where we presume our compound is blocking p75 function (Crowe et al., 1997). The repercussions of oligodendrocyte cell death in these areas of spared axons are therefore more dramatic, since maintaining white matter is critical in preventing further temporal loss of function from degenerative secondary injury mechanisms. As a result, the efficacy of this compound is dependent on the number of spared, demyelinated axons present in the ventro-lateral funiculus. The extent of chronic axonal demyelination in human spinal cord injury is quite heterogeneous; therefore treatments targeting remyelination among human patients will be highly variable depending on the distinctive characteristics of each lesion (Rowland et al., 2008). Collectively, LM11A may function as an effective means of blocking proNGF mediated oligodendrocyte apoptosis in the spared white matter surrounding the ventrolateral tracts, thus preventing degeneration of these descending pathways and result in recovery in motor control of trunk and limbs.

It is necessary to address the potential of non-specific actions related to the dichotomous roles that p75 plays, in that this receptor can regulate opposing pathways in different environments upon binding of context-specific cognate ligands. In correlation with this, distinct spatiotemporal patterns of degenerating fiber tracts were localized to the same regions where p75 expression was upregulated. Specifically, degeneration of
ascending tracts was restricted to regions rostral of the lesion and degeneration of
descending tracts was restricted to regions caudal to the lesion (Crowe et al., 1997). In
addition to observing p75 in the sensory fibers in the dorsal horn and motoneurons in the
ventral horn of the gray matter, we detected p75 expression in the fasciculus gracilis
(ascending pathway tract) rostral to the lesion site after contusion as well (Figure 2.11).
If p75 expression in the fasciculus gracilis governs pain pathways, then LM11A blocking
p75 may block these pathways, which may explain why there is no increased sensitivity
upon treatment in our hands (Figure 2.6). Observing p75 expression in the same regions
where degenerating tracts are localized after spinal cord injury gives further support for
the diversity of p75 function after trauma.

Treatment with LM11A may impinge on p75/NgR (Nogo receptor) mediated
inhibitory pathways in myelin, which may also improve neurite outgrowth of
regenerating axons after injury. In an attempt to disrupt the myelin-derived axon
outgrowth inhibitory action of NgR, the Strittmatter group has published work
demonstrating the use of an antagonist peptide to block the binding of Nogo-66 ligand.
They identified the residues (NEP1-40) that blocked Nogo-66 or CNS myelin mediated
inhibition of chick DRG neurite outgrowth in vitro, suggesting that this peptide functions
as a competitive antagonist to NgR inhibitory function (Grandpre et al., 2002). Further
application of this peptide in vivo exhibited axon sprouting upon local intrathecal
administration after thoracic spinal cord hemisection. Specifically, the authors observed
anterogradely labeled corticospinal tract fibers caudal to the lesion from both the dorsal
lesioned and interestingly, an increased number of ventral unlesioned corticospinal tract
fibers. The authors also demonstrated functional open-field locomotor recovery, in that
BBB scores among NEP1-40 treated mice were higher as soon as four hours after injury (Grandpre et al., 2002). Because this peptide blocked NgR signaling, it potentially inhibited the action of the three myelin inhibitory factors, Nogo-66, OMgp and MAG, making it an effective therapeutic treatment. However, axon sprouting was limited to only the corticospinal and raphespinal tract fibers. Also, intrathecal delivery of this peptide is not feasible for application as complications associated with intrathecal therapy preclude extensive use in humans (Knox et al., 2007; Protopapas et al., 2007). We address these shortcomings with oral administration of our compound as a less invasive mode of delivery. It will be necessary to examine regeneration in these fiber tracts to determine if LM11A treatment prevents p75-mediated inhibitory pathways after injury.

Another attempt to neutralize myelin growth inhibitors include work by the Schwab group, where IN-1 monoclonal antibody raised against a myelin fraction enriched in Nogo-A was been reported to block Nogo-A mediated inhibition of neurite growth. In vivo administration of this antibody induced regeneration of adult rat lesioned corticospinal tract and optic nerve fibers, and overall compensatory sprouting and anatomical reorganization associated with recovery in functional behavior (Bareyre et al., 2002; Brosamle et al., 2000; Raineteau et al., 2001). In disagreement with these in vivo and in vitro experiments, however, IN-1 treatment did not affect outgrowth of cultured adult DRG neurons in the presence of mature, differentiated oligodendrocytes, suggesting that Nogo-A is not the main inhibitory factor that blocks sensory regeneration in adult spinal cord injury models (Oudega et al., 2000). Application of this neutralizing antibody did not promote regeneration of ascending sensory axons across a nerve graft back into the spinal cord of rats and also did not augment NGF-mediated sensory regeneration in
this model as well (Oudega et al., 2000). The authors had previously shown that a conditioning lesion of the peripheral nerve projecting into sensory neurons contributes to sensory neuron regeneration, and this conditioning lesion was most effective when performed one week prior to spinal cord injury (Oudega et al., 1994). Though the authors demonstrate axonal regeneration in this case, a conditioning lesion is not therapeutically viable for human clinical application.

Extensive published research into p75 function has exposed contradictory pathways that depend on environment, cell type and developmental stage. The role of p75 in oligodendrocyte cell death after injury in the adult system is well established, and we defined the mechanistic role of the stress activated protein kinase, JNK3, in mediating this cell death. We aimed to block this apoptotic action by developing an efficient and effective compound that can bind to p75 specifically. Although we observe functional recovery in specific behavioral parameters upon treatment with this compound after contusion injury, we find that this compound may impinge on several other pathways that are governed by p75. It will be important to investigate these pathways further, to ensure that application of this compound does not impinge on unknown facilities that p75 may possess. Likewise, in our study of p75 in development, we discovered a novel interaction between p75 and the neuregulin receptor, ErbB2, further supporting the fact that there is still much more to learn about this receptor. In conclusion, we define the mechanisms by which p75 promotes myelination in the developing PNS and governs oligodendrocyte cell death upon injury in the adult CNS.
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