P74 NTR and its Role in Regeneration: Potential Neuroprotective Effects

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Summary

p75NTR is an important neurotrophin receptor and plays a crucial role in many diverse functions in neurons. In the peripheral nervous system (PNS), p75NTR is known to enhance myelin formation and promote regeneration when it binds to various neurotrophins. However, in the central nervous system (CNS), p75NTR expression affects neuron survival in contrasting fashions depending on the ligand it binds and the other receptors with which it interacts. Recent evidence points to p75NTR as a potential neuron survival promoting receptor when activated in tandem with the apoptotic p75NTR pathway induced by NGF and nitric oxide (NO). Whether p75NTR expression can promote neuron survival or regeneration in a growth inhibitory and apoptotic environment such as the CNS remains unknown. We hypothesize that over-expression of p75NTR will increase neuron survival under otherwise apoptotic and growth inhibitory conditions. To support this hypothesis, we will over- and under-express p75NTR in CNS and PNS neurons both in vitro and in vivo. In vitro experiments will apply PNS and CNS specific neuron cultures with siRNA induced p75NTR under-expression or promoter induced p75NTR over-expression in the presence and absence of p75NTR ligands. In vivo experiments will apply a tetracycline induced Cre/LoxP mouse model to over and under express p75NTR and its ligands in both PNS and CNS. We will measure neuron survival using azino-bis (ethylbenzothiazoline-6-sulphonic acid) enzyme-linked immunoabsorbant assay and the CellTiterGlo ATP assay. Our experiments should reveal a possible means of preventing CNS neuron death in cases of injury or neurodegeneration.

Introduction

Damage of the central nervous system (CNS) is among the most debilitating of ailments, made worse by the inability of tissue to repair itself. Neurons have limited ability to replace damaged axons and dendrites caused by injury or disease. Nervous tissue is virtually incapable of neurogenesis, and adult neurons rarely grow new axons once damaged (Bolsover, Fabes, and Anderson, 2008). An exception is the regrowth and reinnervation of peripheral axon specializations in the skin or at neuromuscular junctions (Temporin, et al., 2008). Three primary barriers restrain regenerative capacity. Foremost, local injury to brain tissue often results in cell death. Second, aggregation of glial cells at the injury site actively inhibits axon growth over long distances. Lastly, adult neural stem cells possess limited ability to divide, migrate, and differentiate. Still, phenomena illustrating nervous system repair lend credence to the scientific pursuit of inducing nervous system regeneration.

Neuroregeneration is the repair of nervous tissues and cells (Zheng, 2005). The mechanisms underlying regeneration include re-myelination, generation of new neurons, glial cells, lengthening of axons, and restoration of synapses. Neuroregeneration differs between the peripheral nervous system (PNS) and the CNS in the cellular mechanisms responsible for repair. In the PNS, regeneration occurs by the formation and growth of axonal sprouts mediated by chemotactic factors secreted from Schwann cells. When injury occurs, macrophages and Schwann cells migrate to the site and clear damaged tissue, permitting new axon outgrowth. In the CNS, myelin-based growth inhibitors (MBGIs) bind receptors such as p75NTR to prevent regeneration.

Damage to the CNS occurs in several ways, including physical trauma, hypoxia, and neurodegenerative diseases such as Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). In these neurodegenerative diseases, there is no regeneration of myelin, which coats the nerve axon, and the axons are unable to grow. Ailments caused by the inhibition of regeneration are often due to factors secreted by glial cells inhibiting axon re-myelination and repair. A limited regenerative response to injury exists in the CNS. Adult CNS axons can regenerate under suitable conditions, such as a growth-permissive peripheral nerve graft (Casaccia-Bonnefil, 1998). However, *in vivo* CNS post-injury conditions predominantly either promote apoptosis, or prevent neurite outgrowth. Additionally, oligodendrocytes express three primary growth inhibitory ligands (MBGIs) in the myelin sheaths they produce around CNS axons (Liu, et al., 2006), while PNS Schwann cells only express one (Domeniconi, 2002). MBGIs include Nogo-A, oligodendrocyte myelin glycoprotein (OMgp), and myelin associated glycoprotein (MAG), which is the only inhibitory ligand expressed in both the CNS and the PNS. Post CNS injury, macrophages fail to effectively clear inhibitory myelin debris. This results in glial cell aggregation around the injury site, which further inhibits outgrowth. Previous studies have shown that antagonizing Nogo-A expression can facilitate minor axon regeneration *in vitro*; however, other MBGIs continue to block growth (Dupuis, et al., 2008).

Identification of the MBGI receptors represents another possible approach towards promoting CNS regeneration. One such receptor is the multi-functional p75 neurotrophin receptor (p75NTR). p75NTR is at the heart of key biological processes involved in neuron survival and death decisions, axonal growth, and responses to environmental stimuli (Brooke, 1999). p75NTR expression impacts neuron survival in contrasting fashions depending on the ligand it binds and the receptors with which it interacts. When p75NTR associates with a tyrosine receptor kinase (Trk), the cell binds nerve growth factor (NGF), which promotes neuron survival (Casaccia-Bonnefil, 1998). Conversely, p75NTR induces apoptosis when activated by NGF and nitric oxide (NO) in tandem (Casaccia-Bonnefil, 1998; Lee et al., 2001). Furthermore, a disparate Nogo receptor (NgR)/p75NTR complex binds the MBGIs (Domeniconi, 2002; Wang et al., 2002). Most recently, Dupuis *et al.* (2008) revealed that crosstalk exists between the activated NGF/NO receptor complex and NgR/p75NTR complex to promote neuron survival under otherwise apoptotic conditions.

Additionally, the p75NTR receptor complex's post-injury activation phenotypes differ between the CNS and PNS. In the PNS, p75NTR/Trk complex is most prevalent and is responsible for promoting neuron survival via neurotrophin binding (Barbacid, 1995). Upon injury to the

*This author wrote the paper for Biology 480: Neural Frontiers taught by Dr. Shubhik DebBurman.

PNS nerve, p75NTR and Trk expression increases on the newly developed growth cone (Roux et al., 1999). However, the absence of OMgp and Nogo-A in the PNS could be responsible for lower NgR/p75NTR complex activation. Clearing of MAG saturated myelin debris by macrophages after nerve injury in the PNS alleviates inhibitory activation of the NgR/p75NTR complex (Domeniconi, 2002). Conversely, the CNS never overcomes MBGI expression to promote regeneration. However, to ensure neuron survival post-injury this could allow eventual recruitment of regeneration-inducing trophic Trk receptors to the site of axon damage. We already know that dual activation of p75NTR by NGF/NO and MBGIs effectively promotes neuron survival (Dupuis, et al., 2008). Thus, we ask whether over-expression of p75NTR augment this neuron survival effect in the CNS and PNS. With this in mind, we hypothesize that the over expression of p75NTR in the CNS and PNS will promote neuron survival in vitro and induce regeneration after injury in vivo.

Relevance

Each year, over two million head injuries occur in the United States. Of these two million, 300,000 require hospitalization, 100,000 must live with permanent disabilities and 56,000 are expected to die. The disabilities and deaths caused by traumatic brain injuries account for about a third of all injury-related deaths in the United States (Sosin, et al., 1991). Many of these permanent disabilities may be prevented if a therapeutic solution promoting CNS regeneration existed. The magnitude of trauma injuries combined with the cost of care and familial loss make regenerative therapy a more economic solution than current treatments. Thus, progress towards a therapeutic treatment benefits both the victims of CNS injury and their families.

To progress in this field, scientists must elucidate the pathways mediating CNS regeneration. From there, clinical trials may guide an effective pharmacological therapy. Our basic research on the value of p75NTR expression in CNS regeneration will pave the road on which to drive development of a therapeutic solution. We firmly believe that p75NTR plays an integral role in facilitating regeneration, and that better understanding its functions will ameliorate the lives of countless brain trauma victims, as well as those of their friends and family.

Specific Aims

This proposal implicates a means to promote CNS neuron survival and regeneration post-injury according to functional PNS regenerative mechanisms. We confidently hold that elucidation of the p75NTR pathway among injured neuronal cells represents a valuable therapeutic target for CNS regeneration. Thus, we hypothesize that over-expression of p75NTR in the CNS and PNS will promote neuron survival in vitro and induce regeneration after injury in vivo.

1. To manipulate expression and activation of p75NTR via injured in vitro CNS neuronal tissues.
P75NTR expression will be knocked-down, knocked-out, and increased in three CNS cell lines, respectively using siRNA and an overactive CMV promoter to express p75NTR. We will co-culture cells with Nogo-A and oligodendrocytes to assess neuron survival in the presence of inhibitory ligands. Neuron survival will be assessed using an enzyme-linked immunoabsorbant assay (ABT/ELISA) and CellTiterGlo ATP assay.

2. To manipulate expression and activation of p75 NTR via injured in vivo CNS mouse models:
In conditional knockout and over-expression mouse models for both p75NTR and Nogo we will determine if p75NTR allows for regeneration in the CNS. Expression will be manipulated using Cre/loxP technology. Neuron survival will be assessed using an enzyme-linked immunoabsorbant assay (ABT/ELISA) and CellTiterGlo ATP assay.

3. To manipulate expression and activation of p75NTR via injured in vitro PNS neuronal tissues:
p75NTR expression will be knocked out and increased in three PNS cell lines, respectively using p75NTR siRNA and an overactive CMV promoter to express p75NTR. We will co-culture neurons with MAG and Schwann cells to assess neuron survival in the presence of inhibitory ligands. Neuron survival will be assessed using an enzyme-linked immunoabsorbant assay (ABT/ELISA) and CellTiterGlo ATP assay.

4. To manipulate expression and activation of p75NTR via injured in vivo PNS mouse models:
In conditional knockout and over-expression mouse models for both p75NTR and Nogo we will determine if p75NTR allows for regeneration in the PNS. Expression will be manipulated using Cre/loxP technology. Neuron survival will be assessed using an enzyme-linked immunoabsorbant assay (ABT/ELISA) and CellTiterGlo ATP assay.

Research Design & Methods

AIM 1: To manipulate p75NTR expression and activation via in vitro CNS cell cultures.

1. Rationale: Damage to the CNS induces apoptosis among the damaged neurons and inhibits regeneration. However, if we over-express p75NTR, neuron survival should increase due to greater amounts of survival promoting crosstalk between apoptotic and growth inhibitory p75NTR pathways. We will support the functionality of this neuron survival mechanism among primary hippocampal and neocortical neuron cultures, as well as an induced hybrid motor neuron cell line, NSC-34. These cultures represent extensively researched CNS culture types of neurons injured by neurodegenerative diseases such as Alzheimer's and multiple sclerosis.
2. Design and Method: We will purify primary hippocampal and neocortical cultures from anesthetized, adult mouse brains (Kerwin, et al., 1993; DeFreitas, McQuillen, and Shatz, 2001) and independently culture NSC-34 cells. A COS cell line, which lacks p75NTR expression, will be used as a negative control. To establish baseline p75NTR expression levels using immunohistochemistry among cell lines, we will perform *in situ* hybridization according to Corriveau, et al. (1998) with rodent specific probes for anti-p75NTR, with an anti-β-actin probe as a positive control (DeFreitas, McQuillen, and Shatz, 2001).

   To produce p75NTR loss of function cells, we will deplete p75NTR transcripts using siRNA sequences and plasmid transfection technique employed by Johnston, et al. (2007). A randomized siRNA sequence vector will serve as a negative control. Preliminary p75NTR siRNA experiments will reveal the appropriate transfection dosage to mimic PNS expression. These siRNA experiments will knockdown p75NTR expressions to PNS levels, and deplete p75NTR. We will confirm transfection by

immunoblotting with an anti-mouse monoclonal antibody after tagging cells with mouse anti-p75NTR, using β-actin as a positive loading control (Johnston, et al., 2007). Gain of function mutants will be produced by transfecting cell lines with p75NTR cDNA plasmids with overactive CMV promoters.

To assess whether over- or under-expression of p75NTR effectively promotes neuron survival, we will co-culture the p75NTR depleted and p75NTR gain of function neurons with exogenous sources of apoptotic factors NGF and NO, as well as either an artificial or natural source of MBGI, soluble Nogo-A or oligodendrocytes, respectively (Dupuis, et al., 2008). The addition of soluble brain derived neurotrophic factor (BDNF) and neurotoxic kainic acid (KA) to cultures are respectively positive and negative cell survival controls (Brooke, 1999; Young, Bilsand, and Harper, 1999). We will assess neuron survival using a 2,3'-azino-bis (ethylbenzothiazoline-6-sulphonic acid) enzyme-linked immunoabsorbant assay (ABT/ELISA) and CellTiterGlo ATP assay. We will conduct two-way analysis of variance (ANOVA) using SPSS 13.0 data analysis software to compare neuron survival between p75NTR expression and ligand co-culture conditions.

Predictions**:** Loss of function mutant cell lines produced by p75NTR siRNA knockout should have no effect on neuron survival under any co-culturing conditions, as no ligand could activate p75NTR. In p75NTR knockdown cell lines mimicking the PNS, reduced neuron survival should occur in all co-cultures. This is expected due to increased probability of NGF and NO binding. However, among p75NTR gain of function cells we expect neuron survival to increase through increased MBGI-p75NTR binding. Contrary to our hypothesis, decreased cell survival upon p75NTR over-expression would suggest greater NGF than MBGI binding to p75NTR. A stronger p75NTR binding affinity by NGF than MBGIs would explain such alternative results.

AIM 2: To manipulate expression and activation of p75 NTR via in vivo CNS mouse models.

1. Rationale: To further test p75NTR's effect on neuron survival, our research uses a conditional knockout and over-expression mouse model for p75NTR and Nogo so as to examine central nervous system neuronal cells *in vivo*. The Cre/loxP system is a way to conditionally knockout or over-express genes in a site specific and time specific fashion to prevent lethality or other complications that occur in mice with conventional knockouts (Neumann, 1998). The Cre/lox system has the advantage of working in almost every type cell demonstrating its complete versatility and usefulness in this experiment.
2. Design and Method:

a) Injury and Analysis: Tetracycline will be injected into the crossed Cre/loxP mouse model one day prior to injury in order to induce knockout of p75NTR. A spinal chord dorsal hemisection (Li and Strittmatter, 2003) will be performed in order to injure the CNS of the Cre/loxP crossed mouse models five days after birth.

To assess whether over- or under-expression of p75NTR effectively promotes neuron survival, we will co-culture the p75NTR depleted and p75NTR gain of function neurons with exogenous sources of apoptotic factors NGF and NO, as well as either an artificial or natural source of MBGI, soluble Nogo-A or oligodendrocytes, respectively (Dupuis, et al., 2008). The addition of soluble brain derived neurotrophic factor (BDNF) and neurotoxic kainic acid (KA) to cultures are respectively positive and negative cell survival controls (Brooke, 1999; Young, Bilsand, and Harper, 1999). We will assess neuron survival three, five and seven days after injury using a 2,3'-azino-bis (ethylbenzothiazoline-6-sulphonic acid) enzyme-linked immunoabsorbant assay (ABT/ELISA) and CellTiterGlo ATP assay. We will conduct multivariate analysis of variance (MANOVA) using SPSS 13.0 data analysis software to compare neuron survival dependence on time, p75NTR expression, and ligand co-culture conditions.

b) Cre/loxP Mouse Models: To produce a conditional knockout of p75NTR in a mouse model, we will use the CNS growth-associated gene promoter (GAP-43; Zhang, 2005) with a tetracycline inducible Cre system (Sun, Chen, and Xiao, 2007) to allow for spatial and temporal control of p75NTR expression *in vivo*. In this case, a conditional knockout of p75NTR should facilitate functional genetic analysis in mouse models. We will conditionally under-express p75NTR using a mouse with Cre recombinase protein mediated by the GAP-43 promoter. The Cre mouse will then be crossed with another mouse containing a loxP flanked p75NTR gene with tetracycline-controlled expression. The mice will be deficient in p75NTR expression, except as mediated by the Cre/loxP system.

In order to create conditional over-expression of p75NTR in an injured neuronal cell, we will use the CNS growth-associated gene promoter (GAP-43; Zhang, 2005) with a tetracycline inducible Cre system (Sun, Chen, and Xiao, 2007) to allow for spatial and temporal control of p75NTR expression *in vivo.* In this case a conditional over-expression of p75NTR should facilitate functional genetic analysis in mouse models. To conditionally over-express p75NTR, a mouse with the Cre recombinase mediated by the GAP-43 promoter will be crossed with a mouse containing a p75NTR gene with the Tet-off system. The coding region of this gene will be separated from its promoter by a floxed 'stop' segment, which would be excised in the presence of active Cre recombinase. In this model, native p75NTR gene is expressed normally by the mouse along with the Cre/loxP gene when activated.

Predictions: Conditional under-expression of p75NTR should promote neuron cell death after injury and conditional over-expression of p75NTR should promote neuron survival after cell death. We project that in tissues in which p75NTR is under expressed we will ascertain that neuron survival is limited or non-existent. In tissues in which p75NTR is over-expressed we will ascertain that neurons are surviving *in vivo*. Lack of regeneration *in vivo*, but presence of neuron survival *in vitro,* would suggest that growth and survival represent independent signaling pathways, the former, p75NTR would not influence. Lack of regeneration and neuron survival would imply greater activation of p75NTR by NGF/NO, as mentioned in *AIM 1* Predictions**.**

AIM 3: To manipulate expression and activation of p75NTR via injured in vitro PNS neuronal tissues.

1. Rationale: Contrary to the CNS, damage to the PNS can be repaired. The expression of p75NTR is implicated in these regenerative capabilities by promoting neuron survival. Hence, if we over-express P75NTR neuron survival should increase relative to baseline and under-expressed levels of p75NTR. We will perform *in vitro* experiments in a primary culture of cervical ganglion (SCG), and induced cultures of muscular dorsal root ganglion (DRG) cells and ciliary ganglia cells. Each culture represents functionally unique regions of the PNS known to express p75NTR.

2. Design and Method: We will independently culture DRG and ciliary ganglia cells. A primary culture of SCG cells will be purified from anesthetized, adult mouse brains. A 3T3 cell line, which lacks p75NTR, will be used as a negative control. To establish baseline p75NTR expression levels using immunohistochemistry, we will perform *in situ* hybridization according to Corriveau, et. al. (1998) with rodent specific probes for anti-p75NTR, with an anti-β-actin probe as a positive control (DeFreitas, McQuillen, and Shatz, 2001).

To produce p75NTR loss of function cells in the PNS, we will deplete p75NTR transcripts using siRNA sequences and plasmid transfection technique employed by Johnston, et al. (2007) as we did in the CNS. A randomized siRNA sequence vector will serve as a negative control. Preliminary p75NTR siRNA experiments will be preformed to determine the appropriate amount necessary to eliminate p75NTR. The cells will be tagged with mouse anti-p75NTR in order to confirm the transfection by immunoblotting with an anti-mouse monoclonal antibody. β-actin will serve as a positive loading control (Johnston, et al., 2007). In order to produce P75NTR gain of function, mutant cell lines will be transfected with p75NTR cDNA plasmids with an overactive CMV promoter. The p75NTR immunoblotting technique will confirm p75NTR over-expression.

To assess whether over- or under-expression of p75NTR effectively promotes neuron survival, we will co-culture each PNS cell line with and without either soluble MAG or pre-cultured Schawnn cells (Tebu-Bio). These will be paired with or without the addition of soluble NGF (Dupuis, et al., 2008). The addition of soluble brain derived neurotrophic factor (BDNF) and neurotoxic kainic acid (KA) to each control culture will serve as respective positive and negative controls for cell survival (Young, et al., 1999; Brooke, et al., 1999). We will assess neuron survival using a 2,3'-azino-bis(ethylbenzothiazoline-6-sulphonic acid) enzyme-linked immunoabsorbant assay (ABT/ELISA) and a CellTiterGlo ATP assay. We will conduct two-way analysis of variance (ANOVA) using SPSS 13.0 data analysis software to compare neuron survival between p75NTR expression and ligand co-culture conditions.

Predictions: It is expected that if p75NTR expression is eliminated, then there will be a significant decrease in neuron regeneration. If p75NTR expression is increased, it is expected that there will be a significant amount of neuron regeneration. However, if there is not a significant amount of neuron survival seen after p75NTR expression is increased, it might mean that there is an alternate pathway that is promoting regeneration.

AIM 4: To manipulate expression and activation of p75NTR via in vivo PNS mouse models.

1. Rationale: To further test p75NTR's effect on neuron survival our research uses a conditional knockout and over-expression mouse model for p75NTR so as to examine peripheral nervous system neuronal cells *in vivo*. The Cre/loxP system is a way to conditionally knockout or over-express genes in a site specific and time specific fashion to prevent lethality or other complications that occur in mice with conventional knockouts (Neumann, 1998). The Cre/lox system has the advantage of working in almost every cell type demonstrating its complete versatility and usefulness in this experiment. Contrary to our hypothesis, decreased cell survival upon p75NTR over-expression would suggest greater NGF than MBGI binding to p75NTR. A stronger p75NTR binding affinity by NGF than MBGIs would explain such alternative results.
2. Design and Method:

a) Injury and Analysis: Tetracycline will be injected into the crossed Cre/loxP mouse model one day prior to injury in order to induce knockout of p75NTR. A spinal chord dorsal hemisection (Li and Strittmatter, 2003) will be performed in order to injure the CNS of the Cre/loxP crossed mouse models five days after birth.

To assess whether over- or under-expression of p75NTR effectively promotes neuron survival, we will co-culture the p75NTR depleted and p75NTR gain of function neurons with exogenous sources of apoptotic factors NGF and NO, as well as either an artificial or natural source of myelin-based growth inhibitor (MBGI), soluble Nogo-A or oligodendrocytes, respectively (Dupuis, et al., 2008). The addition of soluble brain derived neurotrophic factor (BDNF) and neurotoxic kainic acid (KA) to cultures are respectively positive and negative cell survival controls (Brooke, 1999; Young, Bilsand, and Harper, 1999). We will assess neuron survival three, five and seven days after injury using a 2,3'-azino-bis(ethylbenzothiazoline-6-sulphonic acid) enzyme-linked immunoabsorbant assay (ABT/ELISA) and CellTiterGlo ATP assay. We will conduct multivariate analysis of variance (MANOVA) using SPSS 13.0 data analysis software to compare neuron survival dependence on time, p75NTR expression, and ligand co-culture conditions.

b) Cre/loxP Mouse Models: To produce a conditional knockout of p75NTR in a mouse model, we will use the nestin promoter (Kramer, et al., 2006) with a tetracycline inducible Cre system (Sun, Chen, and Xiao, 2007) to allow for spatial and temporal control of p75NTR expression *in vivo*. In this case a conditional knockout of p75NTR should facilitate functional genetic analysis in mouse models. We will conditionally under express p75NTR using a mouse with Cre recombinase protein mediated by the GAP-43 promoter. The Cre mouse will then be crossed with another mouse containing a loxP flanked p75NTR gene with tetracycline-controlled expression. The mice will be deficient in p75NTR expression, except as mediated by the Cre/loxP system.

In order to create conditional over-expression of p75NTR in an injured neuronal cell, we will again use the nestin promoter (Kramer, et

al., 2006) with a tetracycline inducible Cre system (Sun, Chen, and Xiao, 2007) to allow for spatial and temporal control of p75NTR expression *in vivo*. In this case a conditional over-expression of p75NTR should facilitate functional genetic analysis in mouse models. To conditionally over-express p75NTR, a mouse with the Cre recombinase mediated by the GAP-43 promoter will be crossed with a mouse containing a p75NTR gene with the Tet-off system (Sun, Chen, and Xiao, 2007). The coding region of this gene will be separated from its promoter by a floxed 'stop' segment, which would be excised in the presence of active Cre recombinase. In this model, native p75NTR gene is expressed normally by the mouse along with the Cre/loxP gene when activated.

Predictions: Conditional under-expression of p75NTR should promote neuron cell death after injury and conditional over-expression of p75NTR should promote neuron survival after cell death. We project that in tissues in which p75NTR is under-expressed we will ascertain that neuron survival is limited or non-existent. In tissues in which p75NTR is over-expressed we will ascertain that neurons are surviving *in vivo*. Lack of regeneration *in vivo*, but presence of neuron survival *in vitro* would suggest that growth and survival represent independent signaling pathways, the former, p75NTR would not influence. Lack of regeneration and neuron survival would imply greater activation of p75NTR by NGF/NO, as mentioned in *AIM 1* Predictions.

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