EARLY EFFECTS OF ORGANOPHOSPHATE COMPOUNDS ON *IN VITRO* INTRACELLULAR SIGNALING AND LEVELS OF ACTIVE NEUROTROPHIN RECEPTORS, AND ON *IN VIVO* NEUROTROPHIN CONCENTRATIONS

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ABSTRACT

Organophosphorus (OP) compounds are found in household pest control products, plastics, and petroleum. Due to the neurotoxic nature of OP compounds, exposure can cause both acute and delayed symptoms, including organophosphate-induced delayed neuropathy (OPIDN). This syndrome is characterized by Wallerian-like degeneration of nerves in the central and peripheral nervous system after exposure to neuropathic OP compounds. There are many questions surrounding the mechanisms of the onset of OPIDN, including possible alterations in proteins associated with neuronal maintenance and repair. This dissertation investigated the changes in levels of neurotrophins *in vivo* and how *in vitro* levels of neurotrophin receptors and their downstream signaling cascades are affected after exposure to OP compounds. We also characterized the molecular weight of a soluble factor responsible for inducing neurite outgrowth *in vitro* after *in vivo* exposure to a neuropathic OP compound. We evaluated in vivo endpoints using enzyme-linked immunosorbant assays. Results indicated that nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are found in chicken spinal cord but do not increase as a result of exposure to neuropathic OP compounds. This study also noted that NGF, BDNF, and NT-3 concentrations were not altered after exposure to a non-neuropathic OP compound. We evaluated *in vitro* endpoints using Western blots, ultrafiltration, and digital morphometry. These studies revealed that activated forms of high-affinity and low-affinity neurotrophin receptors are present after OP compound exposure, that the ratio of these two receptors to each other is stable after OP compound exposure, and that the activated form of the low-affinity receptor, which can lead to apoptosis, was present in greater levels than the activated form of the high-affinity receptor. Furthermore, OP compound exposure resulted in time-dependent changes of protein levels central to the mitogen-activated kinase and phosopholipase C-γ intracellular pathways. Changes in a third pathway, the protein kinase C pathway, were dependent on the concentration and type of OP compound. Finally, i*n vitro* neurite length was not affected by the type of OP compound administered *in vivo* or when a whole protein fraction was separated by molecular weight. This research has revealed i*n vivo* consequences and early effects on intracellular protein and activated neurotrophin receptor levels after OP compound exposure. These early effects may contribute to the delayed development of neurotoxic effects associated with OP compound exposure.

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DECLARATION OF WORK PERFORMED

I declare that I, Melinda Jane Pomeroy-Black, performed all of the work reported in this dissertation except for that which is reported below.

Chicken brains for neurotoxic esterase and acetylcholinesterase assays were harvested by me and Dr. Bernard S. Jortner and then given to Kristel Fuhrman for assay.

Dan Ward wrote and entered code for statistical analysis and helped interpret results.

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ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
Akt	Serine-threonine kinase
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CREB	Cre-binding protein
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbant assay
FGF	Fibroblast growth factor
GRB2	Growth factor receptor-bound protein 2
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IL	Interleukin
IP_3	Inositol-3 phosphate
JNK	Jun-amino-terminal kinase
kD	Kilodalton
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
Mek	MAP kinase kinase
NGF	Nerve growth factor

NIH	National Institute of Health
NT-3	Neurotrophin-3
NT4/5	Neurotrophin-4/5
NTE	Neurotoxic esterase or neuropathy target esterase
OP	Organophosphorus ester or organophosphate
OPIDN	Organophosphate-induced delayed neuropathy
NRIF	Neurotrophin receptor interacting factor
p75NTR	Common, low-affinity neurotrophin receptor
pp75NTR	Phosphorylated form of the common, low-affinity neurotrophin receptor
pAkt	Phosphorylated form of the Akt
pMek	Phosphorylated form of MAP kinase kinase
pPKC-α	Phosphorylated form of protein kinase C-α
pTrk	Phosphorylated form of the specific, high-affinity neurotrophin receptor with tyrosine kinase activity, subdivided into pTrkA, pTrkB, and pTrkC
PAF	Platelet-activating factor
PI-3K	Phosphoinositol-3 kinase
PKC-α	α isoform of Protein Kinase C
PLC-γ	Phospholipase C-γ
PNS	Peripheral nervous system
PSP	Phenyl saligenin phosphate
RTK	Receptor tyrosine kinase
SHC	Src homology to collagen protein
SLUD	Salivation, Lacrimation, Urination, Defecation
SOS	Son-of-sevenless protein

TBST	Tris-buffered saline + 0.05% (v:v) Tween-20
TOCP	Tri-*ortho*-cresyl phosphate
Trk	Specific, high-affinity neurotrophin receptor with tyrosine kinase activity, subdivided into TrkA, TrkB, and TrkC

PART I.

HYPOTHESIS AND SPECIFIC AIMS

Chapter 1.

HYPOTHESIS

Humans and certain species of animals demonstrate signs of delayed neurotoxicity upon exposure to some organophosphorus (OP) esters (Bradley, 1976; Carboni *et al.*, 1992; Dyer *et al.*, 1992; Johnson, 1993). Clinical symptoms progress from abnormal foot placement and leg weakness to an unwillingness to walk and finally, flaccid paralysis (Ehrich and Jortner, 2001; Lotti, 1992). Literature provides extensive documentation of the pathology and enzyme involvement in organophosphate-induced delayed neuropathy (OPIDN). However, there is little knowledge of the cellular mechanisms involved in the initiation of OPIDN (Carboni *et al.*, 1992; Ehrich and Jortner, 2001).

Within hours of exposure to certain OP esters, neurotoxic esterase (also known as neuropathy target esterase, NTE) is inhibited in the brain, spinal cord, and peripheral nerves of susceptible animal species (Davis and Richardson, 1980; Lotti, 1992). Neuropathic OP compounds inhibit NTE by covalently binding the enzyme at its active serine site. The OP-NTE enzyme may undergo dealkylation, leading to discharge of a leaving group and phosphorylation of the active site. At this point, an intramolecular rearrangement may occur, leaving a negative charge on the enzyme. This reaction, termed "aging," is necessary to induce OPIDN (Ehrich, 1996; Ehrich and Jortner, 2001). Although NTE inhibition occurs within hours of OP exposure, NTE levels return to normal by the time clinical signs appear days to weeks after exposure (Ehrich, 1996; Ehrich and Jortner, 2001). It is interesting to note that young animals appear relatively resistant to the effects of neuropathic OP exposure although they do exhibit NTE

inhibition (Ehrich, 1996; Ehrich and Jortner, 2001; Funk *et al.*, 1994; Peraica *et al.*, 1993).

A soluble factor released within 24 hours of exposure to a neuropathy-inducing OP compound (phenyl saligenin phosphate, PSP) in the cervical spinal cord of adult chickens (*Gallus gallus*) induces neurite outgrowth in undifferentiated SH-SY5Y cells similar to cells treated with nerve growth factor (NGF) (1 μg/ml) after incubation for 6 days. In contrast, morphology of cells treated with spinal cord extracts of chickens exposed to no treatment or vehicle only did not change significantly (Pope *et al.* 1995). This suggests an initial mechanism in the exposed animal which attempts to counteract the neurodegenerative potential of the toxicant. Changes in the concentrations of neurotrophic factors may contribute to degeneration and regeneration within the central nervous system (CNS). This research was designed to clarify the temporal role of neurotrophins and their receptors after OP exposure.

The ratio of cellular expression of high-affinity (Trk) to low-affinity (p75NTR) neurotrophin receptors determines the effect of a particular neurotrophin (Benedetti *et al.*, 1993). The percent of neurotrophin-bound-Trk and of neurotrophin-bound-p75NTR also affects cellular response to a neurotrophin (Yoon *et al.*, 1998). Carlson *et al.* (2000) found that undifferentiated SH-SY5Y cells exposed to a high dose of PSP (100 μM or 1 mM) induced morphological change consistent with necrosis. However, exposure to a lower dose of PSP (10 μM) or to a high dose of a non-neuropathic OP compound (paraoxon, 1 mM) was characterized by apoptosis (Carlson *et al.* 2000). These morphological effects may be determined by the level of the pro-apoptotic neurotrophin receptor, p75, compared to the level of the Trk receptor on the cell membrane. Assessing

the activation state of both receptors will contribute to the understanding of the temporal change of these receptors after neuropathic OP exposure. Finally, the signaling cascade that follows Trk receptor activation may not be sustained at a level sufficient for neurite outgrowth and, therefore, recovery.

The fundamental hypothesis of this research was that there is an immediate increase in neurotrophin release, specifically NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), after exposure to a neuropathy-inducing OP compound, but that regenerative effects of these neurotrophins are not sustained (Figure 1.1). Secondary hypotheses included:

1. The concentration of neurotrophic factors increases shortly after neuropathic OP exposure but this release is not sustained.
2. Expression of the high-affinity NGF receptor, TrkA, increases after exposure to neuropathic OP compound but expression of the low-affinity NGF receptor, p75NTR, does not.
3. The ratio of phosphorylated TrkA to phosphorylated p75NTR is not affected after neuropathic OP exposure.
4. Neuropathic OP compounds interfere with signal transduction in protein cascades that follow neurotrophin-receptor binding, including the MAPK, PLC-γ, and PKC pathways.
5. The increase in neurite outgrowth seen by Pope *et al.* (1995) is due to the release of a soluble factor, which was characterized by molecular weight.

SPECIFIC AIMS

The following specific aims were proposed to test the hypotheses:

Specific Aim #1 (Chapter 4): To examine temporal aspects of known neurotrophin expression, including NGF, BDNF, and NT-3.

Specific Aim #2 (Chapter 5): To evaluate the effect of OP compounds on neurotrophin receptor concentrations

Specific Aim #3 (Chapter 6): To evaluate the effect of OP compounds on intracellular proteins that contribute to cell survival and the initiation of neurite outgrowth.

Specific Aim #4 (Appendix C): To characterize the molecular weight of a neurotrophic soluble factor released from spinal cord of chickens within 24 hours of exposure to cyclic phenyl saligenin phosphate (PSP), an OP compound that induces delayed neuropathy in this animal model

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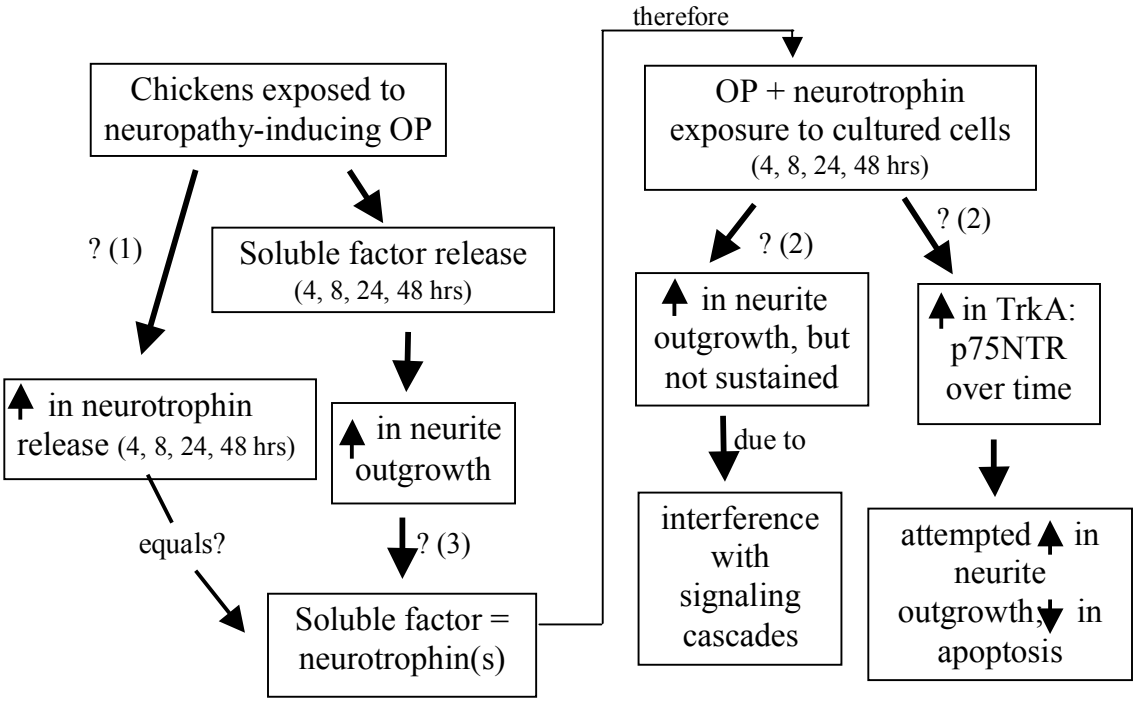

Figure 1.1. The presumed cell response to injury is increased release of neurotrophins. We will determine where signaling pathways are affected by neuropathic OP exposure *in vitro*. Numbers within parentheses reflect a particular specific aim. A "?" followed by a number in parentheses indicates a specific aim.

PART II.

LITERATURE REVIEW

Chapter 2.

LITERATURE REVIEW

Organophosphate Uses and Neurotoxicity

Organophosphorus (OP) esters have several different uses, including residential and agricultural pest control, petroleum additives, and modifiers of plastics (Davis and Richardson 1980; Markowitz 1992). Of all classes of insecticides, the OP esters are the most frequently utilized worldwide. They are also involved in more poisonings than any other class of pesticides (Sultatos 1994). These agents are also commonly used in suicide attempts in developing nations, such as Sri Lanka (Kwong 2002). These agents are also recognized as weapons of terrorism.

The family of OP compounds is neurotoxic and clinical signs may be both acute and delayed. Acute symptoms of cholinergic poisoning are caused by all OP insecticides, and include involuntary salivation, lacrimation, urination, and defecation, or SLUD (Davis and Richardson 1980). Acute cholinergic toxicity is the predominant feature of OP compound exposure. Delayed effects of OP compounds are more likely to be compound specific, and include a neurodegenerative disorder commonly known as organophosphate-induced delayed neuropathy (OPIDN) and, in rare cases, carcinogenesis (Sultatos 1994).

Normally, after a neuron fires and releases the neurotransmitter acetylcholine (ACh) into the synaptic cleft, acetylcholinesterase (AChE) hydrolyzes it such that ACh-induced synaptic transmission ceases. This occurs when ACh binds a hydroxyl group of serine residue 203 on AChE, forming an intermediate enzyme-substrate complex. This

intermediate enzyme goes on to yield hydrolyzed ACh and a regenerated active AChE (Ecobichon 1996).

The OP esters inhibit AChE by phosphorylation of serine residue 203. This occurs when an acidic group on the OP covalently binds a hydroxyl group of AChE (Figure 2.1). The phosphorylated AChE intermediate enzyme complex is more stable and has a lower rate of hydrolysis than the acetylated form. This ultimately results in decreased regeneration of the active enzyme, thereby rendering AChE unable to hydrolyze ACh. Consequently, there is continual stimulation and eventual desensitization of ACh receptors. In mammals, this cholinergic poisoning causes acute clinical signs, including salivation, lacrimation, urination, and defecation (SLUD) and emesis (Ecobichon 1996).

Some phosphorylated OP-AChE intermediate enzymes undergo dealkylation, leading to the discharge of a leaving group and phosphorylation of the active AChE site. At this point, an intramolecular rearrangement may occur, leaving a negative charge on the enzyme. This reaction is termed "aging," at which point AChE is permanently phosphorylated (Kwong 2002; Lotti 1992) (Figure 2.1). After aging occurs, recovery of AChE activity requires synthesis of a new enzyme (Ecobichon 1996). The rate of aging depends on the structure of the OP compound (Sultatos 1994).

Organophosphate-Induced Delayed Neuropathy

Research demonstrates that two different B-esterases play a role in the development of OP neurotoxicity: AChE and neurotoxic esterase (also known as neuropathy target esterase, NTE). Neuropathy-inducing OP compounds inhibit NTE but do not always inhibit AChE. Non-neuropathic OP compounds cause neurotoxicity by

inhibiting AChE without causing the inhibition and aging of NTE that is necessary for onset of OPIDN (Ehrich 1996; Kwong 2002).

Similar to AChE inhibition, within hours of exposure to certain OP esters, NTE is inhibited in the brain, spinal cord, and peripheral nerves of susceptible animal species (Davis and Richardson 1980; Lotti 1992). While NTE is widely considered to be the molecular target of neuropathy-inducing OP compounds, not every OP compound is capable of inhibiting NTE. Some of the OP compounds that have a pentavalent phosphorous in their chemical structure can inhibit NTE (Ehrich and Jortner 2001; Lotti 1992). The development of a strong bond between NTE and the OP compound ("aging") is another essential factor in the initiation of OPIDN (Ehrich 1996; Ehrich and Jortner 2001).

The mechanism of NTE aging is similar to that of AChE aging. Esterase activity of NTE is inhibited when the OP compound covalently phosphorylates NTE. The enzyme may then be cleaved at a covalent bond within the NTE-OP complex, leaving a negative charge on the enzyme (Ehrich 1996; Johnson 1993). The OP residue left attached to NTE determines the potency of that particular OP compound as an NTE inhibitor (Lotti 1992).

Inhibition and aging of 70-80% of available NTE within hours of OP exposure is required for OPIDN initiation (Johnson 1993; Moretto *et al.* 1991). Although NTE inhibition occurs within hours of OP exposure, NTE levels have usually returned to normal levels by the time clinical signs appear days to weeks after exposure (Ehrich 1996; Ehrich and Jortner 2001). Of interest is that young animals appear relatively resistant to the effects of neuropathic OP exposure although they do exhibit NTE

inhibition (Ehrich 1996; Ehrich and Jortner 2001; Peraica *et al.* 1993). Studies also show that when an agent capable of temporarily binding NTE is administered prior to neuropathic OP exposure, OPIDN does not develop. This is termed "protection," as the OP ester is unable to bind NTE and thereby induce subsequent aging and NTE inhibition. Interestingly, a protective agent administered *after* a marginal dose of a neuropathic OP compound can result in OPIDN. This is called "promotion." One explanation is that promotion involves a target other than NTE (Lotti *et al.* 1991; Peraica *et al.* 1993).

Other factors also contribute to the development, severity, and recovery from OP exposure. Dosage, frequency and duration of exposure, and route of administration of the OP compound are obvious contributing factors (Abou-Donia 1981). Although most OP compounds are administered orally to experimental animals, dermal exposure can also induce delayed neurotoxicity (Abou-Donia and Graham 1978). Abou-Donia *et al.* (1979) demonstrated that metabolic activation of an OP compound to a more potent neurotoxic product is also a factor in the development of delayed neurotoxicity. Finally, toxicokinetics of susceptible species differs from that of nonsusceptible species. Susceptible species, such as chickens, eliminate leptophos, a neurotoxic OP compound, slower than non-susceptible species (Abou-Donia 1981).

There are conflicting results concerning the contribution of axonal transport to OPIDN (Abou-Donia 1981; Lotti 1992). Because no damage to the neuronal soma occurs after neuropathic OP exposure, some propose that proteins with energy-producing functions required for normal axonal transport are targeted by the OP compound (Massicotte *et al.* 2005; Moretto *et al.* 1991). Local disruption of axonal transport could cause mitochondrial accumulation within the axon, leading to mitochondrial breakdown.

Calcium release from this breakdown would initiate a chain of destructive cellular events (Abou-Donia 1981). For example, calcium influx activates calpains, which lead to degradation of axonal and muscle cytoskeletal elements. El-Fawal *et al.* (1990) demonstrated that calpain activation occurred within 2-4 days of neuropathic OP exposure in the sciatic nerve and brain, respectively.

The largest incidence of OPIDN was observed in the 1930s during Prohibition in the United States. Nearly 50000 residents were affected by limb weakness and ataxia when tri-*ortho*-cresyl phosphate (TOCP) was used as an alcohol substitute in a product called Ginger Jake. This syndrome was referred to as "Ginger Jake paralysis." Neither the cause nor mechanisms of the disease were understood at the time (Ehrich and Jortner 2001, 2002). Even today, there are many questions surrounding the mechanisms of OPIDN.

Pathology Associated with OPIDN

Humans and other susceptible species of animals, including monkey, rat, cat, ferret, and chickens, demonstrate signs of OPIDN upon significant exposure to NTE-inhibiting OP compounds (Bradley 1976; Carboni *et al.* 1992; Dyer *et al.* 1992; Johnson 1993). Within two weeks of exposure, humans report tingling in the hands and feet, followed by sensory loss. This may progress to bilateral and symmetrical muscle weakness with distal skeletal muscles becoming flaccid. Ataxia may also be noted (Ehrich and Jortner 2001; Lotti 2001). The adult hen (*Gallus gallus*) is the recognized animal model for OPIDN for several reasons. These include the susceptibility of the hen, and that lesions are extensive and distinct, with a lag period of only several days between

exposure and clinical alterations (Ehrich 1996; Ehrich and Jortner 2001). Clinical symptoms in chickens are measurable, progressing from abnormal foot placement and leg weakness to an unwillingness to walk and finally, flaccid paralysis. Humans exhibit similar symptoms (Ehrich and Jortner 2001; Lotti 1992).

Bilateral degeneration in distal levels of myelinated axons is the primary histological lesion of OPIDN. This syndrome primarily affects peripheral and CNS nerve fibers that are larger and/or longer than other fibers, such as those found in the spinocerebellar tract and fasciculus gracilis. Microscopically, these lesions appear as pale-staining, swollen axons with thin myelin sheaths and/or dark-staining fibers with axonal debris in the paranodal region. The affected fiber usually becomes fragmented into myelin ovoids, typical of "Wallerian degeneration" (Ehrich and Jortner 2001; Lotti 1992).

Wallerian-like degeneration can occur within the CNS and peripheral nervous system (PNS) after exposure to a neuropathic OP compound. It is characterized by fragmentation and dissolution of the axon, followed by digestion and removal of the collapsed myelin (Summers *et al.* 1995). Primary axonal degeneration occurs in a retrograde manner, increasing in severity from proximal to distal regions of the axon, with respect to the soma (Davis and Richardson 1980). Myelin degeneration appears to occur subsequent to axonal injury (Abou-Donia 1981).

Consequences of progressive axonal degeneration are severe. As anterograde axonal transport ceases, distal portions of the axon become disengaged from the remaining intact neuron and soma. Retrograde transport of neurotransmitters and neurotrophic factors slows and eventually stops. Cytoskeletal elements and mitochondria

are unable to reach the distal portions of the axon, resulting in organelle accumulation at severed ends of the axon (Griffin *et al.* 1977; Summers *et al.* 1995).

Progressive reduction of retrograde transport has been documented in hens exposed to a neuropathic OP compound. This reduction reached a maximal level approximately 7 days after dosing of experimental animals (Lotti 1992). There is a continuous dying-back of the axon over time and communication with neurons more distal to the CNS ceases. This "dying-back neuropathy" in the CNS presents a medical challenge. This is because, as with other forms of axonal injury, regeneration in the CNS is minimal compared to regenerative capabilities in the PNS (Ehrich and Jortner 2001).

Traumatic Nervous System Injury

There are considerable differences between chemical and traumatic nervous system injury in both the CNS and PNS. The differences include the extent of cellular damage, presence of inflammation and astrocytosis. However in some ways, chemical insult is similar to traumatic injury, in particular when considering some aspects of axonal degeneration.

After massive physical trauma to the spinal cord, neurons in the dorsal horn are affected earlier than those in the ventral horn. Whether impact or compression injury, necrotic neurons appear within the first hour of injury in a random distribution within gray matter. Necrotic cells begin to appear in white matter by 8 hours post-injury (Schwab and Bartholdi 1996). Histologically, necrotic neurons appear as shrunken cells with pyknotic nuclei, central chromatolysis, hyperchromatization, and a loss of Nissl bodies (Hughes 1984; Schwab and Bartholdi 1996). Macroscopically, the physically injured spinal cord appears swollen (Hughes 1984). As with trauma, a toxin that causes

primary injury to the soma (neuronopathy) may also cause axonal degeneration (Jortner 2000).

Within hours after either chemical or physical injury to the CNS and/or PNS, axons that have lost homeostasis begin to swell as a result of disrupted axonal transport and alterations of membranes (Griffin *et al.* 1977; Martinez and Riberio 1998; Schwab and Bartholdi 1996). These reactive swellings, containing densely packed vesicular and membranous organelles, develop at both proximal and distal stumps of transected axons, interrupting the continuity of nerve fibers (Dyck *et al.* 2002; Griffin *et al.* 1977). Retrograde transport of neurotrophic factors is interrupted, leading to deprivation of neurotrophic factors throughout the neuron (Schwab and Bartholdi 1996).

The pathophysiology of axonal damage is similar between trauma and neuropathic OP injury in that excessive calcium that enters the injured neuron is a primary cause of axonal cytoskeletal protein disintegration (El-Fawal *et al.* 1990; Martinez and Riberio 1998). Calcium influx also activates myelinases, leading to demyelination of axons around the lesion site (Young 1993). Disintegrating myelin increases over the next few days, further contributing to conduction failure and consequent loss of function. As with neuropathic OP exposure, large diameter axons are affected at greater proportions than smaller axons after physical trauma (Raineteau and Schwab 2001; Schwab and Bartholdi 1996).

Toxins that affect the axon directly (axonopathy) elicit axonal degeneration that begins at the distal segment of the axon and spreads proximally toward the soma, defined as Wallerian-like degeneration (Graham and Montine 2002; Jortner 2000; King 1999). In advanced stages of Wallerian-like degeneration in either chemical or physical trauma,

myeline degenerates in association with axonopathy, and macrophages digest myelin and axonal debris (Jortner 2000; King 1999). Segemental demyelination of axons in the PNS after toxin exposure is common. This lesion is characterized by inappropriately thin myelin sheaths and shortened internodal distances (Graham and Montine 2002).

Inflammation and Nervous System Trauma

One of the most distinct differences between acute physical trauma and chemical injury in the nervous system is the presence of inflammation and the consequences therein (Hsu *et al.* 1994). It is likely that there is a close association between the degree of inflammation and loss of motor function in traumatic spinal cord injury (Giulian 1994). Features that are particular to the CNS play important roles in CNS trauma, including microglia, astrocytes, and excitotoxicity. However, inflammation in the CNS and PNS shares many characteristics with inflammation elsewhere in the body. There are a number of cells that are involved in acute inflammation: neutrophils, macrophages, endothelial cells, and platelets. It is difficult to decipher the role of each cell type in inflammation because of redundant activating mechanisms between the cells (Hsu *et al.* 1994).

Trauma to the nervous system initially causes an alteration in microvasculature. Petechial hemorrhages occur at the lesion site within minutes (Schwab and Bartholdi 1996). Continued vasodilation and consequent increased blood flow leads to edema as erythrocytes, leukocytes and proteins enter the injured tissue. As plasma enters the tissue, erythrocytes may become concentrated in small vessels, causing slowing of the circulation and eventual stasis of the blood. Blood stasis precedes ischemia, which may

lead to further injury when blood cannot reach tissue around the lesion site (Cotran *et al.* 1999; Parry 1993).

Leukocyte infiltration of the tissue is recognized as the hallmark sign of inflammation that occurs in traumatic CNS and PNS injury (Hsu *et al.* 1994). Upon stasis of the circulation, inflammatory cells are more readily able to migrate through the vascular walls into the interstitial tissue (Cotran *et al.* 1999[Schwab, 1996 #47; Schwab and Bartholdi 1996). Chemoattractants released from injured cells direct infiltrating leukocytes to the injured area. After leukocytes enter the injured tissue, they become active and take on functions such as degranulation and phagocytosis (Hsu *et al.* 1994).

There are two waves of inflammatory cell infiltration. Neutrophils infiltrate the lesion site within the first few hours of injury, peak within 24 hours, and disappear around 3 days (Cotran *et al.* 1999; Schwab and Bartholdi 1996). Monocytes and macrophages dominate the second wave of inflammatory cell migration (Schwab and Bartholdi 1996). Macrophages phagocytize cellular debris from damaged tissue. For example, within an injured section of spinal cord, macrophages provide the primary means by which degraded myelin is disposed (Giulian 1994). Upon phagocytosis, macrophages release cytotoxic factors, such as proteases and free-radicals, which in turn contribute to further cell damage (Giulian 1994; Hsu *et al.* 1994).

Ramified microglia are the primary immune element in the brain. Traumatic injury causes these cells to retract their processes and assume an ameboid shape. At this point, they are termed “reactive microglia” and take on macrophage characteristics, including proliferation and cytokine production. Microglial response to injury varies

widely. Some cells show only surface-membrane changes while others display full-blown phagocytic capabilities (Giulian 1994).

As early as one hour after CNS or PNS traumatic injury, endothelial injury causes cell membrane disruption, which in turn activates membrane phospholipases and initiates lipid peroxidation of cell membranes. Free fatty acids from cell membrane breakdown alter membrane structure, fluidity, and function of surrounding intact cell membranes. This results in the disruption of the Na^+/K^+-ATPase pump (Anderson and Hall 1994). Phospholipase activation also triggers activity of one of two major classes of enzymes, lipoxygenases or cyclooxygenases, which in turn generate arachidonic acid metabolites, or eicosanoids. The lipoxygenase pathway produces leukotrienes, whereas the cyclooxygenase pathway leads to the formation of prostaglandins. Leukotrienes are primarily responsible for chemoattraction and increased vascular permeability. Prostaglandins contribute to vasodilation, and platelet and leukocyte aggregation (Cotran *et al.* 1999; Hsu *et al.* 1994). Bradykinin, a clotting factor activated by protease release from membrane breakdown, further enhances eicosanoid production (Hsu *et al.* 1994).

When blood platelets contact endothelial cells lining the vasculature after traumatic spinal cord injury, both platelets and damaged endothelial cells release platelet-activating factor (PAF). Upon release, PAF induces several events to occur simultaneously. These events include enhanced eicosanoid production, increased leukocyte infiltration to the injured area, and leukocyte aggregation and adhesion (Cotran *et al.* 1999).

Within the CNS, astrocytosis, or reactive gliosis, is a response to trauma, peaking around 14 days postinjury (Kimelberg and Norenberg 1994; Schwab and Bartholdi 1996).

A characteristic response of astrocytes to acute injury is cell swelling. Such swelling occurs due to the release of excitatory amino acids, free fatty acids, and free radicals after injury (Kimelberg and Norenberg 1994). Later, astrocytosis is histologically characterized by cytoplasmic hypertrophy of the astrocyte and decreased cell division. Increased expression of intermediate filaments, including glial fibrillary acidic protein and vimentin, occur within the cell and are localized to the site of the injury (Kimelberg and Norenberg 1994; Schwab and Bartholdi 1996).

Reactive astrocytes accumulate at margins of the lesion within the first week and go on to form a glial scar at the interface between the spinal cord and injury site (Schwab and Bartholdi 1996). The glial scar serves several purposes, perhaps the most important of which is providing a barrier to entry of non-neural tissue (Kimelberg and Norenberg 1994). This barrier may also prevent entry to the diffusion of neurotrophins (Horner and Gage 2000). As the severity of an injury increases, the degree of astrocytosis increases (Hughes 1984; Kimelberg and Norenberg 1994), and in most severe injuries, these changes may be permanent (Kimelberg and Norenberg 1994).

The ultimate consequence of the astrocytic response to acute injury is currently a topic of debate. There is evidence that reactive astrocytes release excitatory amino acids, initiating a chain of destructive events (Kimelberg and Norenberg 1994). Excitotoxicity causes the influx of two ions into the cell: calcium and sodium. Calcium influx causes the initiation of lipid peroxidation by activating proteases, lipases and endonucleases. The consequence of this is neuronal and glial cell death. Excitotoxicity also leads to sodium influx, resulting in cell swelling (Regan and Choi 1994). Astrocyte swelling from sodium influx has several consequences, including failure of neurotransmitter

removal from the extracellular space, dysregulatation of cell volume, and decreased extracellular space (Kimelberg and Norenberg 1994). While some literature points to astrocytosis as an impediment to regeneration, an emerging view is causing reexamination of this. For example, there is evidence that glial scars do not prevent entry of regenerating axons. Furthermore, astrocytes may also possess neurotrophic properties (Kawaja and Gage 1991; Kimelberg and Norenberg 1994).

The role of neurotrophic factors after physical trauma to the CNS or PNS has not yet been precisely defined. Accumulation of nerve growth factor (NGF) at lesion sites has been attributed to both cellular infiltrates and the induction of tissue NGF expression (Leon *et al.* 1994). Using cell culture, Heumann *et al.* (1987) found that macrophages contribute small but significant amounts of NGF upon injury *in vivo*. Other studies have found that even with exogenous administration of neurotrophins, axonal regeneration does not increase significantly compared to controls (Kwon *et al.* 2004).

Activated mast cells may also indirectly upregulate NGF production in surrounding cells when they release cytokines following trauma. These cells may represent a continuous source of NGF. A rapid release of NGF may control immediate modifications to the nervous system after injury, while a prolonged NGF supply contributes to homeostatic functions that allows for reequilibration of the tissue after injury (Leon *et al.* 1994).

Unlike OPIDN, neuronal necrosis often follows physical trauma to the CNS or PNS. Hemorrhage, vasodilation, leukocyte infiltration, and astrocytosis are primary features of such an injury that are not found in OPIDN. However, these two neuropathies

share some similar characteristics as well, such as axonal degeneration with secondary myelin degeneration followed by phagocytosis of myelin debris by macrophages.

Regeneration in the Nervous System

There are striking differences in the capabilities of the CNS and PNS to regenerate. This is evident in both OPIDN and after traumatic spinal cord injury. Evidence suggests an inherent capability of neurons in the CNS and PNS to regenerate. However, inhibitory factors present in the CNS environment prevent regeneration (Brady 1993; Schwab and Bartholdi 1996; Taniuchi *et al.* 1988). For example, when myelin breaks down in the CNS and PNS, neurite outgrowth is inhibited. This contributes to decreased regeneration (Brady 1993). Macrophages entering the PNS after injury engulf and clear myelin debris, whereas in the CNS, macrophage response is limited, leaving myelin debris to accumulate (Avellino *et al.* 1995; George and Griffin 1994). Research has demonstrated that *in vitro* addition of macrophages to transected CNS nerves modifies the non-regenerative nature of the CNS such that neurite outgrowth occurs (David *et al.* 1990). Elongation of axons occurs in experimental myelin-free spinal cords, implicating the accumulation of myelin debris to decreased regeneration in the CNS (Schwab and Bartholdi 1996).

Consequently, the environment of the CNS may be the primary cause of decreased regeneration compared to the PNS (Hagg *et al.* 1993). Glial cells, including oligodendrocytes, inhibit neurite outgrowth after injury, contributing to decreased regeneration in the CNS (Brady 1993). Whereas Schwann cells of the PNS provide bands of Büngner through which regenerating axons can migrate, glial scars in the CNS prevent the progress of growth cones (Taniuchi *et al.* 1988). Furthermore, there is

increased NGF receptor production in the distal stump of axotomized peripheral nerves that accompanies increased production of NGF after trauma (Heumann *et al.* 1987).

Ultimately, the potential for recovery after spinal injury depends upon three factors: 1) redundancy of the affected pathways; 2) potential for reorganization of pathways distal and proximal to the lesion site; and, 3) potential for remyelination (Young 1993). Without redundancy of a pathway, CNS axonal regeneration requires that axons migrate long distances in order to reconnect with an appropriate target (Horner and Gage 2000). Axonal sprouting, observed as neurite outgrowth in cells, is the initial step in the reestablishment of neural pathways. Sprouting appears to be a regulated process rather than a random event (Goldberger *et al.* 1993).

There are two pathways for the initiation of axonal sprouting. If, after injury, the neuron is viable, it can produce numerous axonal sprouts. If a neuron dies, fibers of adjacent neurons sprout to presumably fill vacated postsynaptic sites. This latter type of axonal sprouting is termed "collateral sprouting" and occurs in both the CNS and PNS. Collateral sprouting refers to the expansion of undamaged axons to partially denervated areas (Goldberger *et al.* 1993; Hulsebosch 1987; Raivich and Kreutzberg 1993).

The axon growth cone contains the machinery necessary for elongation of the axonal sprout (Horner and Gage 2000). There are two hypotheses regarding the mechanism of sprouting. One hypothesis highlights the role of cell receptor modification that occurs secondary to denervation. These modifications are postulated to promote new synaptic terminals. A second hypothesis focuses on growth factor release causing neurite outgrowth and synaptogenesis (Goldberger *et al.* 1993). It is widely accepted that an upregulation of growth factors and chemoattractants directs new sprouts to the

appropriate target area. However, the exact nature of participating factors in axonal sprouting remains to be determined (Thallmair *et al.* 1998). The ability of the neuron to reconnect to a proper target determines the success of axonal regeneration (Hagg *et al.* 1993). Literature suggests that if the new axonal fibers do reach their former target(s), functional connections will occur (Schwab and Bartholdi 1996). In both OPIDN and traumatic spinal cord injury, axonal sprouting is the primary means for regeneration in the nervous system. The exact role of growth factors in this process is unclear.

It is important to note that regenerative sprouts do not elongate without the appropriate growth factors (Schnell *et al.* 1994; Schwab and Bartholdi 1996). Research suggests that if an axonal sprout encounters inhibitory factors to elongation during reestablishment of a neural path, the sprout loses the ability to respond to neurotrophins (Cai *et al.* 1999). Consequently, there may be a retraction, or dying back, of the sprout. Histologically, this appears as a bulb at the end of a neurite (Schwab and Bartholdi 1996).

Furthermore, there seems to be a permissive window for regeneration. Younger mammals possess a greater capability of neural regeneration than older mammals (Schwab and Bartholdi 1996). This may be due to higher concentrations of neurotrophic factors in younger animals (Maisonpierre *et al.* 1990).

Neurotrophins and their receptors

In the normal nervous system, neurotrophins play an essential role in neuronal survival, growth, and differentiation (Pope *et al.* 1995). Currently, there are 4 known proteins that compose the neurotrophin family: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (Barker and Shooter 1994; Meakin

and Shooter 1992). These four proteins exist in solution as dimers (Barker and Shooter 1994). During development, there is differential expression of these neurotrophins (Schwab and Bartholdi 1996). Neurons compete for limited amounts of target-derived neurotrophins as they extend processes toward potential targets. Neurons receiving these neurotrophins survive, while those not acquiring neurotrophins undergo "developmental neuronal death" (Barker and Shooter 1994; Hagg *et al.* 1993). Upon traumatic or chemical injury, neurotrophic factors have the capability to protect neurons and restore impaired functions. Neurotrophin effects on cells include initiation of cytoskeletal rearrangement and enhancement of neurotransmitter release (Blöchl *et al.* 1995).

Neurotrophins are synthesized and received by neuronal and nonneuronal cells. A neuron secretes and receives neurotrophins in several locations, including the soma, dendrites, axon, and axon terminals. While neurons generally interact with other neurons, glial cells may interact with other glia, neurons, or release neurotrophins directly into the bloodstream. Thus, glial cells may serve as an intermediate step in neurotrophic signaling between neurons (Fallon and Loughlin 1993) (Figure. 2.2).

Increasing evidence suggests that neurotrophins play a role in synaptic plasticity (Blöchl *et al.* 1995; Brunello *et al.* 1990; Grill *et al.* 1997; Perez-Polo and Werrbach-Perez 1987). Neurotrophin expression rapidly changes in the spinal cord under pathological conditions (Raineteau and Schwab 2001). Retrograde transport of neurotrophins to the neuronal nucleus causes a cascade of cellular events resulting in the activation of transcription factors that control gene expression (Fallon and Loughlin 1993). The investigators noted that a number of cellular events are affected by changes in gene expression, including regulation of neuronal survival or differentiation,

alterations in growth factor synthesis, and regulation of retrograde transport. If neurotrophin transport or reception is interrupted, the cell either dies or sprouts neurites, depending on the dependence of the neuron for that particular growth factor (Hulsebosch 1987).

Production, release, and reception of neurotrophins may be part of the response to injury in neural tissues. These factors likely play an important role in compensatory repair processes after injury (Hagg *et al.* 1993). There are several theories about the role of neurotrophins in injury. Some investigators suggest that neurotrophins stabilize and/or rearrange specific synapses after trauma (Blöchl *et al.* 1995). Other investigators suggest that neurotrophin deficits induced by trauma result in neuronal dysfunction or degeneration by prohibiting axonal regeneration. Hagg *et al.* (1993) demonstrated that administration of exogenous NGF after trauma protected injured neurons against degeneration and promoted axonal regeneration. However, another study showed that exogenous BDNF did not promote axonal regeneration after cervical axotomy, a nonregenerative injury in which a segment of the axon is removed. It is likely that the potential for axonal regeneration is related to the time lapse between injury and administration of the exogenous neurotrophin, such that when weeks pass between trauma and administration of an exogenous neurotrophin, axonal regeneration decreases; whereas administering the exogenous neurotrophin hours after trauma results in increased regeneration (Kwon *et al.* 2004). A third theory suggests that uninjured neurons surrounding an injured neuronal population may find an excess of neurotrophins due to the death of injured neurons. This excess may elicit sprouting into the denervated territory (Hulsebosch 1987). However, one must consider that with the loss of a neuronal

population, there will necessarily be a loss of neurotrophic factors supplied by that population.

Each neurotrophin specifically binds with high-affinity to a receptor with intrinsic tyrosine kinase activity, known as Trk (Barker and Shooter 1994; Hantzopoulos *et al.* 1994). Each neurotrophin has a specific high affinity receptor: NGF binds TrkA, BDNF binds TrkB, and Neurotrophin-3 and 4/5 bind TrkC (Hagg *et al.* 1993; Yin *et al.* 1998). The cytoplasmic region of Trk receptors consists of three well-defined domains: the juxtamembrane region, the kinase catalytic domain, and the C-terminal tail (Figure 2.3). The juxtamembrane domain is the least well conserved among the cytoplasmic regions. The highly conserved tyrosine residue plays a critical role in signal transduction, probably through binding the src homology to collagen protein (SHC) adaptor protein (Barbacid 1995).

There is conflicting evidence regarding the expression of neurotrophin receptors after injury. Some studies indicate that both TrkA and TrkB are upregulated and activated in dorsal root ganglia after spinal cord injury. However, other studies document decreased TrkA, TrkB, TrkC, and p75NTR expression after peripheral nerve axotomy (Kwon *et al.* 2004; Qiao and Vizzard 2002; Zhang *et al.* 2000). Interestingly, TrkA activation can occur in the absence of NGF when this receptor is overexpressed in PC12 neuronal cells. Thus, there is evidence of Trk receptor activation in the absence of neurotrophins (Qiao and Vizzard 2002). These results suggest that neurotrophin action is due to differential expression of their high-affinity receptors. Therefore, the lack of effect of neurotrophins on regeneration may be due to downregulation of high-affinity neurotrophin receptors (Zhang *et al.* 2000). For example, two months after spinal cord

injury, Kwon *et al.* (2004) found decreased expression of the TrkB receptor in axons located at the injury site.

At the same time the neurotrophin binds its respective Trk receptor, it also binds a receptor with low affinity, the p75 neurotrophin receptor (p75NTR) (Bamji *et al.* 1998; Barker and Shooter 1995; Hantzopoulos *et al.* 1994). The active form of p75NTR is believed to exist as a homotrimer and exhibit strong similarity to receptors for the tumor necrosis factor family (Chao 1994). The p75NTR has a wider distribution and is generally expressed at higher levels than Trk receptors (Chao and Hempstead 1995). This receptor interacts with all members of the neurotrophin family with nearly equal affinities (Figure 2.4) (Bamji *et al.* 1998; Barker and Shooter 1995; Hantzopoulos *et al.* 1994).

Research demonstrates that p75NTR expression is required for formation of the high-affinity receptor site (Hempstead *et al.* 1991). Coexpression of Trk and p75NTR receptors likely contributes to increased specificity and sensitivity of neurotrophin binding, leading to greater cellular response to the neurotrophins present in the local environment. For example, binding of NGF to p75NTR increases the affinity of TrkA for NGF, thereby leading to increased cell response (Barker and Shooter 1995; Chao and Hempstead 1995; Kaplan and Miller 1997; Meakin and Shooter 1992).

There are three possible mechanisms by which ligand binding to p75NTR enhances binding to the Trk receptor. One theory suggests that neurotrophin-p75NTR binding induces a conformational change in the Trk receptor, thereby causing a favorable binding condition to the Trk receptor (Barker and Shooter 1995). The second mechanism proposes that ligand binding to p75NTR increases the affinity of the Trk receptor for the

neurotrophin (Chao and Hempstead 1995; Kaplan and Miller 1997; Meakin and Shooter 1992). Lastly, Hantzopoulos *et al.* (1994) suggest that the active form of p75NTR increases neurotrophin concentration in the local environment. Indeed, p75NTR is upregulated on Schwann cells after injury. This response may increase the affinity of the receptor for neurotrophins or play a role in the accumulation of neurotrophins at regenerating axons (Chao 1994).

The ratio of cellular expression of Trk to p75NTR determines the response to a particular neurotrophin, and in turn, the fate of a neuron (Benedetti *et al.* 1993; Bredesen and Rabizadeh 1997; Eggert *et al.* 2000; Yoon *et al.* 1998). Cross-talk between the two receptors is bi-directional. While Trk receptors transmit positive signals such as enhanced survival and growth, p75NTR transmits both positive and negative signals. The signals generated by the two receptors can oppose or augment each other (Kaplan and Miller 2000). Depending on the degree of Trk activity, p75NTR transmits positive (neurite extension) or negative (apoptotic) signals (Bredesen and Rabizadeh 1997; Eggert *et al.* 2000; Yoon *et al.* 1998). For example, high Trk receptor activity abrogates p75NTR-mediated cell death (Kaplan and Miller 1997). Likewise, cell growth mediated by TrkA is inhibited when p75NTR causes selective downregulation of the mitogen-activated protein kinase (MAPK) pathway (Kaplan and Miller 2000). However, when both receptors are highly active, Trk autophosphorylation, and therefore Trk signaling, is enhanced. This results in cell differentiation and survival and suppression of apoptosis (Kaplan and Miller 1997).

Apoptotic actions of p75NTR are induced only when Trk is inactive or suboptimally activated. High Trk activation silences apoptotic p75NTR signaling.

Therefore, neuronal and glial cell apoptosis by p75NTR activation occurs in a Trk-independent fashion. Consequently, in cells expressing low levels of TrkA, such as oligodendrocytes, NGF has the potential to be pro-apoptotic (Kaplan and Miller 2000). However, a lack of Trk receptor expression alone is not sufficient for p75NTR-induced apoptosis (Aloyz *et al.* 1998).

Literature suggests that p75NTR positively or negatively regulates axonal growth depending on the proportion of ligand bound p75NTR present in the local microenvironment (Kaplan and Miller 2000). Upon neuronal injury, TrkA levels decrease but p75NTR and NGF levels increase. This alteration in receptor expression may shift the balance of signaling toward the death pathway as NGF binds the more prevalent p75NTR (Foehr *et al.* 2000). Therefore, it is possible that the ratio of the liganded to unliganded receptors also contributes to neurotrophin response.

Nerve Growth Factor

The best-characterized neurotrophin is nerve growth factor (NGF), a 26 kilodalton (kD) protein. This neurotrophin was initially recognized by neurite stimulation *in vitro*. Other effects of NGF include differentiation of sensory and sympathetic neurons and neuronal survival (Hagg *et al.* 1993).

Initial regeneration of nerve fibers occurs as a result of localized, transient increase in neurotrophic factors at the lesion site. *In vitro* experiments demonstrate that the direction of neurite extension can be guided along an NGF concentration gradient (Hagg *et al.* 1993). Following peripheral nerve injury, neuronal and nonneuronal cells, including Schwann cells, other glial cells, at distal and proximal ends of injured nerve

produce NGF (Brunello *et al.* 1990; Hagg *et al.* 1993; Heumann *et al.* 1987). The upregulation of NGF synthesis and release is accompanied by an upregulation of NGF receptors, particularly p75NTR, on Schwann cells in the distal stump of the nerve (Heumann *et al.* 1987; Taniuchi *et al.* 1988). These events induce Schwann cell migration, which form bands of Bünger around the distal and proximal ends of the injured nerve. The bands of Bünger guide the regenerating axon back to its target (Anton *et al.* 1994). Neurite extension begins at the proximal stump of the injured nerve. Continued extension requires direct exposure of the growth cone, the elongating end of a neurite, to NGF (Hagg *et al.* 1993).

Expression of NGF following peripheral nerve injury occurs in two phases. An initial rapid increase within 24 hours of injury is followed by a second, larger burst days later (Heumann *et al.* 1987). This second burst is likely due to the release of interleukin-1 (IL-1) by infiltrating macrophages. A gradual decrease in NGF at the injury site occurs over the next several weeks as reinnervation progresses in peripheral nerve crush injuries. Because reinnervation is slower after nerve transection, NGF levels remain elevated (Heumann *et al.* 1987; Meyer *et al.* 1992). Therefore, insufficient reception of neurotrophic factors, rather than their unavailability, may explain the failure of sustained regeneration after injury (Hagg *et al.* 1993; Raivich and Kreutzberg 1993).

The above evidence indicates that NGF and NGF receptors play an integral role in the potential for recovery after CNS and PNS injury. The physiological response of the nervous system to injury is increased synthesis and release of NGF. Like other neurotrophins, NGF has the capability to promote axonal regeneration (Hagg *et al.* 1993; Taniuchi *et al.* 1988).

Signal Transduction upon binding of NGF

NGF binds two cell-surface receptors: tyrosine kinase A receptor (TrkA) with high affinity and a common neurotrophin receptor, p75NTR, with low affinity (Bui *et al.* 2002; Hagg *et al.* 1993). Internalization of NGF into the neuron occurs exclusively through the TrkA receptor. Upon internalization, some NGF is rapidly degraded by lysosomes (Hagg *et al.* 1993). Metabolites produced from this degradation may serve as intracellular signals for the action of NGF. However, a significant portion of intact NGF enters cellular compartments (Altin and Bradshaw 1993).

Receptor binding and cell response are two separate steps of neurotrophin action (Chao 1994). When NGF binds TrkA, the receptor dimerizes. This is followed by trans-autophosphorylation of specific tyrosine residues within the transmembrane domain of the receptor (Figure 2.3). Tyrosine kinase activity of TrkA is maximal 5-10 minutes following ligand binding and is attenuated thereafter (Kaplan 1995). Upon NGF-TrkA binding, MAPK activation peaks at 10 minutes and is maintained at a high level for 2 hours (Cohen *et al.* 1992).

Specific intracellular proteins bind different sequence motifs on autophosphorylated TrkA to initiate activation of up to three pathways: the phospholipase C-γ (PLC-γ) pathway, the phosphoinositol-3 kinase (PI-3K) pathway, and the MAPK pathway (Figure 2.5). Initial phosphorylation of SHC initiates a cascade of cellular events that ultimately leads to MAPK or PI-3K activation. Phosphorylated SHC binds and activates GRB2, which then binds the son-of-sevenless (SOS) guanine nucleotide

exchange protein. Activation of SOS induces binding to and activation of a GTP-binding protein, Ras (Kaplan 1995). Ras serves as a branch point directing neurotrophin-initiated signals into multiple signaling pathways (Kaplan and Miller 2000). The duration of Ras activity may determine which cascade is initiated (Kaplan 1995). There are several major roles of Ras including initiating PI-3K pathway activation, suppressing the JNK (Jun amino-terminal kinase)-p53-Bax apoptotic pathway, inducing Raf translocation to the cellular membrane, and initiating the activation of a series of serine/threonine kinases that compose the MAPK pathway (Kaplan 1995; Kaplan and Miller 2000; Mazzoni *et al.* 1999). These serine/threonine kinases include Raf, MAP kinase kinase (Mek) 1 and 2, and Erk 1 and 2. Phosphorylated Ras binds and activates Raf, which then activates Mek 1/2 (Kaplan 1995; Kaplan and Miller 2000). Binding of Mek 1/2 to Erk1/2 induces phosphorylation at specific threonine and tyrosine residues (Figure 2.5). Cohen *et al*. (1992) suggested that dual phosphorylation at these residues acts as a "failsafe" device to prevent MAPK activation by other protein serine/threonine kinases within the cell.

The MAPK pathway is a cell-survival pathway that also plays a role in plasticity and long-term potentiation (Kaplan and Miller 2000). The MAPK pathway contributes to synaptic plasticity by eliciting neurite outgrowth. While the PLC-γ pathway also elicits neurite outgrowth, the MAPK pathway seems to be necessary for complete elaboration of neurites and neurite maintenance (Kaplan 1995).

Although the MAPK pathway is sufficient for cell survival, it is not essential. Research demonstrates that the survival pathway of MAPK protects neurons from death due to injury or toxicity, rather than from trophic factor withdrawal. The MAPK pathway induces survival by stimulating the activity of anti-apoptotic proteins such as CREB and

Bcl-2 (Kaplan and Miller 2000). The MAPK pathway may also promote cell survival by blocking apoptotic pathways initiated by p75NTR. For example, in cultured oligodendrocytes, TrkA-mediated MAPK activation suppressed the JNK pathway of p75NTR, thereby rescuing cells from apoptosis (Yoon *et al.* 1998).

The primary cell survival pathway is the PI-3K pathway. One target of NGF-induced PI-3K activity is the serine/threonine kinase Akt. While Akt is a convergence point for survival signals in neurons, it does not play a role in neurite outgrowth or cell differentiation. Activation of Akt induces survival by inhibiting activation of downstream apoptotic proteins. In the PI-3K pathway, cell survival occurs through phosphorylation of Bad, a pro-apoptotic protein. Phosphorylation of Bad prevents this protein from associating with, and thereby inactivating, the anti-apoptotic transcription factors Bcl-2 and Bcl-XL (Kaplan and Miller 2000). Both of these factors, particularly Bcl-2, promote axonal plasticity and regeneration following injury (Horner and Gage 2000). The PI-3K pathway also plays a role in late phases of neurite outgrowth (Kaplan 1995) (Figure 2.5).

Both the Trk and p75NTR pathways mediate survival promoting effects of NGF (Bui *et al.* 2002; Kaplan and Miller 2000). Activation of p75NTR can mediate neurotrophin binding or induce apoptosis or cell survival (Bredesen and Rabizadeh 1997; Eggert *et al.* 2000; Hempstead *et al.* 1991; Yoon *et al.* 1998). While neurotrophin-p75NTR binding is important for Trk signaling, p75NTR is able to signal independently of Trk activation (Kaplan and Miller 2000).

There are several proteins downstream of p75NTR that have roles in cell survival, cell cycle regulation, and neurite outgrowth. These include TRAF 2, -4, and -6, NRAGE,

SC-1, and Rho (Figure 2.6) (Kaplan and Miller 2000). Activation of TRAF 6 induces NF-κB activation and consequent cell survival. Yamashita *et al.* (1999) demonstrated that p75NTR is a constitutive activator of Rho and that this activation induces rigid actin filament assembly. However, a less rigid form of actin is necessary for neurite outgrowth. When a neurotrophin binds p75NTR, Rho activity is inactivated and actin assembly assumes a less rigid structure, permitting increased neurite outgrowth. Research has documented this effect with NGF, BDNF, and NT-3.

Under conditions of NGF withdrawal, neuronal apoptosis is a p75NTR-mediated process (Aloyz *et al.* 1998). The withdrawal of NGF activates p75NTR, initiating a cascade of cellular events that begins with activation of the TRAF 2/4 protein. Intracellular ceramide levels increase, leading to the activation of the JNK-p53-Bax cell death pathway (Figure 2.6) (Kaplan and Miller 2000; Mazzoni *et al.* 1999). While it is unclear how p75NTR activates this pathway, TrkA activation downregulates it. Phosphorylation of Ras after TrkA activation suppresses the levels and activities of these proteins (Kaplan and Miller 2000; Mazzoni *et al.* 1999). Interestingly, JNK activation appears dependent on the activity of Ras, but not Raf (Kaplan 1995). The NF-κB pathway of p75NTR is unaffected by TrkA activation. These results indicate that high TrkA and p75NTR activity together suppresses the death signal of p75NTR and activates survival signals of the receptor (Yoon *et al.* 1998).

A second p75NTR-dependent apoptotic pathway involves the intracellular signaling protein, neurotrophin receptor interacting factor (NRIF) (Kaplan and Miller 2000). Upon NGF-p75NTR binding during development, NRIF may translocate to the nucleus thereby transducing cell death signals (Casademunt *et al.* 1999). Therefore,

neurotrophins acting as growth inhibitors through p75NTR may provide a mechanism for regulating the specificity and/or density of axonal growth and target innervation (Kaplan and Miller 2000).

The apoptosis-inducing function of p75NTR is important following neural injury (Kaplan and Miller 2000). Expression of the intracellular domain of p75NTR after injury causes death of injured motor neurons (Majdan *et al.* 1997). Neurotrophins released after injury may exacerbate injury through p75NTR (Rudge *et al.* 1998). Some cells will not display the appropriate Trk receptor for neurotrophins released into the injury site. Consequently, these cells may undergo apoptosis. For example, NGF has the potential to be pro-apoptotic in cells expressing low levels of TrkA (Kaplan and Miller 2000).

Brain-Derived Neurotrophic Factor and Neurotrophin-3

Both brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) share just over 50% sequence homology with NGF (Hagg *et al.* 1993; Yin *et al.* 1998; Zhang *et al.* 2000). These neurotrophins also overlap with NGF in biological activity and cells they target (Hagg *et al.* 1993). In the intact nervous system, BDNF and NT-3 serve as maintenance factors, supporting the survival and axonal outgrowth of certain neuronal cells (Cai *et al.* 1998; Landreth 1999). *In vitro*, BDNF and NT-3 have both trophic and neurite-promoting effects (Hagg *et al.* 1993). Along with neurotrophin-4/5, BDNF is specific for the TrkB receptor whereas NT-3 is specific for the TrkC receptor (Yin *et al.* 1998).

Downstream signaling of TrkB is similar to that of TrkA. Upon BDNF-TrkB binding, the SHC protein appears to serve as the primary mediator of growth and

survival. As with TrkA signaling, SHC initiates signaling cascades that result in activation of PI-3K and MEK. There is conflicting literature regarding the role of PLC-γ activation by TrkB. Some research suggests this protein plays a minor role in neurite outgrowth; other studies suggest it is necessary (Atwal *et al.* 2000).

In the intact spinal cord of the adult rat, motor neurons in the lumbar region express TrkB and TrkC. The presence of these receptors suggests that BDNF and NT-3 may serve as possible treatments upon motor nerve injury (Yin *et al.* 1998). However, upon axotomy by transection in the CNS or PNS, both TrkB and TrkC are downregulated (Kwon *et al.* 2004; Qiao and Vizzard 2002). These results differ compared to crush injury in the PNS. While TrkB is upregulated after crush lesion in the PNS, TrkC is downregulated. These results have two implications: NT-3 may not contribute to regeneration to the degree BDNF does after peripheral nerve injury (Zhang *et al.* 2000), and both BDNF and NT-3 will have decreased efficacy after axotomy compared to their effectiveness in the intact nervous system. Contrary to these results, Schnell *et al*. (1994) found that exogenous NT-3 injected into the transected spinal cord of adult rats increased regenerative sprouting. This effect was greater than that seen after injection of exogenous NGF and BDNF.

During ensuing inflammation after trauma, platelets are the initial source of BDNF. Anterograde transport of BDNF is enhanced in nerves following peripheral nerve injury (Zhang *et al.* 2000). In the CNS, oligodendrocytes and their progenitors may serve as a primary and continuing source of increased BDNF and NT-3 after injury (McTigue *et al.* 1998).

Since oligodendrocytes are responsible for myelin formation in the CNS, the loss of these cells upon injury to the spinal cord contributes to demyelination. Endogenous, highly motile oligodendrocyte progenitors present in the adult CNS form mature oligodendrocytes that replace those lost due to injury. Because oligodendrocytes express TrkA, -B, and -C receptors, NGF, BDNF and NT-3 may contribute to oligodendrocyte motility. This interaction would, in turn, contribute to myelinogenesis (McTigue *et al.* 1998).

The roles and expression of BDNF and NT-3 differ from that of NGF during degeneration and regeneration in the PNS. For example, BDNF and NT-3 appear to contribute to neurite outgrowth and myelinogenesis to a greater degree than NGF (Grill *et al.* 1997; McTigue *et al.* 1998). Both BDNF and NGF are normally expressed in low levels in the adult PNS and are induced in peripheral nerves during Wallerian degeneration (Meyer *et al.* 1992; Schwab and Bartholdi 1996). However, the increase in BDNF expression differs from that of NGF expression in three ways: localization, time-course, and peak levels. Increased BDNF mRNA is localized to Schwann cells and distal segments of the lesioned nerve (Meyer *et al.* 1992; Zhang *et al.* 2000). Furthermore, BDNF mRNA peaks at levels 10 times higher than NGF mRNA (Meyer *et al.* 1992).

The above evidence indicates that degeneration and regeneration of nerve fibers after physical or chemical injury is dependent on the presence of neurotrophic factors and their appropriate high-affinity receptor. Determining the roles of neurotrophins, their high- and low-affinity receptors, and the signaling cascades after neurotrophin-receptor binding can contribute to our understanding of the mechanism of OPIDN. Finally, many events occur within hours after physical injury in the PNS and CNS. Similar early events

may occur after exposure to a neuropathic OP compound. Investigating these early changes may elucidate the mechanism of delayed neuropathy.

Summary

Organophosphorus compounds are found in household pest control products, plastics, and petroleum. The ubiquitous nature of OP compounds allows for regular human contact with them (Davis and Richardson 1980; Markowitz 1992). However, OP compounds are neurotoxic, and can cause both acute and delayed clinical signs (Davis and Richardson 1980). One of these delayed effects is the development of OPIDN, characterized by Wallerian-like degeneration of nerves in the CNS and PNS (Ehrich and Jortner 2001). While pathological changes that accompany OPIDN are well-characterized, there are many questions surrounding the mechanisms of the onset of OPIDN.

The pathophysiology of axonal damage is similar between trauma and neuropathic OP injury. Both types of injuries exhibit Wallerian-like degeneration and differing degrees of regeneration depending on the location of the injury (CNS or PNS). Axonal sprouting, seen as neurite outgrowth *in vitro*, is the primary means for regeneration in the nervous system. Production, release, and reception of neurotrophins may be part of the response to injury in neural tissues (Hagg *et al.* 1993). The exact role of neurotrophins in regeneration and axonal sprouting is unclear.

Literature identifies a role for neurotrophic factors after traumatic injury to the nervous system, but concentrations of neurotrophins and neurotrophin receptors after exposure to a neuropathic OP compound has not been examined. Neurotrophins and their

receptors contribute to regeneration within the CNS (Schnell *et al.* 1994; Schwab and Bartholdi 1996). This study clarifies the temporal role of neurotrophins and their receptors after OP exposure. *In vitro* studies examine the role of neuropathy-inducing OP compounds in signal transduction of cellular pathways that promote neurite outgrowth, which may prevent sustained axonal regeneration. The results of this study provide information that may elucidate early mechanisms of neuropathic OP exposure.

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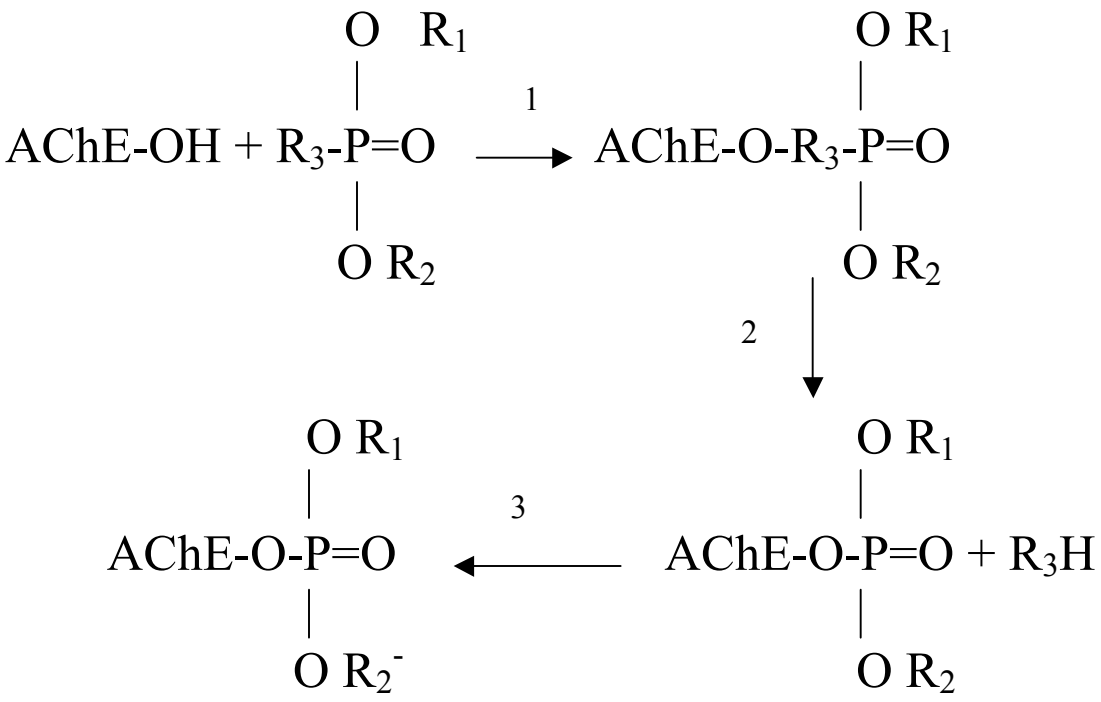

Figure 2.1. Aging of Acetylcholinesterase. The hydroxyl group of AChE covalently reacts with acidic group of a neuropathy-inducing organophosphate (reaction 1). Dealkylation leads to discharge of a leaving group from the AChE-OP complex (reaction 2). Intramolecular rearrangement results in a permanent negative charge on the enzyme (reaction 3). R groups bound to O atoms can be either the same or different and can include $–CH_2$, $-C_2H_5$, or a 6-carbon ring.

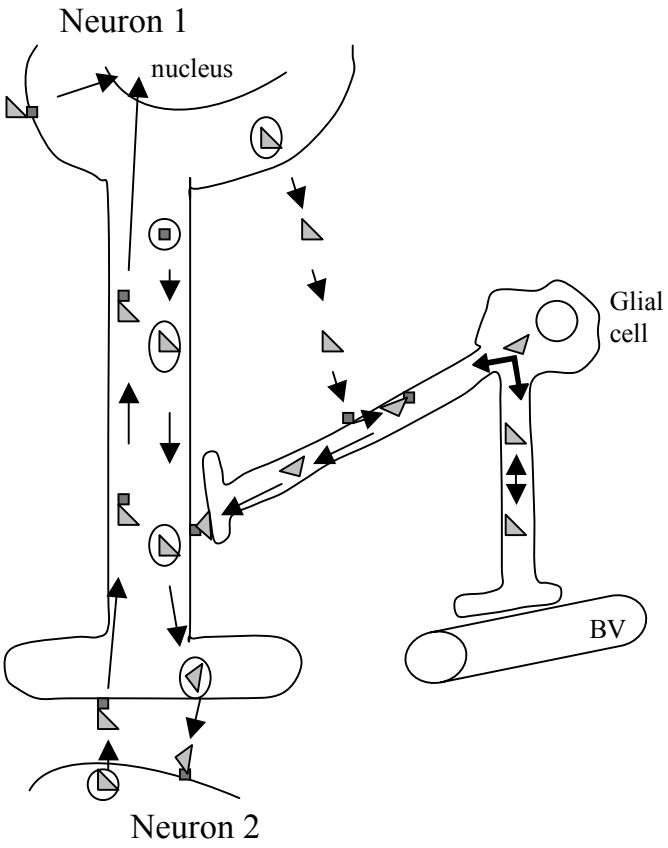

Figure 2.2. Neurotrophic factor activity in neuronal and non-neuronal cells. Neurotrophins from glial cells bind their respective receptor on a neuron and are retrogradely transported to the nucleus, resulting in activation of transcription factors. Anterograde and retrograde transport of neurotrophins and receptors allows bi-directional communication between Neuron 1 and Neuron 2. Glial cells both receive and release neurotrophic factors from neurons and blood vessels. BV= blood vessel; ▪ = receptor; ◣ = neurotrophin. Vesicles are indicated by circles (○). Diagram adapted from Fallon and Loughlin (1973).

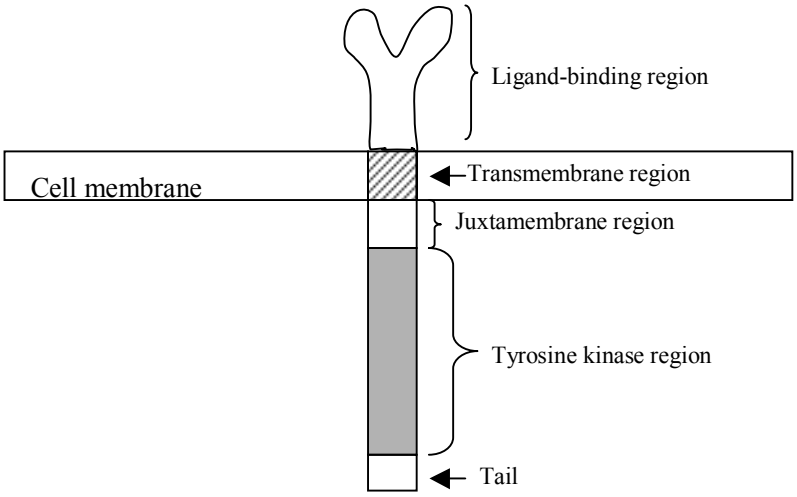

Figure 2.3. Representation of a Trk receptor. The juxtamembrane domain contains the src homology to collagen (SHC) region and is the least well-conserved within the cytoplasmic region between the Trk receptors. The tyrosine kinase domain is essential for signal transduction.

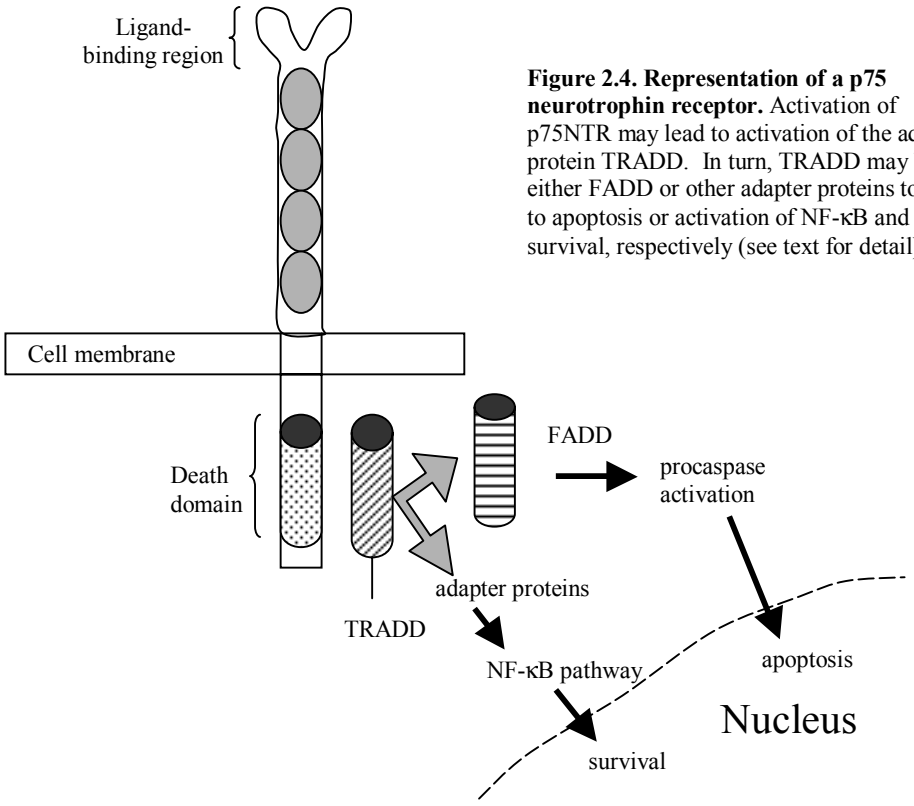

Figure 2.4. Representation of a p75 neurotrophin receptor. Activation of p75NTR may lead to activation of the adapter protein TRADD. In turn, TRADD may bind either FADD or other adapter proteins to lead to apoptosis or activation of NF-κB and survival, respectively (see text for detail).

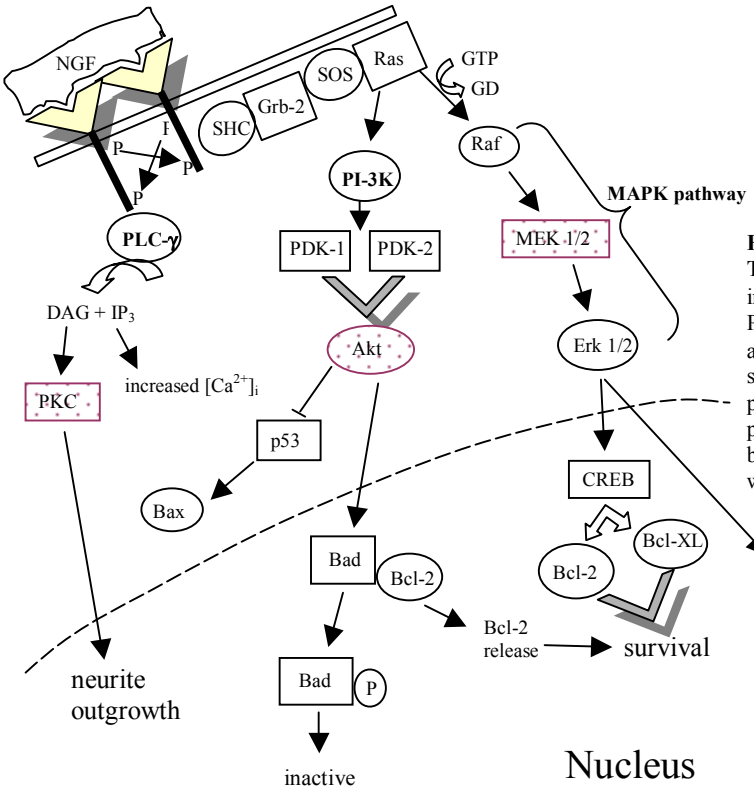

Figure 2.5. Signal transduction of the TrkA receptor. The 3 main pathways include PLC-γ, PI-3K, and MAPK. The PKC pathway results in neurite outgrowth and PLC-γ pathway results in cell survival. The MAPK pathway is able to produce both these effects. →= pathway permitted; —┤= pathway blocked. Dotted proteins are proteins that will be targeted.

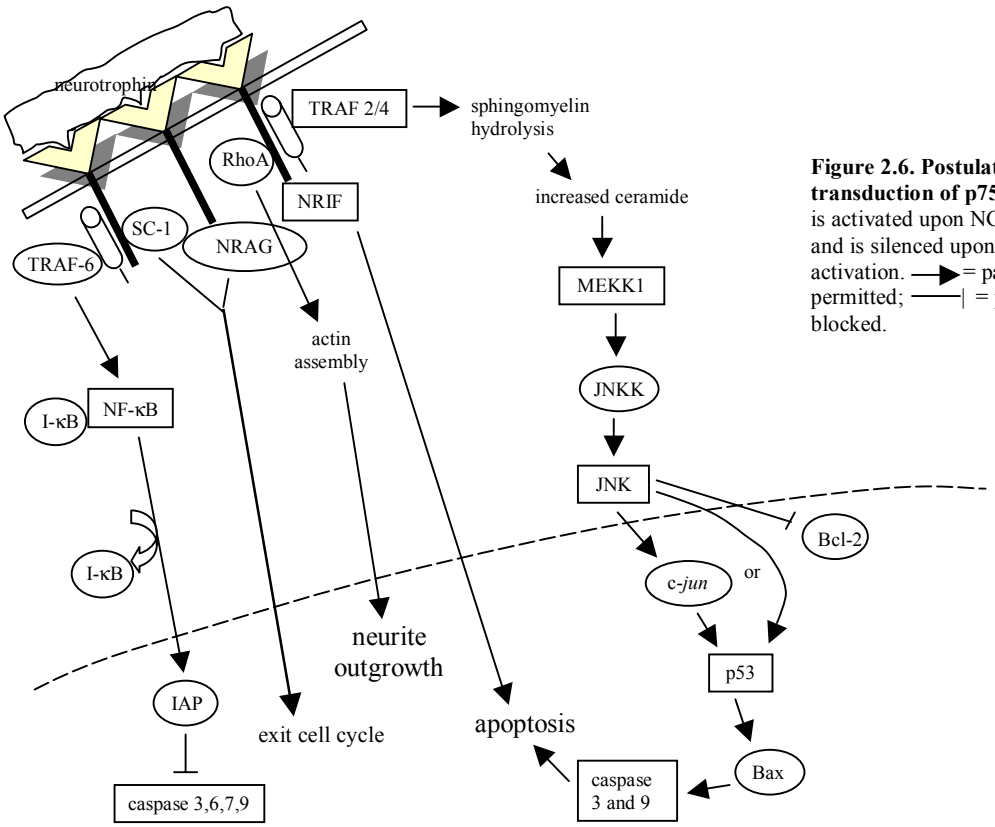

Figure 2.6. Postulated signal transduction of p75NTR. Traf 2/4 is activated upon NGF withdrawal and is silenced upon TrkA activation. → = pathway permitted; —| = pathway blocked.

PART III.

MATERIALS AND METHODS

Chapter 3.

EXPERIMENTAL METHODS AND PROTOCOLS

In vivo and *in vitro* experiments in this dissertation tested the hypothesis that an immediate increase in growth factor release, specifically nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), after *in vivo* exposure to neuropathy-inducing organophosphorous (OP) compounds does not elicit axonal regeneration. I tested second and third hypotheses that neuropathic OP compounds interfere with the ratio of activated high-affinity and low-affinity neurotrophin receptors and that neuropathic OP compounds inhibit activation of intracellular proteins downstream of these receptors shortly after exposure. Finally, I characterized a soluble factor released in the spinal cord of chickens exposed to a neuropathic OP compound on the basis of molecular weight and charge.

Chapter 4. This study examined the concentrations of neurotrophins, specifically nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), after exposure to neuropathic and non-neuropathic OP compounds *in vivo*. The neuropathic OP compound was dissolved in DMSO (127 mg PSP in 25.4 ml DMSO providing a 5 mg/ml concentration; a 2.5 mg/kg dose was provided in a 0.5 ml/kg volume). The dose of 2 mg/kg PSP is sufficient to cause OPIDN in adult hens (Jortner and Ehrich, 1987). The non-neuropathic OP compound was dissolved in ethanol (13.76 mg paraoxon in 1 ml ethanol providing a 13.76 mg/ml concentration; the paraoxon was further diluted in saline such that a 0.5 mg/kg dose was provided in a 0.1 ml/kg volume). This dose of paraoxon is sufficient to produce at least 70% inhibition of AChE without

significantly affecting NTE (McCain *et al.,* 1996; McCain *et al.,* 1995). The negative control was 0.5 ml/kg DMSO. Three endpoints were assessed: neurotrophin concentration, neurotoxic esterase (NTE) activity, and acetylcholinesterase (AChE) activity.

1. I determined NGF and NT-3 concentration determined using the NGF and NT-3 E_{max} ImmunoAssay System (Promega Corporation, Madison, WI), respectively.
2. I determined BDNF concentration using the ChemiKine™ BDNF kit (Chemicon, Temecula, CA).
3. NTE and AChE activity were determined by the spectrophotometric assay outlined in Correll and Ehrich (1991).

Chapter 5. This study examined ratio of the activated high-affinity receptor for NGF (pTrkA) to the activated low-affinity receptor for all neurotrophins (pp75) in SH-SY5Y human neuroblastoma cells differentiated with retinoic acid and treated with a neuropathic and non-neuropathic OP compounds. Prior to treating cells with an OP compound, we differentiated them with 10^{-5} M retinoic acid (RA). Differentiating these cells with RA yields similar results to differentiation accomplished using NGF, including a morphological appearance similar to that of mature neurons. Differentiation with RA also induces p75 expression, decreased cell proliferation, expression of TrkA and TrkB receptors and a cholinergic phenotype (Abemayor *et al.* 1989; Cosgaya *et al.* 1996; Kaplan *et al.* 1993; Påhlman *et al.* 1984). Because we are interested in a toxicant that affects the cholinergic system, differentiation with RA was more appropriate for our purposes than differentiation with NGF (Adem *et al.*, 1987). We initially dissolved the

neuropathic OP compound, PSP, in DMSO (11.4 mg PSP (mw = 262) in 435 µl DMSO). To make a stock 100 µM PSP solution, I dissolved 1 µl of PSP dissolved in DMSO in 999 µl acetone. From the stock solution, I dissolved 2 ml PSP in 20 ml media, followed by dissolving 4.5 ml PSP in 45 ml media. This yielded a 1 µM PSP solution, which served as the high dose of the neuropathic OP compound. I then dissolved 500 µl of the 1 µM PSP solution in 50 ml media for a 0.1 µM PSP solution, followed by dissolving 500 µl of the 0.1 µM PSP solution in 50 ml media to yield a 0.01 µM solution of PSP. This served as the low dose of the neuropathic OP compound. Concentration of DMSO ranged from 0.00001% - 0.000000001%. For the non-neuropathic OP compound (paraoxon), I dissolved paraoxon in ethanol for a 50 mM paraoxon solution. For 100 µM paraoxon, I dissolved 100 µl 50 mM paraoxon in 50 ml media, giving an ethanol concentration of 0.005%. Control cells were treated with medium only. We used medium only as the negative control because the concentration of vehicle in PSP- and paraoxon-treated cells was so low.

At 10^{-7} mol/L, PSP inhibits >80% of available NTE in SH-SY5Y cells (Ehrich *et al.,* 1997). This NTE inhibition level is similar to the 70-80% threshold inhibition of NTE required before the development of OPIDN *in vivo* (Ehrich *et al.,* 1993; Johnson 1993; Moretto *et al.,* 1991). At 10^{-4} mol/L, paraoxon inhibits nearly 100% of available AChE in SH-SY5Y cells differentiated with RA without causing significant NTE inhibition (Ehrich *et al.*, 1997).

I determined the amount of pTrkA and pp75 using Western blot analysis and completed densitometry using NIH Image (v. 1.63).

Chapter 6. This study examined the effects of neuropathic and non-neuropathic OP compounds on the activation of specific SH-SY5Y human neuroblastoma intracellular proteins downstream of the activated TrkA receptor that play a role in cell survival and/or neurite outgrowth. I measured three endpoints associated with three different signaling pathways: phosphorylated protein kinase C-α (pPKC-α), phosphorylated mitogen activated kinase (pMek1/2), and phosphorylated Akt (pAkt). I determined the amount of pPKC-α, pMek1/2, and pAkt using Western blot analysis. I completed densitometry using NIH Image (v. 1.63).

Statistics. We determined the statistical validity of all data ($p < 0.05$) using the MIXED procedure in SAS (version 8.2, SAS Institute, Cary, NC).

1. We performed an analysis of variance (ANOVA) followed by Bonferroni corrected comparisons for ELISA data.
2. We performed an ANOVA followed by Tukey's t-test for neurotrophin receptor ratio and receptor level data.
3. We performed an ANOVA followed by Tukey's t-test for intracellular protein level data.
4. We performed an ANOVA for neurite outgrowth data.

Appendix C. This study characterized the molecular weight of a soluble factor released in the lumbar spinal cord of chickens exposed to a neuropathic OP compound that elicits neurite outgrowth in SH-SY5Y human neuroblastoma cells. Prior to treating the cells with supernatant from birds exposed to OP compounds, I differentiated the cells with 10^{-5}

M RA. Although Pope *et al.* (1995) used NGF to differentiate SH-SY5Y cells, literature suggested that differentiation by either RA or NGF yields similar phenotypic characteristics. Furthermore, because we are interested in a toxicant that affects the cholinergic system, differentiation with RA was more appropriate for our purposes than differentiation with NGF (Adem *et al.*, 1987). I determined the molecular weight range of this factor using ultrafiltration and digitized images of formalin-fixed cells. Using a morphometry program (Image Processing Tool Kit, v.5, Reindeer Graphics, Asheville, NC), I traced the lengths of neurites.

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PART IV.

RESULTS

Chapter 4

Neurotrophic Factors Are Not Increased as an Early Effect of Exposure to Neuropathy-Inducing Organophosphates

ABSTRACT

Organophosphorous (OP) esters are used as insecticides, petroleum additives and modifiers of plastics (Davis and Richardson, 1980), and have also been employed as weapons of terrorism. Exposure to OP compounds that inhibit neurotoxic esterase (NTE) induces a delayed neuropathy (OPIDN) characterized by Wallerian-like degeneration of long axons in certain animals, including humans. Pope *et al*. (1995) found that neurite outgrowth occurred following the addition of spinal cord extracts from chickens with active OPIDN to SH-SY5Y cells. This suggested growth factors might be expressed during the neuropathy. Support for this comes from other studies, which document increased levels of nerve growth factor (NGF) in the spinal cord after traumatic injury (Heumann *et al.*, 1987a; Raineteau and Schwab, 2001), a condition in which individual fiber degeneration resembles that of OPIDN. We hypothesized that the level of neurotrophic factors NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) would increase in susceptible regions of the nervous system shortly after exposure to a neuropathy-inducing OP compound as the central nervous system (CNS) attempts to recover from the toxic insult, but that this release cannot be sustained. To test this hypothesis, we exposed juvenile chickens to a neuropathy-inducing OP (PSP, 2.5 mg/kg), an OP compound that does not induce neuropathy (paraoxon, 0.10 mg/kg), and vehicle (DMSO, 0.5ml/kg) intramuscularly. By day 8, all PSP-treated birds demonstrated clinical signs of OPIDN. We sacrificed chickens by pentobarbital overdose at 4, 8, 24, and 48 hours, and 5 and 10 days and confirmed NTE inhibition in birds treated with PSP for 4 and 24 hours and performed enzyme-linked immunosorbant assays to determine the concentrations of NGF, BDNF, and NT-3 in lumbar spinal cord. Our data indicate that NGF, BDNF, and NT-3 are found in chicken spinal cord but do not increase as a result of exposure to neuropathic OP compounds. Additionally, BDNF and NT-3 levels demonstrated decreasing and increasing trends, respectively, from 4 hours to 10 days post-exposure in all treatment groups. These results establish the presence of NGF, BDNF, and NT-3 after neuropathic OP compound exposure and suggest that these neurotrophins alone do not contribute to a sustained regenerative effort in the CNS.

INTRODUCTION

Between days and weeks after exposure to neuropathy-inducing organophosphates, certain animal species develop clinical signs that progress from abnormal foot placement and leg weakness to an unwillingness to walk and, finally, flaccid paralysis. Susceptible species include humans, cats, and chickens. (Ehrich and Jortner, 2001; Lotti, 1992). Neurotoxic esterase (NTE) is an enzyme that is inhibited in the brain, spinal cord, and peripheral nerves of these susceptible species within hours of exposure to certain OP esters (Davis and Richardson, 1980; Lotti, 1992). The development of a strong bond between NTE and the OP compound ("aging") is one essential factor in the initiation of OPIDN (Ehrich, 1996; Ehrich and Jortner, 2001). Sufficient doses of certain OP compounds, including PSP, can inhibit and age 70-80% of available NTE within hours of OP exposure (see Literature Review) (Ehrich, 1996; Johnson, 1993; Moretto *et al.*, 1991). This inhibition and aging of NTE ultimately results in lesions consistent with the development and progression of OPIDN in both the spinal cord and peripheral nerves (Jortner and Ehrich, 1987). It is interesting to note that young animals appear relatively resistant to the effects of neuropathic OP exposure although they do exhibit NTE inhibition (Ehrich, 1996; Ehrich and Jortner, 2001; Funk *et al.*, 1994; Peraica *et al.*, 1993).

Histopathology reveals the primary lesion of OPIDN to be bilateral degeneration in distal levels of myelinated axons. This syndrome primarily affects large and/or long peripheral nerve fibers and fibers in the central nervous system (CNS). Microscopically, these lesions appear as pale-staining, swollen axons with thin myelin sheaths or collapsed axons, both progressing to fiber degeneration (Ehrich and Jortner, 2001; Jortner, 2000;

Lotti, 1992). Toxins that affect the axon directly elicit axonal degeneration (axonopathy) that begins at the distal segment of the axon and spreads proximally toward the soma. This lesion is referred to as a dying-back axonopathy, or Wallerian-like degeneration (Graham and Montine, 2002; Jortner, 2000; King, 1999).

Dying-back of nerve fibers in the CNS presents a medical challenge. This is because axonal regeneration does not occur in the CNS as it does in the peripheral nervous system (PNS) (Brady, 1993; Ehrich and Jortner, 2001). Evidence suggests that CNS neurons have an inherent capability to regenerate, but this does not occur in the CNS environment due to the presence of inhibitory factors, such as those produced by neuroglia (Brady, 1993; Schwab and Bartholdi, 1996; Taniuchi *et al.*, 1988). Glial cells, including oligodendrocytes and myelin, contribute to inhibition of neurite outgrowth in the CNS, resulting in decreased regeneration in the CNS compared to the PNS (Brady, 1993).

Published information identifies a role for neurotrophic factors in traumatic injury to the nervous system (see Literature Review). Accumulation of nerve growth factor (NGF) at lesion sites has been attributed to both cellular infiltrates and the induction of tissue NGF expression following physical trauma. Macrophages and activated mast cells may also indirectly upregulate NGF production in surrounding cells when they release cytokines following trauma. An initial rapid release of NGF may control immediate modifications to the nervous system after injury, while a prolonged NGF supply contributes to homeostatic functions allowing for reequilibration of the tissue after injury (Leon *et al.*, 1994). Expression of NGF following peripheral nerve injury occurs in two phases. An initial rapid increase within 24 hours of injury is followed by a second, larger

burst days later (Heumann *et al.*, 1987b). This second burst is apparently due to the effects of IL-1 released by infiltrating macrophages (Heumann *et al.*, 1987b; Meyer *et al.*, 1992).

In both OPIDN and traumatic spinal cord injury, axonal sprouting is the primary means for regeneration in the nervous system. Increasing evidence suggests that neurotrophins play a role in the establishment of new axonal sprouts, which is one form of synaptic plasticity. It is this plasticity that permits the limited regeneration seen in the CNS (Blöchl *et al.*, 1995; Brunello *et al.*, 1990; Grill *et al.*, 1997; Perez-Polo and Werrbach-Perez, 1987). Production, release, and reception of neurotrophins may be part of the response to injury in neural tissues. These factors likely play an important role in compensatory repair processes after injury (Hagg *et al.*, 1993). The exact role of growth factors in this process is unclear, but it is known that neurotrophin concentration rapidly changes in the spinal cord under pathological conditions (Raineteau and Schwab, 2001). An upregulation of growth factors and chemoattractants appears to direct new sprouts to the appropriate target area (Thallmair *et al.*, 1998). Initial regeneration of nerve fibers occurs as a result of localized, transient increase in neurotrophic factors at the lesion site (Hagg *et al.*, 1993). It is important to note that regenerative sprouts do not elongate without the appropriate growth factors (Schnell *et al.*, 1994; Schwab and Bartholdi, 1996).

In the normal nervous system, the family of neurotrophins plays an essential role in neuronal survival, growth, and differentiation (Kaplan and Miller, 2000; Maisonpierre *et al.*, 1990; Pope *et al.*, 1995). Currently, there are 4 proteins that compose the neurotrophin family: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3

(NT-3), and neurotrophin 4/5 (Barker and Shooter, 1994; Meakin and Shooter, 1992). The best-characterized neurotrophin is NGF, a 26 kilodalton (kD) protein. *In vitro* experiments document that the direction of neurite extension can be guided along an NGF concentration gradient (Hagg *et al.*, 1993). This upregulation induces Schwann cell migration. These migrating cells form bands of Bünger around the distal and proximal ends of the injured nerve. The bands of Bünger go on to guide the regenerating axon back to its target (Anton *et al.*, 1994).

Both BDNF and NT-3 share just over 50% sequence homology with NGF (Hagg *et al.*, 1993; Yin *et al.*, 1998; Zhang *et al.*, 2000). These neurotrophins also overlap in biological activity and target cells with NGF (Hagg *et al.*, 1993). In the intact nervous system, BDNF and NT-3 serve as maintenance factors, supporting the survival and axon outgrowth of certain populations of neurons (Cai *et al.*, 1998; Landreth, 1999). During ensuing inflammation after trauma, platelets serve as the initial source of BDNF. Anterograde transport of BDNF is enhanced in nerves following peripheral nerve injury (Zhang *et al.*, 2000). Oligodendrocytes and their progenitors may serve as a primary and continuing source of increased BDNF and NT-3 after injury (McTigue *et al.*, 1998).

There is evidence that the roles and concentrations of BDNF and NT-3 differ from that of NGF during degeneration and regeneration of peripheral nerves (Grill *et al.*, 1997; McTigue *et al.*, 1998). Time-related concentration of neurotrophins and neurotrophin receptors has not been examined after exposure to neuropathy-inducing OP compounds. We hypothesized that the level of neurotrophic factors NGF, BDNF, and NT-3 would increase shortly after exposure to a neuropathy-inducing OP compound as the CNS attempts to recover from the toxic insult, but that this release cannot be

sustained. Consequently, Wallerian-like degeneration would affect axons and eventually clinical signs of OPIDN emerge.

SPECIFIC QUESTIONS AND RATIONALE

1. Are the relative concentrations of NGF, BDNF, and NT-3 in the lumbar spinal cord of chickens exposed to a neuropathic OP different when compared to relative concentrations of NGF, BDNF, and NT-3 in the lumbar spinal cord of chickens exposed to a non-neuropathic OP?

We wanted to determine how the concentrations of neurotrophin proteins change, if at all, after exposure to phenyl saligenin phosphate (PSP) and paraoxon, neuropathic and non-neuropathic OP compounds, respectively. The vehicle, DMSO, served as the negative control. Proteins are the functional end-products of gene expression. This experiment allowed us to determine the concentrations of NGF, BDNF, and NT-3 proteins after treatment with PSP, paraoxon, and DMSO.

The adult hen (*Gallus gallus*) is the U.S. Environmental Protection Agency recognized animal model for OPIDN, in part because there is a lag period of only several days between exposure and clinical alterations, and lesions are extensive and distinct (Ehrich, 1996; Ehrich and Jortner, 2001; U.S.EPA, 1991). Similar to humans, clinical symptoms in chickens progress from abnormal foot placement and leg weakness to an unwillingness to walk and finally, flaccid paralysis (Ehrich and Jortner, 2001; Lotti, 1992). The use of an *in vivo* model is desired because it accounts for cell interactions that may occur in the physiological environment and allows development of clinical signs. Such interactions may not be detected in an *in vitro* environment where only one type of cell is cultured.

We used the lumbar portion of the spinal cord because research demonstrates that axonal degeneration occurs in the lumbar spinal cord of chickens after neuropathic OP

exposure. The most susceptible portions of the lumbar cord to OPIDN are the fasciculus gracilis and medial pontine spinal tract (Jortner, 2000; Jortner and Ehrich, 1987).

We used PSP (Figure 4.1a.) as the neuropathy-inducing OP compound because it produces lesions in the chicken consistent with the development and progression of OPIDN in both the spinal cord and peripheral nerves (Jortner and Ehrich, 1987). Furthermore, PSP does not cause concurrent cholinergic poisoning associated with significant acetylcholinesterase (AChE) inhibition, and it does inhibit neurotoxic esterase (NTE) without need for biotransformation (Ehrich and Correll, 1998; Ehrich *et al.*, 1993). Inhibition of NTE is required before OPIDN can occur (Ehrich and Jortner, 2001). The dose of PSP we used (2.5 mg/kg) induces at least 70% inhibition of NTE without significantly affecting the level of AChE. In order to compare the effects of a neuropathic compound with a non-neuropathic OP compound, we used paraoxon. Paraoxon (Figure 4.1b) inhibits AChE but not NTE in the chicken and therefore, does not induce the delayed locomotor deficits that are seen in OPIDN (McCain *et al.*, 1996; McCain *et al.*, 1995).

At least two methods are appropriate for quantifying proteins in spinal cord extract: Western blotting and enzyme-linked immunosorbant assays (ELISAs). We used the ELISA method since neurotrophic factors are present in quantities too small to be detected by Western blots. Cross-reactivity of commercially-available NGF and NT-3 antibodies (Promega Corp., Madison, WI) with chicken was established in preliminary experiments (D. Parran, unpublished observations). Further preliminary experiments established cross-reactivity of commercial BDNF antibodies (Chemicon International Inc., Temecula, CA) with chicken (M. Pomeroy, unpublished observation).

2. Do the concentrations of NGF, BDNF, and NT-3 in the lumbar spinal cord of chickens exposed to a neuropathic OP compound or a non-neuropathic OP compound change with time from 4 hours to 10 days post-exposure when compared to chickens exposed to vehicle only?

We chose several timepoints to examine neurotrophin protein concentration, including 4, 8, 24, and 48 hours, and 5 and 10 days post-exposure. We investigated NGF, BDNF, and NT-3 concentrations early after OP exposure, prior to the appearance of clinical signs. We also measured the concentrations of these neurotrophins after the onset of clinical signs. We expected that early changes in neurotrophin concentration could contribute to development of the neuropathy at later timepoints.

We based this hypothesis on previously published results. Studies have observed decreased neurite outgrowth after exposure to neuropathic OP compounds (Henschler *et al.*, 1992; Nostrandt *et al.*, 1992). For example, using SH-SY5Y cells, Hong *et al.* (2003) found no morphometric effect on cells 4-8 days after direct exposure of the neuropathic OP mipafox (0.05 mM), but found neurite length reduced at 8-12 days. They suggested that this morphological effect was similar to the dying-back neuropathy that characterizes OPIDN (Hong *et al.*, 2003). Therefore, if neurotrophic factors are active *in vivo* shortly after exposure to OP compounds but their effects decrease soon thereafter, the timepoints we use should help explain the morphological changes seen both early and later following exposure to neuropathy-inducing OP compounds.

MATERIALS AND METHODS

Experimental Design and Analysis

For the ELISA study, we used a two-way factorial treatment structure with a generalized randomized block design. Treatment factors were compound (PSP, paraoxon, and DMSO) and time (4, 8, 24, 48 hours, 5 and 10 days post-exposure). The study was divided into 3 blocks to overcome logistical problems of sacrificing numerous birds. There were 2-3 chickens within each block*compound*time combination with a total of 8-9 chickens per treatment group. Using the MIXED procedure in SAS (version. 8.2, SAS Institute, Cary, NC), we performed an analysis of variance (ANOVA) for time and compound main effects and their interactions. Pre-planned comparisons of the interactions were performed using Bonferroni corrected comparisons of each compound at each timepoint. For NGF analysis, data were log-transformed to stabilize variances.

Treatments and Clinical Assessment

The sample studied consisted of 140 mixed-sex 10-week-old White Leghorn (*Gallus gallus*) chickens. We selected this age based on a previous study that suggested neurotrophin-related effects after neuropathic OP compound exposure (Pope *et al.*, 1995). All chickens were housed together in a temperature-controlled environment at 23^{o}C with free access to water and feed through 10 weeks of age. Three days before treatment, we moved chickens to cages for acclimation. Chickens were allocated by random selection from the entire group to compound*time combinations. On day 0, we administered 2.5 mg/kg PSP (Oryza Laboratories, Inc., Chelmsford, MA), 0.10 mg/kg paraoxon (ChemService Inc., West Chester, PA), or 0.5 ml/kg DMSO (Sigma, St. Louis, MO) to

chickens by intramuscular injection. The dose of 2 mg/kg is sufficient to cause OPIDN in adult hens (Jortner and Ehrich, 1987). The dose of paraoxon used is sufficient to produce at least 70% inhibition of AChE without significantly affecting NTE (McCain *et al.,* 1996; McCain *et al.,* 1995).

Using an established procedure, we observed chickens daily for clinical signs from days 1-4, and days 1-10 for chickens sacrificed at 5 and 10 days post-exposure, respectively (Dyer *et al.*, 1991; Ehrich *et al.*, 1993; Jortner and Ehrich, 1987; Pope *et al.*, 1995). Signs of ataxia were recorded as: 0 = no clinical signs; 1-2 = slight, infrequent hindlimb incoordination; 3-4 = moderate but definite incoordination; and, 5-6 = frequent difficulties in standing erect and walking.

Brain esterase measurements

We removed whole brains of chickens euthanized at 4 and 24 hours post-exposure to PSP and paraoxon for esterase determination. After placing the brain in a labeled tube, we froze the brain samples on dry ice. Upon finishing all sacrifices for a particular timepoint, we stored the samples at –80^{o}C. Within one month of extraction, we determined brain NTE and AChE activity using a spectrophotometric assay (Correll and Ehrich, 1991).

Enzyme-linked immunosorbant assays

We euthanized chickens at 4, 8, 24, 48 hours, and 5 and 10 days post-exposure by intra-venous pentobarbital overdose and immediately removed the lumbar spinal cord. After removing the glycogen body, we wrapped the lumbar spinal cord in aluminum foil,

placed it in a labeled scintillation tube, and froze it on dry ice. After all sacrifices for a particular timepoint, we froze the spinal cords at –80^{o}C.

Within 2 weeks of extraction, we weighed the entire spinal cord and suspended two-thirds of the cord 1:10 (w:v) in cold lysis buffer [150 mM NaCl , 20 mM Tris-HCl, 10% glycerol (v:v), 1% Triton X-100 (v:v), 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1:200 dilution of Protease Inhibitor Cocktail Set III (Calbiochem, La Jolla, CA)]. We used approximately one-fourth of the total wet weight of the cord for the BNDF ELISA, and one-third for NGF and NT-3 ELISA. The wet weight of the cord in BDNF ELISAs ranged from 50-70 mg in all blocks (Table 1). The wet weight of spinal cord used in NGF and NT-3 ELISAs ranged from 110-140 mg in block 1, 110-130 mg in block 2, and 90-120 mg in block 3 (Table 1).

After homogenizing lumbar spinal cord using an ultrasonic homogenizer (W-225R model, Heat Systems-Ultrasonics, Inc., Farmingdale, NY), we centrifuged the homogenate at 4^{o}C at 14000 x g for 30 minutes. We removed the supernatants, placed each respective supernatant in a labeled 2 ml microcentrifuge tube, and maintained them at –20^{o}C until assayed.

We determined the protein concentration of each supernatant using the Bio-Rad *DC* Protein Assay (Bio-Rad, Hercules, CA), which is similar to the Lowry assay (Lowry *et al.*, 1951). Prior to each assay, we diluted each sample 1:5 with lysis buffer. We prepared standard dilutions in the range of 0 mg/ml – 2.0 mg/ml. For each 96-well plate, we added 60 µl reagent S to 3 ml reagent A to make reagent A'. To each well, we added 5 µl of diluted samples to respective wells, followed by 25 µl of reagent A', and finally 200 µl reagent B. After agitating the plate for five seconds, it was incubated at room

temperature for 15 minutes. Absorbance was read at 750 nm by a SPECTRAmax Plus^{384} microplate spectrophotometer and Softmax Pro software (Molecular Devices, Sunnyvale, CA).

We measured BDNF concentrations using 96-well plate and ChemiKine™ BDNF kit (Chemicon, Temecula, CA). For each assay, we performed serial dilutions for a standard curve in the range of 0 pg/ml – 500 pg/ml. We added samples in triplicate (100 µl per well) and incubated the plate on a shaker (100 rpm) at 4^{o}C overnight. The following day, we washed the plate four times with 1X Wash Buffer and added 100 µl of biotinylated mouse anti-BDNF to each well. After incubating the plate on a shaker at room temperature for 2.5 hours, we washed the plate four times with 1X Wash Buffer, added 100 µl horseradish peroxidase (HRP) conjugated streptavidin to each well and incubated the plate on a shaker at room temperature for one hour. We washed the plate four times with 1X Wash Buffer and added 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) to each well in order to detect the presence of HRP. Following a 15 minute incubation period at room temperature, we added 100 µl of HCl to each well in the same order the TMB was added and read the absorbance at 450 nm with a SPECTRAmax Plus^{384} microplate spectrophotometer and Softmax Pro software (Molecular Devices). We calculated the concentration of BDNF and expressed it as µg BDNF per mg protein.

We measured NGF and NT-3 levels on 96-well plates using the NGF and NT-3 E_{max} ImmunoAssay System (Promega, Madison, WI). For each assay, we coated wells with 100 µl of anti-NGF or anti-NT-3 carbonate coating buffer and incubated the plate overnight at 4^{o}C. The next day, after washing wells with 200 µl TBST Wash Buffer (Tris-buffered saline + 0.05% (v:v) Tween-20), we blocked the plate for one hour at room

temperature. Following blocking, we again washed the wells with 200 µl TBST Wash Buffer and performed serial dilutions for a standard curve in the range of 0 pg/ml – 250 pg/ml for NGF and 0 pg/ml – 300 pg/ml for NT-3. After diluting samples to be used in the NT-3 ELISAs (1:1) in saline, we added samples in triplicate (100 µl per well). We did not dilute samples used in NGF ELISAs. After incubating the plate on a shaker (~500 rpm) at room temperature for 6 hours, we washed the plate 5 times with TBST Wash Buffer and incubated it with anti-NGF or anti-NT-3 overnight at 4^{o}C. The following day, we washed the plate 5 times with TBST Wash Buffer and added 100 µl IgG-HRP to each well. After incubating the plate for 2.5 hours on a shaker (~500 rpm) at room temperature, we washed the plate 5 times with TBST Wash Buffer and added 100 µl TMB to each well. We incubated the plate for 15 minutes on a shaker (~500 rpm) at room temperature, added 100 µl 1N HCl to each well in the same order TMB was added, and read the absorbance at 450 nm with a SPECTRAmax Plus^{384} microplate spectrophotometer and Softmax Pro software (Molecular Devices). We calculated the concentrations of NGF and NT-3 and expressed the concentrations as µg NGF or NT-3 per mg protein.

RESULTS

Clinical Signs and Neurochemistry

Clinical signs were not detected before day 4 in any PSP-treated chickens. Chickens treated with PSP and sacrificed at 5 days had a clinical score of 1.0 +/- 0.58 (mean +/- SD) prior to sacrifice, which progressed to 3.5 +/- 1.5 (mean +/- SD) on day 10. No effect was seen with DMSO or paraoxon-treated birds (Figure 4.2).

Brain NTE and AChE assays confirmed the inhibition of NTE and AChE by PSP and paraoxon, respectively (Figures 4.3 and 4.4). Greatest NTE inhibition occurred 4 hours post-dosing with PSP ($p < 0.0001$), with some recovery at 24 hours (Figure 4.3). Inhibition of AChE was greatest at 4 hours post-dosing with paraoxon ($p < 0.0001$) with significant recovery at 24 hours ($p < 0.05$) (Figure 4.4).

Relative concentrations of NGF, BDNF, and NT-3 in the lumbar spinal cord of chickens exposed to a neuropathic or non-neuropathic OP compound

Concentrations of NGF ($p = 0.81$), BDNF, ($p = 0.70$), and NT-3 ($p = 0.86$) were not affected by the compound administered (Figures 4.5, 4.6, 4.7).

Temporal concentrations of NGF, BDNF, and NT-3 in the lumbar spinal cord of chickens exposed to a neuropathic or non-neuropathic OP compound

There was no effect on NGF concentrations due to time ($p = 0.16$). There was not a significant effect due to the interaction of compound administered (PSP, paraoxon, DMSO) and time of sacrifice ($p = 0.74$). We found a trend toward increase in NGF concentration between 8 hours and 24 hours among all groups of chickens, such that NGF concentration is greater at 24 hours than at 8 hours ($p = 0.063$) (Figure 4.5).

There was a trend toward decrease in BDNF concentration from 4 hours post-treatment to 10 days post-exposure ($p = 0.09$). We found no significant effect from the interaction of compound administered (PSP, paraoxon, DMSO) and time of sacrifice on BDNF concentrations in the spinal cord ($p = 0.19$) (Figure 4.6).

We found no significant effect from the interaction of compound administered (PSP, paraoxon, DMSO) and time of sacrifice on NT-3 concentrations in the spinal cord ($p = 0.60$). The concentration of NT-3 increased from 4 hours post-treatment to 10 days post-exposure among all compounds administered ($p = 0.045$) (Figure 4.7).

DISCUSSION

These results established the presence of NGF, BDNF, and NT-3 in the spinal cord of control chickens and chickens treated with PSP, a neuropathy-inducing compound. Concentration of each of these neurotrophins did not change significantly from levels in control chickens after exposure to this neuropathy-inducing OP compound or to a non-neuropathy-inducing OP compound (paraoxon). Previous studies show an increase in NGF after trauma, suggesting that NGF contributed to recovery efforts after trauma (Brunello *et al.*, 1990; Hagg *et al.*, 1993; Heumann *et al.*, 1987a). Because there was no upregulation of these neurotrophins, our data suggest that NGF, BDNF, and NT-3 alone do not play a significant role in recovery efforts of the CNS or PNS after exposure to neuropathy-inducing OP compounds.

In relation to neuronal injury, NGF, BDNF, and NT-3 are the representative members of the neurotrophin family that historically have been the most studied. Another less studied member of the neurotrophin family, NT-4/5, promotes survival of spinal ganglion neuronal cultures equivalent to the survival seen after BDNF administration and greater than that post-NT-3 administration. Interestingly, NGF showed no detectable effect on neuronal survival (Ebadi *et al.*, 1997). While we did not include NT-4/5 in our study, but it would be interesting to observe the effects of neurotoxicants on expression of this neurotrophin as well.

We are interested in the concentrations of neurotrophins after exposure to neuropathic OP compounds because Pope *et al.* (1995) discovered a soluble factor in the supernatant of homogenized cervical spinal cords of birds exposed to a neuropathy-inducing compound that elicited neurite outgrowth in SH-SY5Y cells. Observations in

our laboratory confirm that this effect is not seen at 4 or 8 hours post-dosing, but is seen at 24 hours post-dosing (M. Pomeroy-Black, unpublished observations). We did not see increases in BDNF or NT-3 in the supernatant of homogenized spinal cords between 8 and 24 hours post-dosing. This suggests that neither BDNF nor NT-3 is the soluble factor responsible for neurite outgrowth noted by Pope *et al.* (1995). Although we did find an increasing trend in NGF concentration between 8 and 24 hours, this occurred among all treatments and not only in birds treated with a neuropathic OP compound.

Consequently, if Pope's proposed soluble factor were one of the three neurotrophins we examined, it is likely we would have seen significant increased concentration of one or all of the neurotrophins in spinal cords of birds treated with PSP compared to vehicle and paraoxon. Therefore, the soluble factor eliciting neurite outgrowth in the Pope *et al.* (1995) study is not likely to be NGF, BDNF, or NT-3.

Concentration of NGF showed a trend toward increasing between 8 hours and 24 hours post-treatment ($p = 0.063$). These data might suggest that the soluble factor observed in Pope's study (1995) is NGF itself. However, because the birds in this study were juveniles and because this trend occurred across all treatments, we must consider that increased levels of NGF between 8 and 24 hours may be a developmental effect. For example, we discovered temporal changes in both BDNF and NT-3 concentrations between 4 hours and 10 days post-dosing. Concentration of NT-3 increased slightly ($p = 0.045$) over this time period, while BDNF concentration showed a trend of decreasing slightly ($p = 0.09$). Therefore, future studies using adult birds, which are more susceptible to OPIDN (Moretto *et al.*, 1991), may confirm that the increase in NGF seen is an effect of treatment and not age.

These data support findings of another study that suggests other factors in the spinal cord, and perhaps in the cerebrospinal fluid, are necessary for regenerative efforts after exposure to OP compounds (Funk *et al.*, 1994). Younger animals demonstrate higher levels of neurotrophins than adult animals. Some studies have suggested that these higher concentrations allow the young to better cope with toxic insults compared to adults (Ehrich and Jortner, 2001; Funk *et al.*, 1994; Peraica *et al.*, 1993).

Our study suggests that it is unlikely that high concentrations of neurotrophins are the essential factors that assist regenerative efforts of young animals' central nervous systems. A combination of factors may collectively appear as a single "soluble factor" that induces neuronal regeneration, represented by neurite outgrowth in cell culture. In particular, the cytokine family, including interleukins, tumor necrosis factors, interferons, neuropoietins, colony-stimulating factors, leukemia inhibitory factor (LIF), and fibroblast growth factors (FGF), plays a role in neuronal recovery after insult (Ebadi *et al.*, 1997). Glial cells serve as the immune system of the CNS, playing an integral role in sustaining the CNS after injury or exposure to viruses, fungi, or bacteria (Cotran *et al.*, 1999). These cells give the CNS its own cytokine network (Ebadi *et al.*, 1997). Studies have detected the production of various cytokines after brain injury, in neurodegenerative disease, and in viral and bacterial infections (Junier *et al.*, 1998).

While the neurotrophins we examined are constitutively produced, cytokines are induced upon injury. For example, ciliary neurotrophic factor and LIF, also known as cholinergic differentiation factor, are induced upon peripheral nerve injury and possess similar activity to NGF, BDNF, and NT-3 (Ebadi *et al.*, 1997; Landreth, 1999). However, high concentrations of LIF appear to inhibit neurite outgrowth *in vitro* (Edoff

and Jerregard, 2002). Other studies implicate FGF as a role-player in the promotion of axonal regeneration (Landreth, 1999). Exogenous basic FGF applied to sensory neurons supports neurite outgrowth after axotomy (Grothe and Nikkhah, 2001). Glial cell line-derived neurotrophic factor is a survival factor for motoneurons (Landreth, 1999). Finally, research demonstrates that treating neuronal cells with interleukins such as IL-6 and –12 enhances neurite outgrowth (Lee *et al.*, 2000; Lin *et al.*, 2000). It is likely that, in response to neuropathic-OP exposure, non-neuronal cells release cytokines and that these cytokines work collaboratively with constitutively present neurotrophins.

Most of the cytokines noted above have similar signaling pathways as neurotrophins, particularly NGF, BDNF, and NT-3, because they bind receptor tyrosine kinases (RTKs) (Landreth, 1999). A common pathway that is activated by proteins that bind RTKs is the mitogen-activated protein kinase (MAPK) pathway (see Literature Review). One result of activation of the MAPK pathway is neurite outgrowth and cell survival (Kaplan and Miller, 2000). Consequently, it is not unreasonable to suggest that one or more of the above mentioned factors contribute to the neurite outgrowth seen in Pope *et al.* (1995). Therefore, one or more of these factors may be essential in regenerative efforts, visualized as neurite outgrowth *in vitro*, seen after neuropathic-OP compound exposure.

Finally, the results of this study emphasize the fact that repair mechanisms of the CNS after exposure to an OP compound differ from repair mechanisms after trauma. By virtue of the injury itself, trauma induces inflammation and the resulting consequences thereof (see Literature Review). Toxic insult does not induce inflammation. Whereas several studies show that trauma results in an upregulation of NGF (Brunello *et al.*, 1990;

Hagg *et al.*, 1993; Heumann *et al.*, 1987a; Kawaja and Gage, 1991), we did not see definitive upregulation of NGF, BDNF, or NT-3 in response to neuropathic-OP toxicity.

The presence of the neurotrophins NGF, BDNF, and NT-3 in the avian model of OPIDN is clearly established with this study. However, it is unlikely that any of these three neurotrophins contribute to a sustained regenerative effort within the CNS after exposure to a neuropathy-inducing OP compound. Our data suggest that NGF may play a transient role in regenerative efforts between 8 and 24-hours after exposure, but that this release is not sustained.

While our data suggest that NGF may be involved in regenerative efforts in the CNS, it is likely that NGF collaborates with other factors in the CNS to attempt regeneration. *In vitro* studies using antibodies to block NGF in lumbar spinal cord supernatants of birds exposed to a neuropathic OP can clarify whether this neurotrophin plays a role in regeneration. Incubating supernatants separately with antibodies to known *in vivo* factors, such as NGF, BDNF and NT-3, may reduce neurite outgrowth seen by Pope *et al.* (1995). Future studies can also examine incubation of antibodies to these three neurotrophins together. These methods can further decipher if these neurotrophins work collectively or individually to initiated neurite outgrowth *in vitro*.

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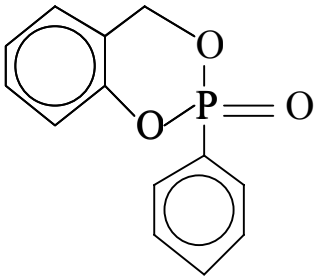
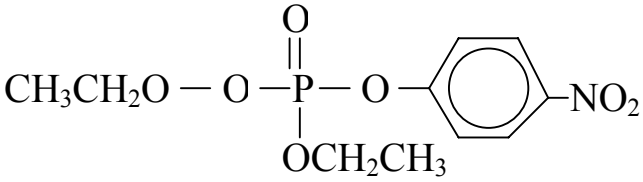
(a) PSP



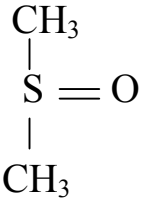
PSP causes NTE inhibition and minimal AChE inhibition.

(b) paraoxon



Paraoxon causes AChE inhibition but not NTE inhibition.

(c) DMSO



DMSO was the vehicle for PSP in this study.

Figure 4.1. Structures of phenyl salignen phosphate (PSP), paraoxon and dimethyl sulfoxide (DMSO).

Table 4.1. Mean wet weights and standard deviations of lumbar spinal cord tissue used in nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 enzyme-linked immunosorbant assays.

Neurotrophin	Block	N	Mean (mg)	SD (mg)
BDNF	1	51	62.4	6.5
	2	46	63.3	6.3
	3	37	57.8	7.1
NGF/ NT-3	1	51	123.1	9.3
	2	46	120.7	7.1
	3	37	112.2	6.3

Cord weights for NGF/NT-3 ELISAs are approximately double that used in BDNF ELISAs due to logistics of ELISA kits. Means and standard deviations of cord weights are given.

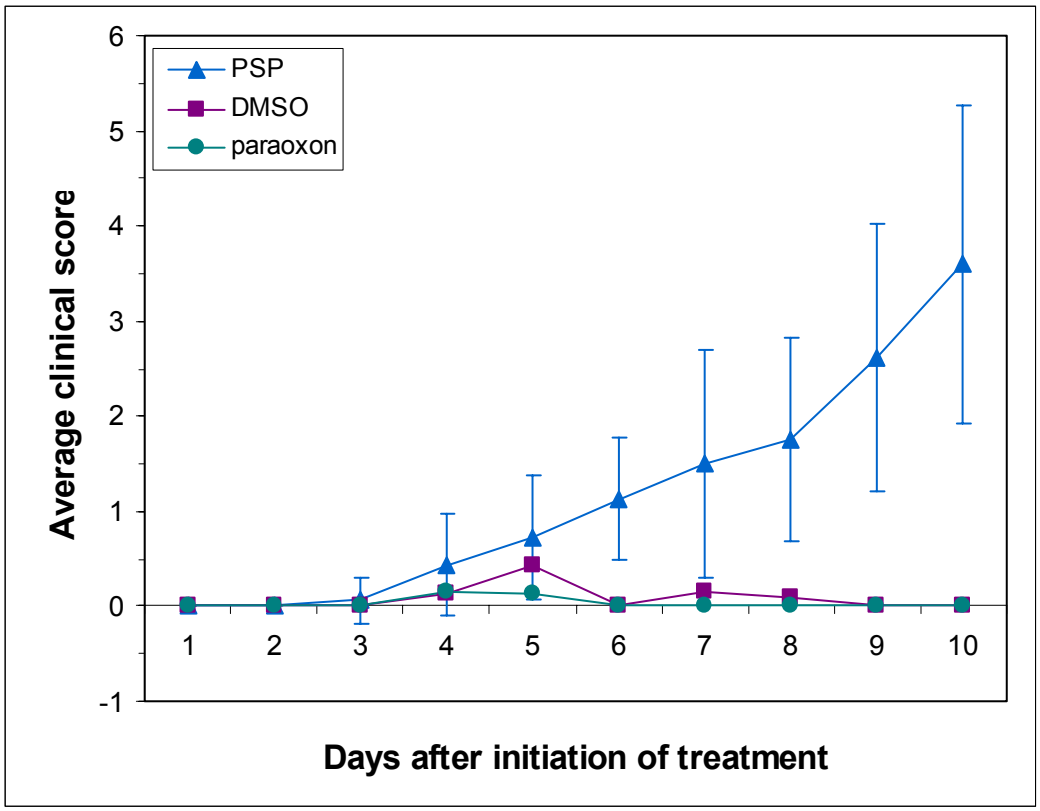


Figure 4.2. Average daily clinical score after intramuscular treatment with a neuropathic esterase inhibitor (PSP, 2.5 mg/kg) (n = 8 at 5 and 10 days), non-neuropathic esterase inhibitor (paraoxon, 0.10 mg/kg) (n = 7 at day 5, n=8 at day 10), or vehicle (DMSO, 0.5 ml/kg) (n = 6, at 5 and 10 days). All birds treated with PSP showed clinical signs of neuropathy by day 8 post-treatment. Means and standard deviation (represented by error bars) are shown. Birds treated with DMSO or paraoxon demonstrated variability less than what could be displayed on the graph due to the size of the square or circle.

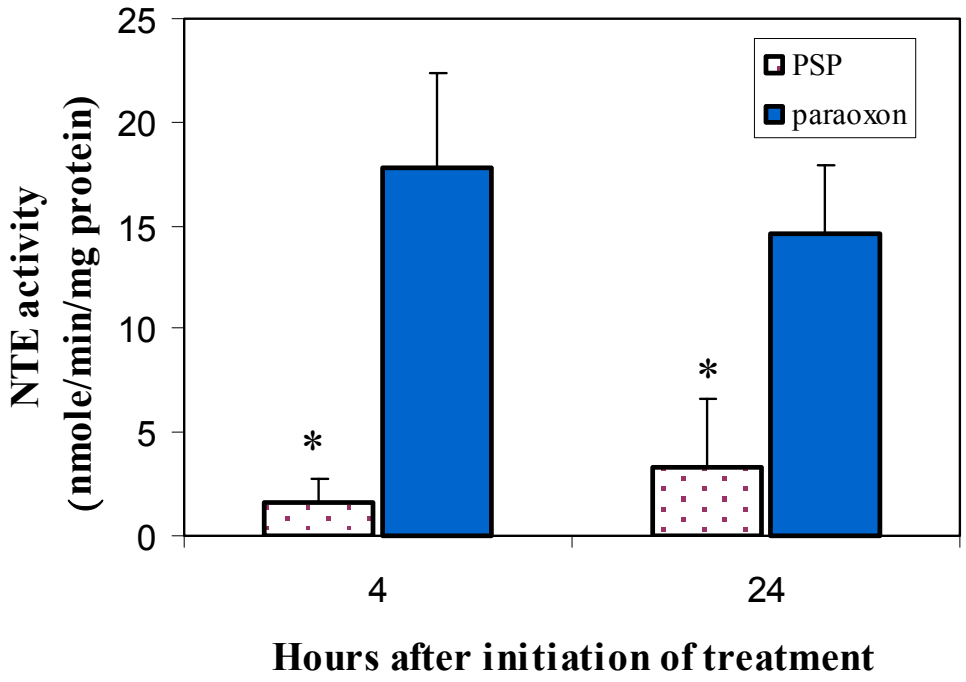


Figure 4.3. Neurotoxic esterase activity in the brain of chickens *(Gallus gallus)* 4 hours and 24 hours post-dosing IM with a neuropathic esterase inhibitor (PSP, 2.5 mg/kg) (n=8) or a non-neuropathic esterase inhibitor (paraoxon, 0.10 mg/kg) (n = 8). Means and standard deviations (represented by error bars) are shown. Means within each timepoint were compared using a t-test (GraphPad Prism)[1]. Birds treated with PSP had significantly more NTE inhibition than paraoxon treated birds at both timepoints ($p < 0.0001$), as indicated by an "*". Historical control data of adult birds treated with DMSO were 20.39 +/- 5.27 nmole/min/mg protein (mean +/- SD, n = 4) and 25.3 +/- 1.1 nmole/min/mg protein (n = 28) 4 and 24 hours post-dosing, respectively (Ehrich *et al.* 1995; Fox *et al.* 2003).

[1] version 1.0, GraphPad Software, Inc., San Diego, CA

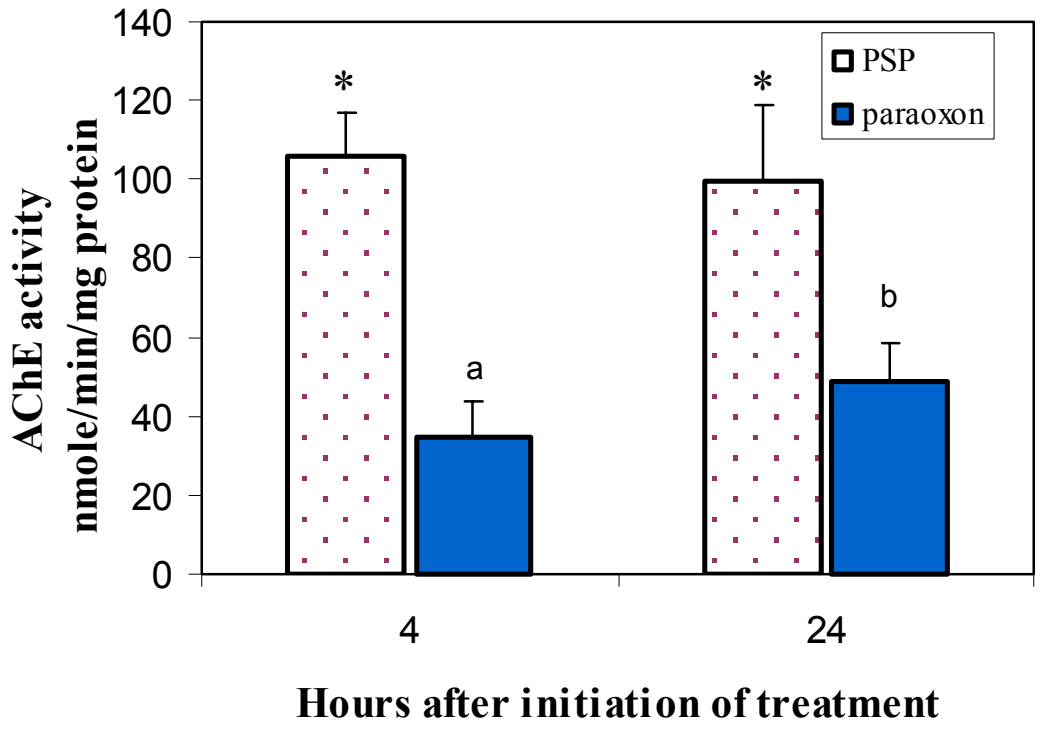


Figure 4.4. Acetylcholinesterase activity in the brain of chickens (***Gallus gallus***) 4 hours and 24 hours post-dosing IM with a neuropathic esterase inhibitor (PSP, 2.5 mg/kg) (n=7-8) or a non-neuropathic esterase inhibitor (paraoxon, 0.10 mg/kg) (n = 7-8). Means and standard deviations (represented by error bars) are shown. Means within each timepoint were compared using a t-test (GraphPad Prism)[2]. Birds treated with PSP had significantly less AChE inhibition than paraoxon treated birds at both timepoints at the $\alpha = 0.0001$ level, as indicated by an "*". There was a slight recovery in AChE inhibition in paraoxon-exposed birds between 4 and 24 hours post-treatment ($p < 0.05$). Treatments with different letters were significantly different ($p < 0.05$). Historical control data of adult birds treated with DMSO were 76.8 +/- 27.0 nmole/min/mg protein (mean +/- SD, n

[2] version 1.0, GraphPad Software, Inc., San Diego, CA

= 4) and 246 +/- 13 nmole/min/mg protein (n=28) 4 and 24 hours post-dosing, respectively (Ehrich *et al.* 1995; Fox *et al.* 2003).

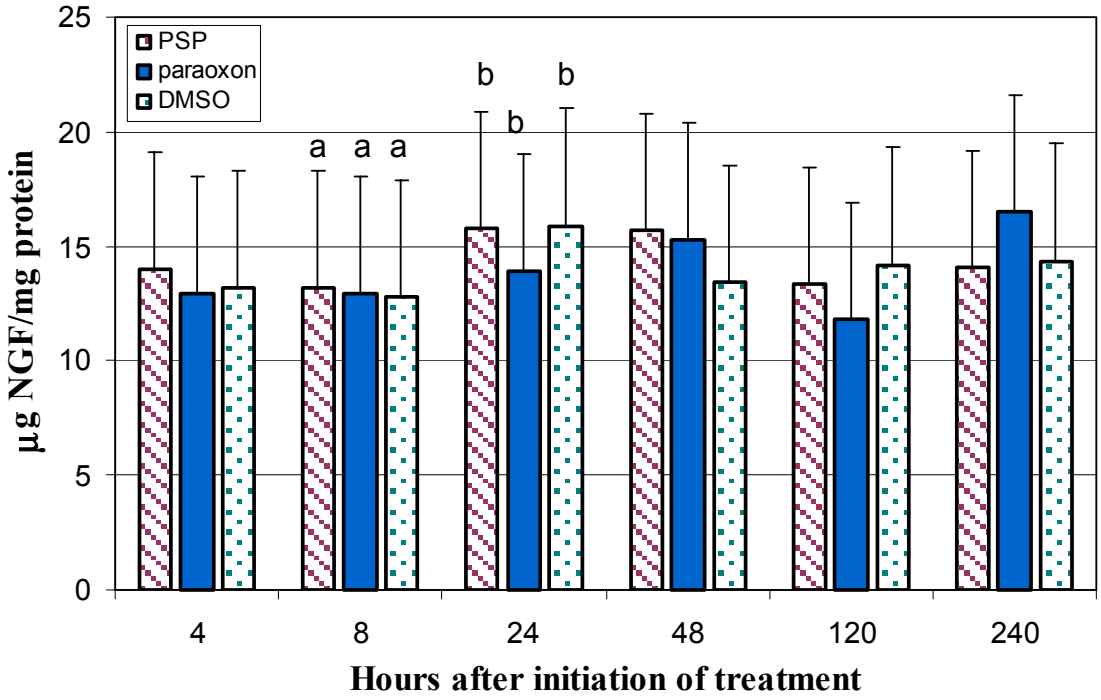


Figure 4.5. Least square means and standard error (represented by error bars) of nerve growth factor (NGF) concentrations in lumbar spinal cord of chickens treated with a neuropathic esterase inhibitor (PSP, 2.5 mg/kg) (n = 8-9 per timepoint), non-neuropathic esterase inhibitor (paraoxon, 0.10 mg/kg) (n = 7-8 per timepoint), and vehicle (DMSO, 0.5 ml/kg) (n = 6-7 per timepoint) 4-48 hours and 5 and 10 days post-dosing. Treatments with similar letters were *not* significantly different ($p < 0.05$). Treatments with different letters showed a trend toward significance ($p \leq 0.06$), such that all treatment groups showed a trend toward increase between 8 and 24 hours post-treatment. Concentrations of NGF were not affected by compound administered ($p = 0.81$) or by time ($p = 0.16$). There was no significant compound*time interaction ($p = 0.74$).

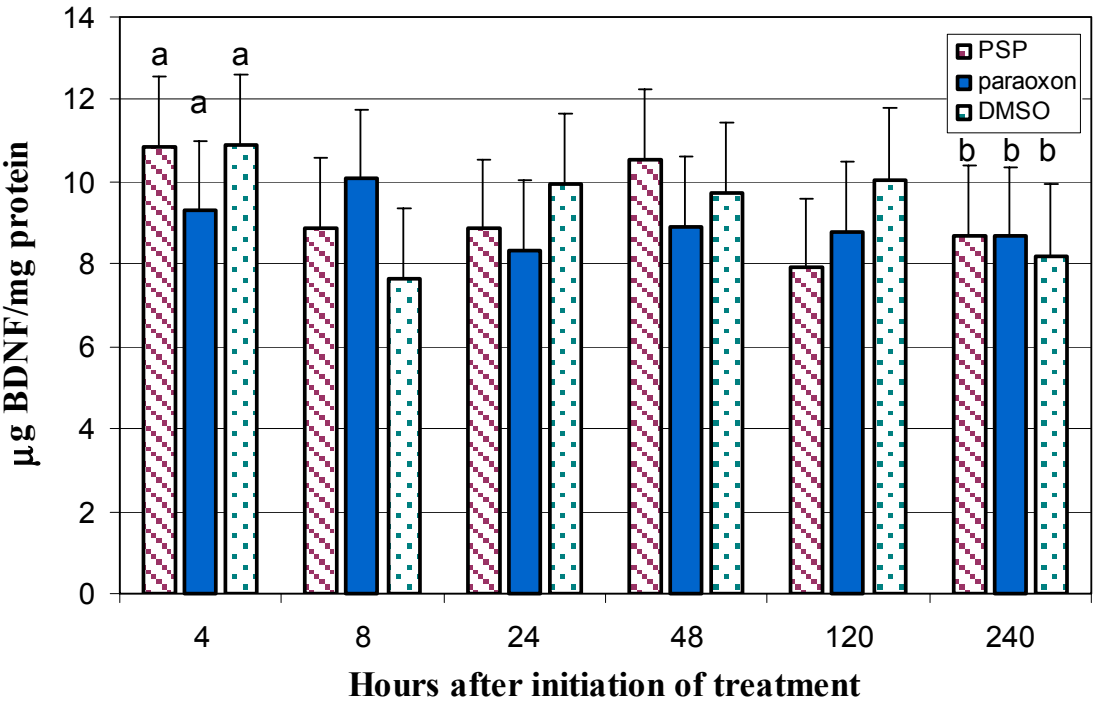


Figure 4.6. Least square means and standard error (represented by error bars) of brain derived neurotrophic factor (BDNF) concentrations in lumbar spinal cord of chickens treated with a neuropathic esterase inhibitor (PSP, 2.5 mg/kg) (n = 7-9 per timepoint), non-neuropathic esterase inhibitor (paraoxon, 0.10 mg/kg) (n = 7-8 per timepoint), and vehicle (DMSO, 0.5 ml/kg) (n = 6-7 per timepoint) 4-48 hours and 5 and 10 days post-exposure. Treatments with similar letters were *not* significantly different ($p < 0.05$). Treatments with different letters showed a trend toward significance ($p < 0.10$), such that there was a gradual decrease from 4 hours post-exposure to 10 days post-exposure among all treatment groups. Concentrations of BDNF were not affected by compound administered ($p = 0.70$). There was no significant compound*time interaction ($p = 0.19$).

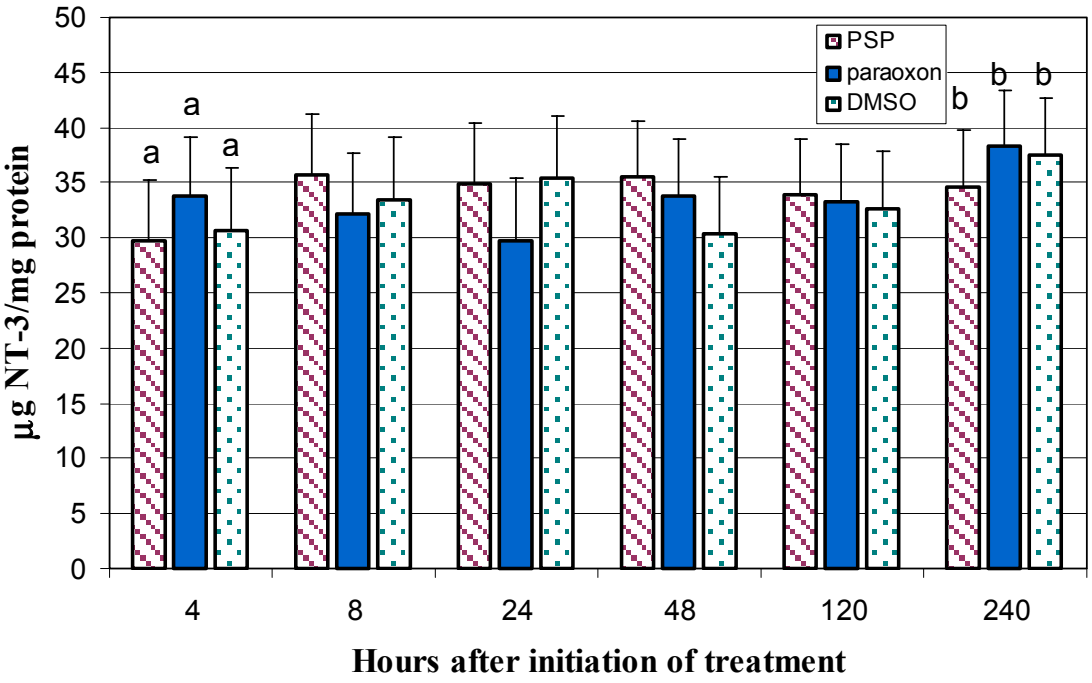


Figure 4.7. Least square means and standard error (represented by error bars) of neurotrophin-3 (NT-3) concentrations in lumbar spinal cord of chickens treated with a neuropathic esterase inhibitor (PSP, 2.5 mg/kg) (n = 5-9 per timepoint), non-neuropathic esterase inhibitor (paraoxon, 0.10 mg/kg) (n = 4-8 per timepoint), and vehicle (DMSO, 0.5 ml/kg) (n = 4-7 per timepoint) 4-48 hours and 5 and 10 days post-exposure. Treatments with similar letters were *not* significantly different ($p < 0.05$). Treatments with different letters were significantly different ($p < 0.05$). Therefore, the concentrations of NT-3 are significantly lower at 4 hours compared to 10 days among all treatments. Concentrations of NT-3 were not affected by compound administered ($p = 0.86$). There was no significant effect due to compound*time interaction ($p = 0.60$). The concentrations of NT-3 are significantly lower at 4 hours compared to 10 days among all treatment groups.

Chapter 5

Levels of Active Neurotrophin Receptors in SH-SY5Y Cells after Exposure to Neuropathy-Inducing Organophosphates

ABSTRACT

Bi-directional crosstalk between the two types of neurotrophin receptors (Trk and p75) allows generated signals to oppose or augment each other. For example, depending on the degree of TrkA activity, p75NTR transmits positive (neurite extension) or negative (apoptotic) signals (Bredesen and Rabizadeh 1997; Chao and Hempstead 1995). We hypothesize that a neuropathy-inducing organophosphate (OP) compound interferes with neurotrophin-receptor binding when compared to a non-neuropathic OP compound. To test this hypothesis, we exposed SH-SY5Y human neuroblastoma cells to a neuropathic OP compound (PSP; 0.01 μM, 0.1 μM, 1.0 μM), a non-neuropathic OP compound (paraoxon; 100 μM), a neuropathic OP compound with nerve growth factor (1.0 μM PSP + 1 ng/ml NGF), and medium only for 4, 8, 24, and 48 hours. We performed Western blots on cell lysates to determine the level and ratio of activated high-affinity NGF receptor (pTrkA) to activated low-affinity common neurotrophin receptor (pp75). Our data demonstrate that both receptors were present in SH-SY5Y cells and that pp75 is expressed in higher levels after exposure to a non-neuropathic OP compound. The higher levels of pp75 relative to pTrkA in other treatment groups early after exposure may contribute to cell death by apoptosis (Dobrowsky *et al.* 1994; Rabizadeh *et al.* 1993). Our data also indicate that there is no change in the ratio of pTrkA to pp75 shortly after exposure to neuropathic or non-neuropathic OP compounds. Therefore, the decrease in neurite outgrowth seen after neuropathic OP exposure cannot be attributed solely to a change in the ratio of activated neurotrophin receptors.

INTRODUCTION

In the normal nervous system, the family of neurotrophins plays an essential role in neuronal survival, growth, and differentiation (Landreth 1999). Currently, there are four proteins that compose the neurotrophin family: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (Barker and Shooter 1994; Meakin and Shooter 1992). Upon traumatic or chemical injury, neurotrophic factors have the capability to protect neurons and restore impaired functions when they bind coexpressed high- and low-affinity receptors (Blöchl *et al.* 1995; Hagg *et al.* 1993).

There is increasing evidence that neurotrophins play a role in neural plasticity, or regeneration (Blöchl *et al.* 1995; Brunello *et al.* 1990; Grill *et al.* 1997; Perez-Polo and Werrbach-Perez 1987). Retrograde transport of neurotrophins to the nucleus causes a cascade of cellular events resulting in activation of transcription factors that control gene expression. Changes in gene expression can contribute to the potential for neuronal survival and regeneration after insult (Fallon and Loughlin 1993). Literature suggests that failure of sustained regeneration in the central nervous system may result from insufficient reception of neurotrophins, rather than unavailability of neurotrophic factors (Hagg *et al.* 1993; Raivich and Kreutzberg 1993).

Each neurotrophin binds specifically to a high-affinity receptor ($K_d \approx 10^{-11}$ M) with intrinsic tyrosine kinase activity (Trk) (Barker and Shooter 1994; Hantzopoulos *et al.* 1994). The cytoplasmic region of Trk receptors consists of three distinct domains: the juxtamembrane region, the kinase catalytic domain, and the C-terminal tail (Figure 5.1). The highly conserved tyrosine residue plays a critical role in signal transduction,

probably through binding the src homology to collagen adaptor protein (SHC) (Barbacid 1995).

Because neurotrophins exist as dimers, they are able to simultaneously bind 2 different receptors on the cell (Barker and Shooter, 1994). At the same time the neurotrophin binds its Trk receptor, it binds a receptor with low affinity (p75; $K_d \approx 10^{-9}$ M). This receptor interacts with all members of the neurotrophin family with nearly equal affinities (Bamji *et al.* 1998; Barker and Shooter 1995; Hantzopoulos *et al.* 1994) (Figure 5.2). Expression of p75 is essential to the formation of the high-affinity receptor site and can modify Trk activity, partially by concentrating neurotrophins in the local environment (Kaplan and Stephens 1994). Coexpression of the p75 receptor with Trk enhances the affinity with which a neurotrophin binds Trk as well as the cellular response to neurotrophins present in the local environment (Barker and Shooter 1995; Chao and Hempstead 1995; Greene and Kaplan 1995; Hantzopoulos *et al.* 1994; Hempstead *et al.* 1991; Kaplan and Miller 1997; Meakin and Shooter 1992).

There is conflicting evidence regarding the expression of neurotrophin receptors after injury. Some studies indicate that expression of certain Trk receptors is upregulated in dorsal root ganglia after spinal cord injury; other studies document decreased Trk receptor expression after peripheral nerve axotomy (Qiao and Vizzard 2002; Zhang *et al.* 2000). The decreased effect of a particular neurotrophin on regeneration may be due to downregulation of its Trk receptor (Zhang *et al.* 2000).

The cellular expression level of Trk and p75 has been shown to determine the fate of a neuron (Bredesen and Rabizadeh 1997; Eggert *et al.* 2000; Yoon *et al.* 1998). The intracellular signals generated by the two receptors can oppose or augment each other.

While Trk receptors transmit positive signals for enhanced survival and growth, p75 transmits both positive (neurite outgrowth) and negative (apoptotic) signals. The result of neurotrophin-mediated p75 binding is related to the expression level of Trk receptors (Bredesen and Rabizadeh 1997; Eggert *et al.* 2000; Kaplan and Miller 1997; Yoon *et al.* 1998). For example, neuronal and glial cell apoptosis is induced by p75 only when Trk is inactive or suboptimally activated (Kaplan and Miller 2000). High Trk receptor activity silences p75-mediated cell death pathways (Kaplan and Miller 1997, 2000). High p75 activity inhibits cell survival or outgrowth mediated by suboptimally activated Trk. When both receptors are highly active, Trk activity is enhanced. This results in cell differentiation and survival, and consequently, suppression of apoptosis (Kaplan and Miller 1997).

The apoptosis-inducing function of p75 is important following neural injury (Kaplan and Miller 2000). Expression of the intracellular domain of p75 after injury causes death of injured motor neurons (Majdan *et al.* 1997). Some research suggests that neurotrophins released after injury may exacerbate damage when they bind p75 (Rudge *et al.* 1998). Cells exposed to a particular neurotrophin that do not carry the specific Trk receptor for that neurotrophin undergo apoptosis (Kaplan and Miller 2000).

There are two possible mechanisms by which ligand binding of p75 enhances neurotrophin binding to the Trk receptor. The first assumes that ligand binding to p75 induces a conformational change that results in a favorable binding condition to the Trk receptor. This increases the affinity of the Trk receptor for the neurotrophin (Chao and Hempstead 1995; Kaplan and Miller 1997; Meakin and Shooter 1992). The second mechanism suggests that p75 acts to concentrate the neurotrophin in the local

environment (Hantzopoulos *et al.* 1994). Studies have shown that peripheral nerve lesions can influence the expression of neurotrophin receptors. As an example, after peripheral nerve injury, p75 is upregulated on Schwann cells. This response may increase the capacity of the Trk receptor for neurotrophins or play a role in the accumulation of neurotrophins at regenerating axons (Chao 1994).

As TrkA levels decrease, the balance of TrkA to p75 changes such that p75 levels exist in greater proportions on the cell membrane. Literature suggests that p75 positively or negatively regulates axonal growth depending on the proportion of unliganded to liganded p75 present in the local environment (Kaplan and Miller 2000). For example, TrkA expression decreases upon neuronal cell injury, but p75 expression and NGF level increase. This alteration in receptor expression may shift the balance of signaling toward the death pathway as NGF binds the more prevalent p75 (Foehr *et al.* 2000). Other factors that may contribute to changes in the ratio of these two receptors to each other include ligand-receptor conformational changes, the affinity and/or capacity of the receptors for their respective ligands, as well as the presence of either phosphatases or kinases in the environment.

We hypothesized that the level of activated p75 does not change after neuropathic OP compound exposure compared to non-neuropathic OP compound exposure. We based this hypothesis on previously published results. Carlson *et al.* (2000) demonstrated that SH-SY5Y cells exposed to non-neuropathic OP compounds undergo apoptosis whereas those exposed to a neuropathic OP compound undergo cell death that more closely resembles necrosis. This suggests that the p75 receptor is activated after OP exposure. We also hypothesize that the ratio of liganded TrkA to liganded p75 decreases

after exposure to neuropathic OP compounds, contributing to decreased neurite outgrowth after neuropathic OP compound exposure. Further, we believed that the addition of NGF to a higher level of a neuropathic OP compound will not affect the ratio of liganded TrkA to liganded p75 or the level of pp75.

SPECIFIC QUESTIONS AND RATIONALE

1. Does the relative level of activated p75 receptors in SH-SY5Y cells differ following exposure to a neuropathic OP compound compared to a non-neuropathic OP compound?

We wanted to determine how the level of activated p75 receptors changes, if at all, after exposure to 0.01 μM PSP, 0.1 μM PSP, and 1.0 μM PSP, 100 μM paraoxon, 1 μM PSP + NGF, and medium only. We selected these levels of PSP and paraoxon based on preliminary cytotoxicity experiments.

We used SH-SY5Y human neuroblastoma cells for these studies because they are a neural line of human origin. Furthermore, studies have shown that acetylcholinesterase (AChE) and neurotoxic esterase (NTE) are inhibited in this cell line at lower levels of OP compounds than required for cytotoxicity by neuropathic compounds (Ehrich *et al.* 1997). Inhibition of NTE occurs quickly after OP exposure in these cells, which is similar to inhibition that occurs in chicken brain (Adem *et al.* 1987; Nostrandt and Ehrich 1992; Påhlman *et al.* 1995). We treated the cells with retinoic acid (RA) prior to exposing them to OP compounds because Påhlman *et al.* (1984) demonstrated that SH-SY5Y cells could be made to assume a morphological appearance similar to that of mature neurons by including RA in the culture medium. Taken together, these facts indicate that SH-SY5Y cells can be used to study events at the cellular level after esterase inhibition (Ehrich *et al.* 1997).

We hypothesized that the level of activated p75 receptors would not change after neuropathic OP compound exposure compared to non-neuropathic OP compound exposure. We based this hypothesis on previously published results. Carlson *et al.*

(2000) demonstrated that SH-SY5Y cells undergo apoptosis in response to OP compound exposure. This indicates that the p75 receptor is active after exposure to OP compounds. However, cells exposed to high levels of neuropathic OP compounds underwent a form of cell death that cannot be characterized as traditional apoptosis, but more closely resembles necrosis (Carlson *et al.* 2000). Because the p75 receptor is not involved in the induction of necrosis, we did not expect that a high dose of a neuropathic OP compound will significantly affect the level of this receptor.

2. Are the relative levels of activated TrkA to activated p75 receptors in SH-SY5Y cells exposed to a neuropathic OP compound different from that seen in these cells exposed to a non-neuropathic OP compound?

We wanted to determine how the ratio of activated TrkA and p75 receptors changes, if at all, after exposure to 0.01 μM PSP, 0.1 μM PSP, and 1.0 μM PSP, 100 μM paraoxon, 1 μM PSP + NGF, and medium only. We examined the ratio of these receptors at 4, 8, 24, and 48 hours after exposure to these OP compounds.

We treated the cells with retinoic acid (RA) prior to exposing them to OP compounds because Påhlman *et al.* (1984) demonstrated that SH-SY5Y cells could be made to assume a morphological appearance similar to that of mature neurons by including RA in the culture medium. Treatment with RA also induces a cholinergic phenotype (Adem *et al.* 1987; Påhlman *et al.* 1995) and induces SH-SY5Y cells to express TrkA and TrkB receptors and extend neurites (Kaplan *et al.* 1993).

The effect of a neurotrophin on a neuronal cell depends on the ratio of high- and low-affinity receptors expressed (Benedetti *et al.* 1993). Therefore, the ratio of neurotrophin-bound TrkA and of neurotrophin-bound p75 may contribute to overall cell response. By assessing the phosphorylation of the TrkA and p75 receptors, we had the opportunity to examine how the ratio of these activated receptors is affected during OP exposure. We hypothesized that neuropathy-inducing OP compounds interfered with neurotrophin-receptor binding as demonstrated by a decreased ratio of activated TrkA to activated p75, such that there is less activated TrkA compared to activated p75.

3. Do the levels of activated TrkA to activated p75 receptors in SH-SY5Y cells exposed to a neuropathic OP compound or a non-neuropathic OP compound change with time from 4 hours to 48 hours post-exposure compared to SH-SY5Y cells exposed to medium only?

We chose several timepoints to examine receptor level, including 4, 8, 24, and 48 hours. By examining the phosphorylation states of these two receptors, we can demonstrate the activation of TrkA and p75 over time during OP exposure. We hypothesized that early inhibition of the phosphorylation of TrkA compared to the phosphorylation of p75 contributes to morphological changes seen in a delayed neuropathy after neuropathic OP compound exposure (organophosphate induced delayed neuropathy, OPIDN), as suggested by Hong *et al.* (2003).

We based this hypothesis on previously published results. Studies have reported decreased neurite outgrowth within hours and days after exposure to neuropathic OP compounds (Hong *et al.* 2003; Nostrandt *et al.* 1992). Also, after direct exposure of SH-SY5Y cells to the neuropathic OP compound mipafox (0.05 mM), Hong *et al.* (2003) found no morphometric effect on cells 4-8 days after exposure, but found neurite length reduced at 8-12 days. They suggested that this morphological effect was similar to the dying-back neuropathy that characterizes OPIDN (Hong *et al.* 2003). The timepoints we selected are earlier than those used by Hong *et al.* (2003). However, they should demonstrate an effect that ultimately results in morphological changes seen following exposure to neuropathy-inducing OP compounds.

MATERIALS AND METHODS

Experimental Design and Analysis

We used a complete randomized block design. Treatment factors were compound (PSP, PSP + NGF, paraoxon, and medium) and time (4, 8, 24, and 48 hours post-exposure). The study was divided into 2 blocks. We performed 3-4 Western blots within each block*compound*time combination with a total of 6-7 blots per treatment group. Using the MIXED procedure in SAS (version 8.2, SAS Institute, Cary, NC), we performed an analysis of variance (ANOVA) for time and compound main effects and their interactions. Pre-planned comparisons of the interactions were performed using Tukey's t-test of each compound at each timepoint. For pTrkA analysis, data were log transformed to stabilize variances.

Cell Culture and Differentiation

We maintained SH-SY5Y human neuroblastoma cells (passages 46-53**,** ATCC, Rockville, MD) at 37^{o}C in a humidified atmosphere with 5% CO_2 and cultured cells in 75 cm^2 flasks (Corning, Acton, MA) containing 15 ml medium, replacing the medium every 2-3 days. The medium was composed of Ham F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 1% (v/v) antibiotic/antimycotic solution (Sigma, St. Louis, MO), and 1% (v/v) L-glutamine (Mediatech, Herndon, VA). Every 7-10 days, we passaged cells using 0.25% trypsin-EDTA (Invitrogen). At 80% confluency, we treated the cells with 10^{-7} M retinoic acid (RA; Sigma) in fresh medium for 3 days.

Treatment

After differentiation, we treated cells with one of 6 treatments: 0.01 µM PSP (Oryza Laboratories, Inc., Chelmsford, MA), 0.1 µM PSP, 1.0 µM PSP, 1.0 µM PSP + 1 ng/ml NGF (Calbiochem, La Jolla, CA), 100 µM paraoxon (ChemService Inc., West Chester, PA), or medium only. A previous study demonstrated that the intermediate and high concentrations of PSP were sufficient to inhibit NTE, an esterase indicative of potential to cause delayed neuropathy. The concentration of paraoxon we used was greater than the concentration required for AChE inhibition, and just under the concentration required for NTE inhibition (Ehrich *et al.* 1997). We included NGF in the high concentration of PSP as a treatment because neurotrophic factors can protect neurons and restore impaired functions after traumatic or chemical injury (Blöchl *et al.* 1995). To obtain stock solutions of PSP and paraoxon, we diluted compounds in acetone to yield 100 µM and 13.76 mg/ml, respectively, and stored each solution at -20^{o}C. We diluted the stock solutions in medium to their respective concentrations immediately prior to treating cells. We diluted 1 mg of NGF in 1 ml of medium for a stock solution of 1 mg/ml NGF. For a final concentration of 1 ng/ml NGF, we diluted a stock solution of 1 mg/ml NGF in 1.0 µM PSP in medium. After sterile filtering each solution with a 0.2 µm filter, we added 15 ml of each treatment to labeled flasks. We tested for viability using the Trypan Blue method. Cells with compromised membranes turned blue and were considered dead. Intact cells prevented the Trypan Blue from entering the cell and consequently, did not turn blue. At least 50 cells were counted for each treatment.

Western Blot

We homogenized cells in 1.0 ml lysis buffer [150 mM NaCl , 20 mM Tris-HCl, 10% glycerol (v:v), 1% Triton X-100 (v:v), 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1:200 dilution of Protease Inhibitor Cocktail Set III (Calbiochem)] on ice for 30 minutes. After passing cells through a 21-gauge needle three times to further shear the cell membranes, we placed cells on a gentle rocker at 4°C for 30 minutes. We then centrifuged cells at 10,000 x *g* for 10 minutes at 4°C. In order to determine protein concentration, we removed an aliquot of the supernatant; we diluted remaining supernatant with an equal volume of Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% β-mercaptoethanol. After heating the samples to 95°C for 5 minutes, we stored them at -80°C.

We determined the protein level of each cell lysate using the Bio-Rad *DC* Protein Assay (Bio-Rad), which is similar to the Lowry assay (Lowry *et al.* 1951). After preparing standard dilutions in the range of 0 mg/ml – 2.0 mg/ml, we diluted each sample 1:5 with saline. Absorbance was read at 750 nm by a SPECTRAmax Plus^{384} microplate spectrophotometer and Softmax Pro software (Molecular Devices, Sunnyvale, CA).

We loaded 50 µg of sample protein in duplicates on 7.5% SDS-PAGE polyacrylamide gels (Bio-Rad) and included a positive control for the TrkA protein (H4 cell lysate; Santa Cruz, Santa Cruz, CA) with each gel. After electrophoretically transferring gels onto nitrocellulose membranes (Bio-Rad) in 48 mM Tris, pH 6.8, 39 mM glycine, 0.00375% SDS (v:v), and 20% methanol (v:v), we washed the membranes in TBS (50 mM Tris, 0.9% NaCl (v:v), pH 7.5) for 10 minutes. We blocked membranes in TBS containing 5% non-fat dry milk and 0.03% Tween- 20 (v:v) for 1 hour prior to

incubating membranes overnight with commercially available rabbit or mouse polyclonal antibodies recognizing the following epitopes: TrkA (1:500; Santa Cruz), p75 (1:200; Santa Cruz), pp75 (1:500; Promega, Madison, WI), and pTrkA (1:200; mouse-derived, Santa Cruz). After the membranes equilibrated to room temperature, we washed the membranes in TBS and incubated them for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG, respectively (1:5000; Santa Cruz). We used a chemiluminescent system for detection of the proteins (Pierce, Rockford, IL). We measured the presence of these proteins by the number of pixels present in scanned blots using NIH Image/Image J (v. 1.34, National Institutes of Health, Bethesda, MD).

RESULTS

Cell viability

Viability of cells treated with 0.01 μM, 0.1 μM, and 1.0 μM PSP ranged from 90-100% at all timepoints. Cells treated with 1 μM PSP + 1 ng/ml nerve growth factor (NGF) were 100% viable at all timepoints. Among all timepoints, 94.7% and 100% of cells treated with 100 μM paraoxon were viable and 93.75-100% of negative control cells were viable (Table I).

Relative levels of activated TrkA and p75 receptors in SH-SY5Y cells after exposure to a neuropathic and non-neuropathic OP compound

There were no significant effects on the levels of pTrkA due to treatment ($p = 0.28$), time ($p = 0.48$) or to the interaction of time and treatment ($p = 0.98$). There were no significant effects on the level of pp75 receptors due to time ($p = 0.77$) or to the interaction of time and treatment ($p = 1.0$).

There was a significant effect on the level of pp75 receptors due to treatment ($p = 0.012$). Cells treated with 100 μM paraoxon had significantly higher levels of pp75 receptors compared to cells treated with 0.01 μM PSP and 1.0 μM PSP + 1 ng/ml NGF ($p = 0.0095$ and 0.039, respectively) (Figure 5.3 and 5.4). Other treatment differences were not significant at $\alpha = 0.05$.

Relative and temporal levels of activated TrkA receptors to activated p75 receptors in SH-SY5Y cells after exposure to a neuropathic and to a non-neuropathic OP compound

The relative level of activated TrkA (pTrkA) receptors to activated p75 (pp75) receptors was not affected by the compound administered ($p = 0.39$) (Figure 5.5).

There was no effect on the ratio of pTrkA receptors to pp75 receptors due to time ($p = 0.59$). There was not a significant effect due to the interaction of compound administered and time ($p = 0.99$) (Figure 5.5).

DISCUSSION

This study indicates that both TrkA and p75 are expressed in a phosphorylated state after exposure to both neuropathic and non-neuropathic OP compounds. Our data indicate that exposure to paraoxon resulted in higher levels of pp75 than either a high concentration of a neuropathic OP compound supplemented with nerve growth factor (NGF), or a low dose (0.01 μM PSP) of a neuropathic OP compound alone. However, the ratio of pTrkA and pp75 receptors does not change shortly after exposure to doses of neuropathic or non-neuropathic OP compounds compared to controls. We suggest that the lower relative levels of pp75 observed after exposure to a high dose of a neuropathic OP compound supplemented with NGF are similar to effects seen with a low dose of a neuropathic OP compound alone. However, neither could alter the ratio of pTrkA to pp75. Together, these data suggest that the ratio of pTrkA and pp75 does not contribute to decreased neurite outgrowth seen after neuropathic OP compound exposure (Hong *et al.* 2003), and that these receptors are capable of ligand-induced phosphorylation after exposure to both neuropathic and non-neuropathic OP compounds. The difference in p75 activation between neuropathic and non-neuropathic OP compounds may suggest an inherent difference in the induction of cell death between these two types of compounds.

The relative level of pp75 on cells exposed to 100 μM paraoxon is greater than that of cells exposed to 0.01 μM PSP. This concentration of PSP was lower than the concentration of 0.03 μM PSP (0.028, 0.18 μM standard deviation) required for 50% neurotoxic esterase (NTE) inhibition in differentiated SH-SY5Y cells. The concentration of paraoxon was high, inhibiting acetylcholinesterase by more than 90%, but not capable of inhibiting NTE (Ehrich *et al.* 1997). Our data suggest, therefore, that exposure to OP

compounds at levels with little to no effect on NTE could result in different changes in neuroblastoma cells than NTE-inhibiting concentrations of such toxicants.

Exposure to a higher level of a neuropathic OP compound (1 μM PSP) supplemented with NGF also results in lower levels of pp75 compared to paraoxon-treated cells. Nerve growth factor typically protects neurons against injury and promotes axonal regeneration after injury, but it can also be pro-apoptotic if p75 levels are greater than TrkA levels (Hagg *et al.* 1993; Kaplan and Miller 2000). Our data demonstrate that pp75 levels are indeed greater than pTrkA levels in SH-SY5Y cells. However, necrosis, not apoptosis, is the form of cell death likely seen after exposure to high doses of a neuropathic OP compound (Fioroni *et al.* 1995). We suggest that this is due in part to activation of p75.

A third form of cell death, labeled autophagic apoptosis or oncosis, may be the cause of cell death in cells treated with high doses of a neuropathic OP compound (Boix *et al.* 1997; Majno and Joris 1995). High levels of neuropathic OP compounds have been noted for inducing a form of cell death that cannot be characterized as the traditional form of apoptosis (Carlson *et al.* 2000). When a high dose of a neuropathic OP compound is supplemented with NGF, autophagic apoptosis may result, with high levels of pp75 versus pTrkA as a contributing factor. Supporting this suggestion are several studies that suggest the binding of NGF to p75 induces apoptosis (Bamji *et al.* 1998; Dobrowsky *et al.* 1994; Hirata *et al.* 2001; Yoon *et al.* 1998). In fact, Schwann cells can modulate their response during peripheral nerve repair after injury by changing the expression level of p75. Schwann cells that do not suppress p75 expression in the

presence of NGF undergo a form of cell death that does not include nuclear condensation and other characteristics typical of apoptosis (Hirata *et al.* 1998; Hirata *et al.* 2001).

It is interesting to note that we did not detect a significant difference in relative pp75 between cells exposed to these OP compounds versus controls. In fact, all treatments in this study, including controls, resulted in higher levels of pp75 compared to pTrkA levels. However, cell death is not observed in cells treated with medium only (controls) whereas it has been observed in undifferentiated SH-SY5Y cells treated with OP compounds (Carlson *et al.* 2000). While a contributing factor to cell survival in this study may be our use of differentiated SH-SY5Y cells, we suggest that activation of p75 is another of several factors required for the induction of cell death, particularly apoptosis (Hirata *et al.* 2001; Majdan *et al.* 1997).

We are interested in the ratio of pTrkA and pp75 coexpression after OP compound exposure because the ratio of the unphosphorylated forms of these receptors determines the fate of a cell and can lead to a greater cellular response to neurotrophins present in the local environment (Barker and Shooter 1995; Chao and Hempstead 1995; Kaplan and Miller 1997; Meakin and Shooter 1992). However, it is the phosphorylated states of these receptors, as induced by neurotrophin binding, which initiates cellular events. A previous study demonstrated that neurotrophins are present in the spinal cord of chickens exposed to OP compounds, but the levels of these neurotrophins does not increase after insult (M. Pomeroy-Black, submitted to *Neurotox Res*). *In vitro* experiments demonstrated that undifferentiated SH-SY5Y cells undergo rapid cell death in response to sufficient levels of certain OP compounds, some of which were higher than those used in this study (Carlson *et al.* 2000). Therefore, while neurotrophins are present

in vivo, this study demonstrates that pp75 is present in higher levels than pTrkA up to 48 hours after neuropathic and non-neuropathic OP exposure. Consequently, early cell death of SH-SY5Y cells exposed to 100 μM paraoxon or higher concentrations of neuropathic OP compounds than were used in this study may be the result of a greater level of p75 on the cell membrane compared to Trk receptors because it is activation of this receptor that contributes to cell death (Carlson *et al.* 2000; Kaplan and Miller 1997).

Not only does the ratio of unliganded Trk to unliganded p75 on the cell membrane determine cell fate, but this ratio likely contributes to which pathway (cell survival or death) is activated after p75 receptor phosphorylation (Greene and Kaplan 1995). Researchers have detected an increase in p75 in motor neurons within 24 hours of axotomy *in vivo* (Friedman *et al.* 1995; Raivich and Kreutzberg 1993). This upregulation occurs in advance of an increase in neurotrophin levels after axotomy, which is first detected 3 days after injury (Friedman *et al.* 1995). Neurons in the injured area overexpressing p75 prior to an increase in neurotrophin levels undergo apoptosis (Dobrowsky *et al.* 1994). However, in the presence of decreased neurotrophins, the contribution of the p75 receptor to neuronal survival is enhanced when p75 expression increases relative to that of TrkA (Rydén *et al.* 1997). Although we did not detect an *increase* in pp75 level compared to pTrkA over time, our data indicate that the level of pp75 on the cell membrane is consistently greater than pTrkA after OP compound exposure. Therefore, we suggest that the greater level of pp75 on the cell membrane compared to pTrkA causes *in vitro* cell death seen after OP compound exposure.

This study indicates that the high-affinity receptor for NGF and the common low-affinity neurotrophin receptor are active after exposure to both neuropathic and non-

neuropathic OP compounds. This suggests that these receptors are functional and downstream signaling from these receptors may be initiated. Our data indicate that the ratio of pTrkA and pp75 does not cause decreased neurite outgrowth seen after neuropathic OP compound exposure (Hong *et al.* 2003). Finally, there is a difference in the activation of the p75 receptor after exposure to neuropathic OP compounds compared to non-neuropathic OP compounds. Exposure to a non-neuropathic OP compound increases relative activation of p75 compared to exposure to a high dose of a neuropathic OP compound supplemented with NGF or exposure to a low dose of a neuropathy-inducing OP compound. This may indicate that neuropathic OP compounds induce cell death in a manner different than non-neuropathic OP compounds.

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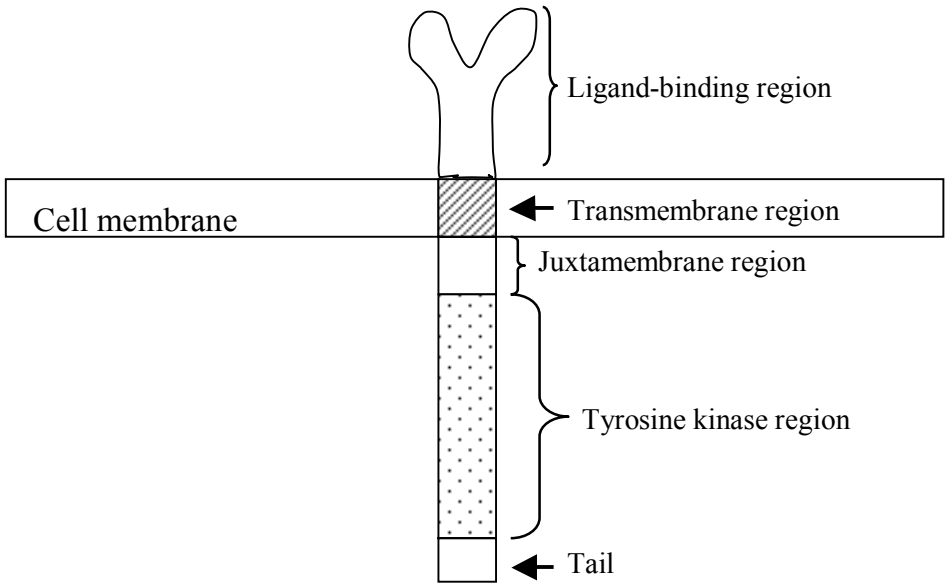

Figure 5.1. Representation of a Trk receptor. The juxtamembrane domain contains the src homology to collagen (SHC) region and is the least well-conserved within the cytoplasmic region between the Trk receptors. The tyrosine kinase domain is essential for signal transduction.

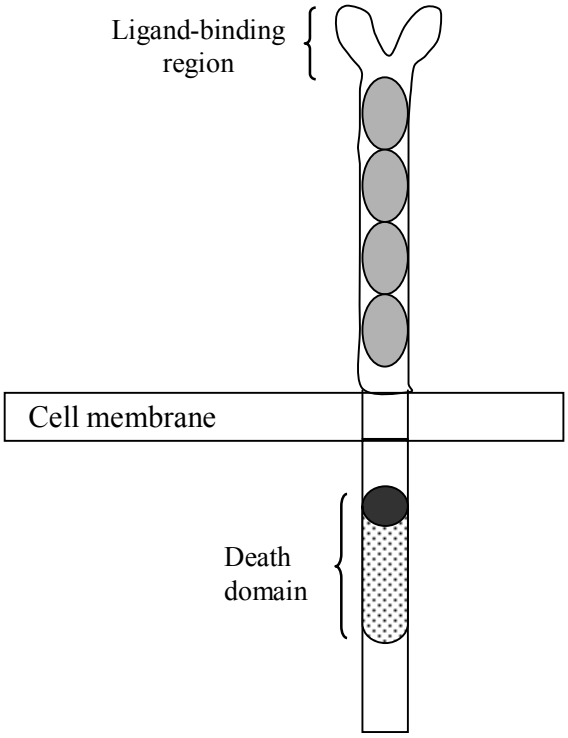

Figure 5.2. Representation of a p75 neurotrophin receptor.

Time	Treatment	% Viability
4 hours	0.01 μM PSP	100
	0.1 μM PSP	100
	1.0 μM PSP	100
	100 μM paraoxon	100
	1.0 μM PSP + 1 ng/ml NGF	100
	negative control	93.8
8 hours	0.01 μM PSP	90
	0.1 μM PSP	100
	1.0 μM PSP	100
	100 μM paraoxon	100
	1.0 μM PSP + 1 ng/ml NGF	100
	negative control	100
24 hours	0.01 μM PSP	100
	0.1 μM PSP	100
	1.0 μM PSP	100
	100 μM paraoxon	94.7
	1.0 μM PSP + 1 ng/ml NGF	100
	negative control	100
48 hours	0.01 μM PSP	94.4
	0.1 μM PSP	100
	1.0 μM PSP	100
	100 μM paraoxon	100
	1.0 μM PSP + 1 ng/ml NGF	100
	negative control	95.4

Table 5.1. Viability of differentiated cells treated with a neuropathic esterase inhibitor (0.01 μM, 0.1 μM, or 1.0 μM PSP), a non-neuropathic esterase inhibitor (100 μM paraoxon), a neuropathic esterase inhibitor plus nerve growth factor (NGF) (1.0 μM PSP + 1 ng/ml NGF) or medium only.

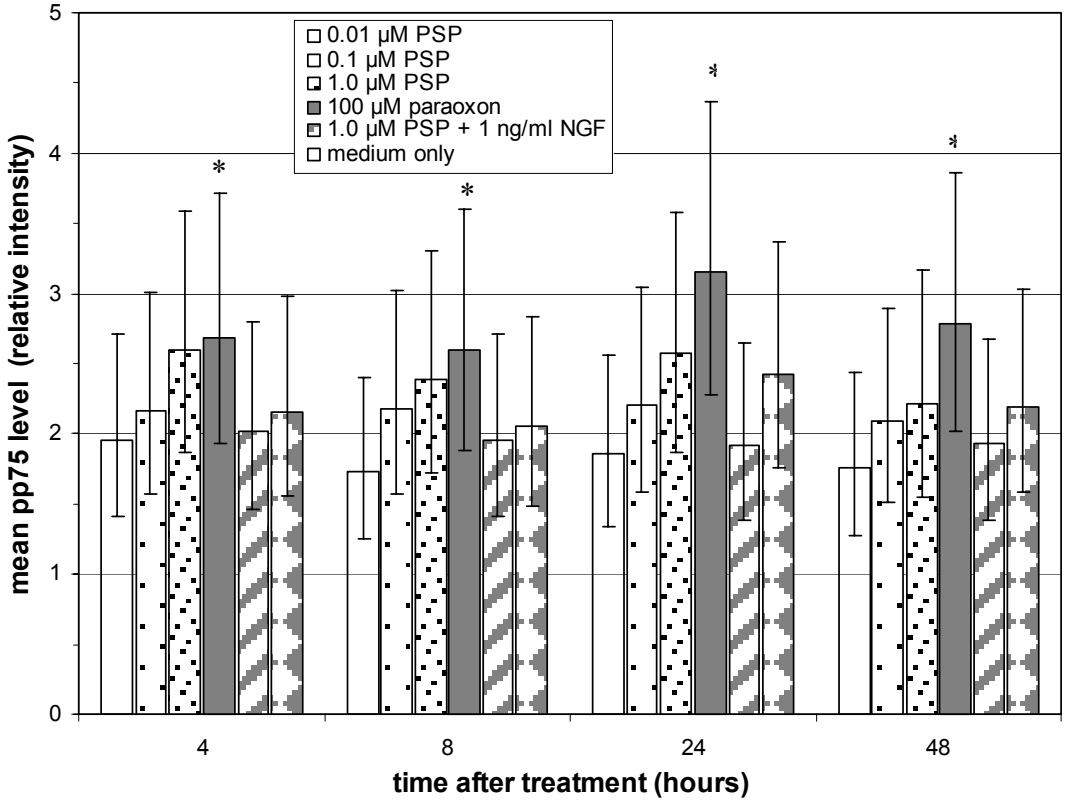

Figure 5.3. Least square means and standard error (represented by error bars) of the level of phosphorylated p75 (pp75) receptors in cells treated with a neuropathic esterase inhibitor (PSP; 0.01 µM, 0.1 µM, and 1.0 µM) (n = 6-7 per timepoint), a non-neuropathic esterase inhibitor (paraoxon, 100 µM) (n = 6-7 per timepoint), a neuropathic esterase inhibitor plus NGF (1.0 µM PSP + 1 ng/ml NGF) (n = 6-7 per timepoint), or medium alone (n = 6-7 per timepoint). The level of pp75 receptors was significantly affected by compound administered such that cells treated with 100 µM paraoxon had significantly higher levels of pp75 than cells treated with 0.01 µM PSP and with 1.0 µM PSP + 1 ng/ml NGF at the $\alpha = 0.05$ level, as indicated by an *. The level of pp75 receptors was not significantly affected by time ($p = 0.77$). There was no significant compound*time interaction ($p = 1.0$). Controls (medium only) had mean pp75 levels of 2.05 – 2.43.

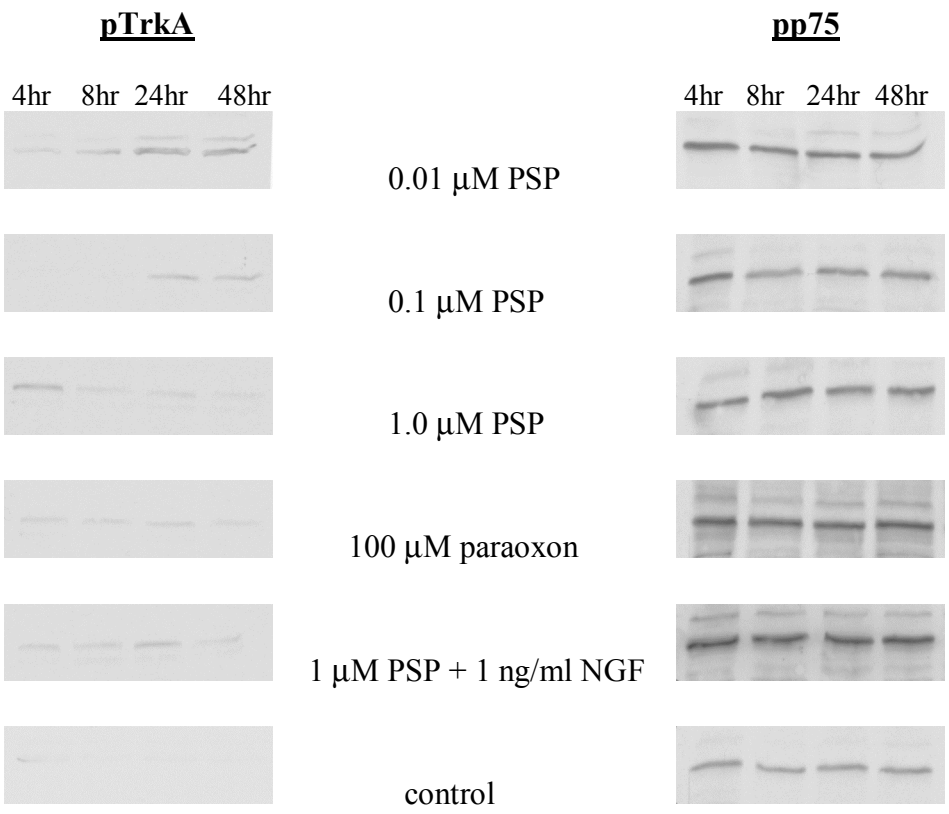

Figure 5.4. Representative total phosphorylated TrkA (pTrkA) and pp75 Western blots of SH-SY5Y cells exposed to a neuropathic OP compound (0.01 µM, 0.1 µM, and 1.0 µM PSP), a non-neuropathic OP compound (100 µM paraoxon), a neuropathic OP compound supplemented with nerve growth factor (1 µM PSP + 1 ng/ml NGF) and medium only (control).

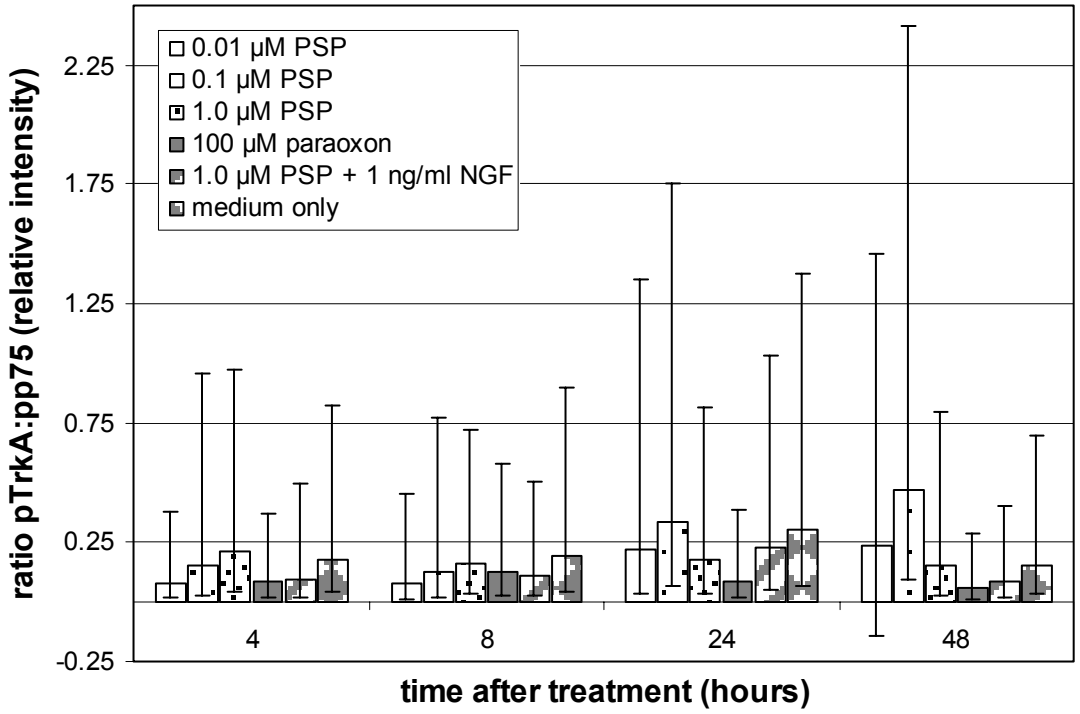

Figure 5.5. Least square means and standard error (represented by error bars) of the ratio of the level of pTrkA receptors to the level of pp75 receptors in cells treated with a neuropathic esterase inhibitor (PSP; 0.01 µM, 0.1 µM, and 1.0 µM) (n = 6-7 per timepoint), a non-neuropathic esterase inhibitor (paraoxon, 100 µM) (n = 6-7 per timepoint), a neuropathic esterase inhibitor plus nerve growth factor (NGF) (1.0 µM PSP + 1 ng/ml NGF) (n = 6-7 per timepoint), or medium only controls (n = 6-7 per timepoint). The ratio of pTrkA to pp75 was not significantly affected by compound administered ($p = 0.39$) or by time ($p = 0.59$). There was no significant compound*time interaction ($p = 0.99$). Controls had a mean relative intensity pTrkA:pp75 ratio of 0.18 (range 0.14 – 0.82) at 4 hours, 0.2 (0.15 – 0.9) at 8 hours, 0.3 (0.24 – 1.38) at 24 hours, and 0.15 (0.12 – 0.7) at 48 hours.

Chapter 6

Early Intracellular Signaling in SH-SY5Y Cells after Exposure to Neuropathy-Inducing Organophosphates

ABSTRACT

The binding of a neurotrophin to its specific high-affinity receptor initiates activation of up to three intracellular pathways: the phospholipase C-γ (PLC-γ) pathway, the phosphoinositol-3 kinase (PI-3K) pathway, and the mitogen-activated protein kinase (MAPK) pathway (Kaplan 1995). While the PLC-γ and PI-3K pathways contribute primarily to cell survival, the MAPK pathway is primarily responsible for neurite outgrowth *in vitro*. We hypothesize that a neuropathy-inducing organophosphate (OP) compound interferes with the activation of specific proteins in these intracellular pathways, specifically PKC-α, Akt, and Mek 1/2, respectively, when compared to a non-neuropathic OP compound. As a result, neurite outgrowth of cells exposed to a neuropathic OP compound is inhibited. To test this hypothesis, we exposed SH-SY5Y human neuroblastoma cells to a neuropathic OP compound (PSP; 0.01 μM, 0.1 μM, 1.0 μM), a non-neuropathic OP compound (paraoxon; 100 μM), a neuropathic OP compound with nerve growth factor (1.0 μM PSP + 1 ng/ml NGF), and medium only for 4, 8, 24, and 48 hours. We performed Western blots on cell lysates to determine the level of activated PKC-α (pPKC-α), Akt (pAkt), and Mek1/2 (pMek1/2). Our data indicate that cells treated with 100 μM paraoxon have higher levels of pMek1/2 compared to all other treatments. Furthermore, cells harvested 4 hours post-treatment have higher levels of pMek1/2 than cells harvested 8 hours post-treatment. Cells treated with 0.1 μM PSP or 100 μM paraoxon had higher levels of pAkt than controls ($p = 0.03$ and 0.048, respectively). Finally, pPKC-α levels in cells treated for 4 hours or 8 hours is significantly more than cells treated for 48 hours ($p = 0.0021$ and 0.035, respectively). Together, these findings suggest that non-neuropathic OP compounds cause increased activity of the MAPK pathway compared to neuropathic OP compounds and that the PI-3K pathway is upregulated by both types of OP compounds.

INTRODUCTION

The family of neurotrophins mediates cell survival, differentiation, growth, and apoptosis by binding two types of cell-surface receptors on neurons (Kaplan and Miller 2000). Activation of the neurotrophin receptors initiates one or more intracellular cascades of protein signaling to promote neurotrophic actions (Kaplan and Miller 2000). Each neurotrophin binds specifically to a high-affinity receptor: nerve growth factor (NGF) binds the TrkA receptor, brain-derived neurotrophic factor (BDNF) binds TrkB, and neurotrophin-3 (NT-3) binds TrkC (Atwal *et al.* 2000; Huang and Reichardt 2003; Kaplan 1995). At the same time a neurotrophin binds its specific high-affinity receptor, it also binds a common neurotrophin receptor with low affinity (p75) (Bui *et al.* 2002; Hagg *et al.* 1993).

Internalization and downstream signaling among the Trk receptors is similar (Atwal *et al.* 2000; Huang and Reichardt 2003). Tyrosine kinase activity of TrkA is maximal 5-10 minutes following ligand binding and is attenuated thereafter (Kaplan 1995). Specific intracellular proteins bind different sequence motifs on autophosphorylated TrkA to initiate activation of up to three pathways: the phospholipase C-γ (PLC-γ) pathway, the phosphoinositol-3 kinase (PI-3K) pathway, and the mitogen-activated protein kinase (MAPK) pathway (Figure 6.1). Initial phosphorylation of src homology to collagen (SHC) protein initiates a cascade of cellular events, leading to the activation of a GTP-binding protein, Ras (Kaplan 1995). The Ras protein directs neurotrophin-initiated signals into multiple signaling pathways (Kaplan *et al.* 2000). The duration of Ras activity may determine which cascade is initiated (Kaplan 1995). Major roles of Ras include initiating PI-3K pathway activation, suppressing the JNK (Jun

amino-terminal kinase)-p53-Bax apoptotic pathway, induction of Raf translocation to the cellular membrane, and initiating a series of serine/threonine kinases that compose the MAPK pathway, including Raf, MAP kinase kinase (Mek) 1 and 2, and Erk 1 and 2 (Erk 1/2) (Figure 6.2) (Kaplan 1995; Kaplan *et al.* 2000; Mazzoni *et al.* 1999).

The MAPK pathway plays several roles in neuronal cells, including plasticity, long-term potentiation, and survival (Kaplan *et al.* 2000). The primary result of MAPK pathway activation is neurite outgrowth. Therefore, this pathway appears necessary for complete elaboration of neurites and neurite maintenance (Kaplan 1995). Although the activation of the MAPK pathway is sufficient for cell survival, it is not necessary. This pathway promotes survival by stimulating activity of anti-apoptotic proteins such as Bcl-2 and CREB. Importantly, the MAPK pathway appears to protect neurons from death due to injury or toxicity, rather than from trophic factor withdrawal. (Kaplan *et al.* 2000).

The primary survival-promoting pathway is the PI-3K pathway. Induction of PI-3K activates the serine/threonine kinase Akt. While Akt acts as a convergence point for survival signals in neurons, it does not play a role in neurite outgrowth. Activation of Akt induces cell survival by inhibiting activation of downstream apoptotic proteins. When Akt phosphorylates Bad, a pro-apoptotic protein, Bad is unable to associate with, and thereby inactivate, the anti-apoptotic transcription factors Bcl-2 and Bcl-XL (Figure 6.3) (Kaplan *et al.* 2000). Consequently, these transcription factors remain active, preventing apoptosis. Research demonstrates that Bcl-2 also plays a role in axonal plasticity, promoting regeneration after injury (Horner *et al.* 2000).

Phosphorylation and activation of PLC-γ induces hydrolysis of phosphoinositol 4,5-biphosphate to the second messengers diacylglycerol (DAG) and inositol

triphosphate. In turn, DAG activates protein kinase C (PKC) (Kaplan 1995) (Figure 6.4). After activation, PKC translocates to the plasma membrane where it phosphorylates cytoskeletal components (Keenan and Kelleher 1998). There are several isoforms of PKC, including PKC- α, -βII, -δ, and-ε. Previous research demonstrated that these isoforms play differing roles in promoting cell growth and survival, and neurite outgrowth (Zeidman *et al.* 1999). In particular, PKC-α contributes to neurite outgrowth (Parrow *et al.* 1995).

Studies indicate that these three pathways do not act independently of each other, but are redundant (Stephens *et al.* 1994). Specifically, the PI-3K and MAPK pathway appear to act concurrently in the induction of neurite outgrowth (Greene and Kaplan 1995). Signaling activity of the MAPK pathway is also regulated by the family of PKC proteins (Roberson *et al.* 1999). Specifically, PKC-α can activate the MAPK pathway through the Mek1/2 protein (Choe *et al.* 2002). Therefore, each pathway may contribute to specific functions of the neuron to differing degrees after injury or toxic insult.

Studies demonstrate that neurite outgrowth is initiated in SH-SY5Y cells directly exposed to neuropathy-inducing OP compounds (Hong *et al.* 2003; Li *et al.* 1998). However, neurite length decreases over time whereas neurite length in control cells continues to increase (Hong *et al.* 2003). This indicates that initial binding of the neurotrophin to its receptor and signal transduction occurs, but is not maintained at a level sufficient for neurite outgrowth. Therefore, we hypothesize that cessation of sustained neurite outgrowth in cultured cells after OP exposure occurs as a result of interference with the MAPK signaling cascade and the PKC signaling cascade.

SPECIFIC QUESTIONS AND RATIONALE

1. Is there a difference in the relative expression of specific proteins in the mitogen-activated protein kinase (MAPK) cascade, the phosphoinositol-3 kinase (PI-3K) cascade, and the protein kinase C (PKC) cascade of Trk receptors of SH-SY5Y neuroblastoma cells treated with increasing doses of a neuropathic OP compound?

We wanted to determine how the expression of intracellular proteins involved in neurite outgrowth and/or cell survival changes, if at all, after exposure to 0.01 μM, 0.1 μM, and 1.0 μM PSP. We selected these concentrations of OP toxicants based on preliminary cytotoxicity experiments. There are at least three pathways involved in neurite outgrowth and/or cell survival after a neurotrophin binds its high-affinity Trk receptor, including the MAPK pathway, the PI-3K pathway, and the PKC pathway (Kaplan 1995). Therefore, we wanted to examine different proteins in each of these cascades. The proteins we chose to examine included phosphorylated Mek1/2 (pMek1/2) in the MAPK pathway, phosphorylated Akt (pAkt) in the PKC pathway, and phosphorylated PKC-α (pPKC-α.) in the PLC-γ pathway. We used a non-neuropathic OP compound (100 μM paraoxon) as a control.

We used SH-SY5Y human neuroblastoma cells for these studies because they are a neural line of human origin. Furthermore, studies have shown that esterases involved in neurotoxicity (AChE and NTE) are inhibited in this cell line at lower concentrations than required for cytotoxicity (Ehrich *et al.* 1997). We treated the cells with retinoic acid (RA) prior to exposing them to OP compounds because Påhlman *et al.* (1984) demonstrated that SH-SY5Y cells could be made to assume a morphological appearance

similar to that of mature neurons by including RA in the culture medium. Treatment with RA also induces a cholinergic phenotype (Adem *et al.* 1987; Påhlman *et al.* 1995) and induces SH-SY5Y cells to express TrkA and TrkB receptors and extend neurites (Kaplan *et al.* 1993). Taken together, these facts indicate that SH-SY5Y cells can be used to study events at the cellular level after esterase inhibition (Ehrich *et al.* 1997).

Studies demonstrate that neurite outgrowth is initiated in SH-SY5Y cells directly exposed to neuropathy-inducing OP compounds (Hong *et al.* 2003; Li *et al.* 1998). However, neurite length decreases over time whereas neurite length in control cells continues to increase (Hong *et al.* 2003). This indicates that initial binding of the neurotrophin to its receptor and signal transduction occurs, but is not maintained at a level sufficient for neurite outgrowth. Therefore, we hypothesize that cessation of sustained neurite outgrowth in cultured cells after OP exposure occurs as a result of interference with the MAPK signaling cascade and the PKC signaling cascade.

2. Are the relative expressions of specific proteins in the MAPK cascade, PI-3K cascade, and PKC cascade of Trk receptors affected differently in SH-SY5Y cells exposed to a neuropathic OP compound compared to the relative expressions of the same proteins in SH-SY5Y cells treated with a non-neuropathic OP compound?

We wanted to determine how the expression of specific intracellular proteins involved in neurite outgrowth and/or cell survival, changes, if at all, after exposure to 0.01 μM PSP, 0.1 μM PSP, 1.0 μM PSP, 100 μM paraoxon, 1 μM PSP + 1 ng/ml NGF, and medium alone. We used paraoxon as the non-neuropathic OP compound. We added 1 ng/ml NGF to the high dose of PSP because neurotrophic factors can protect neurons and restore impaired functions after traumatic or chemical injury (Blöchl *et al.* 1995), and therefore, should counteract PSP-induced effects. The proteins we chose to examine included pMek1/2 in the MAPK pathway, pAkt in the PI-3K pathway, and pPKC-α in the PLC-γ pathway. We selected these proteins because Mek1/2 and PKC-α are involved in neurite outgrowth and pAkt is involved in cell survival (Encinas *et al.* 1999; Kaplan 1995; Parrow *et al.* 1995). We hypothesized the pMek1/2 level decreases after exposure to a neuropathic OP compound when compared to a non-neuropathic OP compound. Because Akt is an essential protein in cell survival and cells exposed to low doses of a neuropathic OP compound have been shown to survive this toxic insult (Hong *et al.* 2003; Nostrandt *et al.* 1992), we hypothesized that pAkt level increases after exposure to a neuropathic OP compound. We also hypothesized that pAkt level decreases in cells exposed to a non-neuropathic OP compound because previous studies in our laboratory demonstrate that undifferentiated SH-SY5Y cells exposed to paraoxon (1mM) undergo apoptosis (Carlson *et al.* 2000).

3. Does the relative expression of specific proteins in the MAPK cascade, PI-3K cascade, and PKC cascade of Trk receptors of SH-SY5Y cells exposed to a neuropathic OP compound or a non-neuropathic OP compound change with time from 4 hours to 48 hours post-exposure compared to SH-SY5Y cells exposed to medium only?

We wanted to determine how the expression of specific activated intracellular proteins involved in neurite outgrowth and/or cell survival, including pMek1/2, pAkt, pPKC-α, changes, if at all, over the course of time after exposure to 0.01 μM PSP, 0.1 μM PSP, 1.0 μM PSP, 100 μM paraoxon, 1 μM PSP + 1 ng/ml NGF, or medium alone. We chose several timepoints to examine the levels of these intracellular proteins, including 4, 8, 24, and 48 hours post-exposure. We hypothesized that inhibition of pMek1/2 and pPKC-α at 4, 8, 24, and 48 hours contributes to development of a delayed neuropathy in animals exposed to certain OP compounds (organophosphate induced delayed neuropathy, OPIDN), as evidenced by clinical signs at later timepoints.

Studies have observed decreased neurite outgrowth after exposure to neuropathic OP compounds (Henschler *et al.*, 1992; Nostrandt *et al.*, 1992). For example, using SH-SY5Y cells, Hong *et al.* (2003) found no morphometric effect on cells 4-8 days after direct exposure of the neuropathic OP mipafox (0.05 mM), but found neurite length reduced at 8-12 days. They suggested that this morphological effect was similar to the dying-back neuropathy that characterizes OPIDN (Hong *et al.*, 2003).

On average, transcription, translation, and processing of a large protein takes no more than an hour (Lodish *et al.* 2000). However, early changes in intracellular protein levels may not manifest themselves morphologically for hours or days. We selected early

timepoints in order to measure early changes in protein levels that may ultimately result in morphological changes seen following exposure to neuropathy-inducing OP compounds.

MATERIALS AND METHODS

Experimental Design and Analysis

We used a complete randomized block design. Treatment factors were compound (PSP, PSP + NGF, paraoxon, and medium) and time (4, 8, 24, and 48 hours post-exposure). The study was divided into 2 blocks. We performed 3-4 Western blots within each block*compound*time combination with a total of 6-7 blots per treatment group. Using the MIXED procedure in SAS (version 8.2, SAS Institute, Cary, NC), we performed an analysis of variance (ANOVA) for time and compound main effects and their interactions. Pre-planned comparisons of the interactions were performed using Tukey's t-test of each compound at each timepoint.

Cell Culture and Differentiation

We maintained SH-SY5Y human neuroblastoma cells (passages 46-53**,** ATCC, Rockville, MD) at 37^{o}C in a humidified atmosphere with 5% CO_2 and cultured cells in 75 cm^2 flasks (Corning, Acton, MA) containing 15 ml medium, replacing the medium every 2-3 days. The medium was composed of Ham F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 1% (v/v) antibiotic/antimycotic solution (Sigma, St. Louis, MO), and 1% (v/v) L-glutamine (Mediatech, Herndon, VA). Every 7-10 days, we passaged cells using 0.25% trypsin-EDTA (Invitrogen). At 80% confluency, we treated the cells with 10^{-7} M retinoic acid (RA; Sigma) in fresh medium for 3 days.

Treatment

After differentiation, we treated cells with one of 6 treatments: 0.01 µM PSP (Oryza Laboratories, Inc., Chelmsford, MA), 0.1 µM PSP, 1.0 µM PSP, 1.0 µM PSP + 1 ng/ml NGF (Calbiochem, La Jolla, CA), 100 µM paraoxon (ChemService Inc., West Chester, PA), or medium only. A previous study demonstrated that the intermediate and high doses of PSP were sufficient to inhibit NTE, an esterase indicative of potential to cause delayed neuropathy. The dose of paraoxon we used was greater than the dose required for AChE inhibition, and just under the dose required for NTE inhibition (Ehrich *et al.* 1997). We included NGF in the high dose of PSP as a treatment because neurotrophic factors can protect neurons and restore impaired functions after traumatic or chemical injury (Blöchl *et al.* 1995). To obtain stock solutions of PSP and paraoxon, we diluted compounds in acetone to yield 100 µM and 13.76 mg/ml, respectively, and stored each solution at -20^{o}C. We diluted the stock solutions in medium to their respective concentrations immediately prior to treating cells. We diluted 1 mg of NGF in 1 ml of medium for a stock solution of 1 mg/ml NGF. For a final concentration of 1 ng/ml NGF, we diluted a stock solution of 1 mg/ml NGF in 1.0 µM PSP in medium. After sterile filtering each solution with a 0.2 µm filter, we added 15 ml of each treatment to labeled flasks. We harvested cells at 4, 8, 24, and 48 hours after exposure.

Western Blot

We homogenized cells in 1.0 ml lysis buffer [150 mM NaCl , 20 mM Tris-HCl, 10% glycerol (v:v), 1% Triton X-100 (v:v), 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1:200 dilution of Protease Inhibitor Cocktail Set III (Calbiochem)] on ice for 30 minutes.

After passing cells through a 21-gauge needle three times to further shear the cell membranes, we placed cells on a gentle rocker at 4°C for 30 minutes. We then centrifuged cells at 10,000 x *g* for 10 minutes at 4°C. In order to determine protein concentration, we removed an aliquot of the supernatant, then diluted remaining supernatant with an equal volume of Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% β-mercaptoethanol. After heating the diluted samples to 95°C for 5 minutes, we stored them at -80°C.

We determined the protein concentration of each cell lysate using the Bio-Rad *DC* Protein Assay (Bio-Rad), which is similar to the Lowry assay (Lowry *et al.* 1951). After preparing standard dilutions in the range of 0 mg/ml – 2.0 mg/ml, we diluted each sample 1:5 with saline. Absorbance was read at 750 nm by a SPECTRAmax Plus^{384} microplate spectrophotometer and Softmax Pro software (Molecular Devices, Sunnyvale, CA).

We loaded 50 µg of sample protein in triplicates on 7.5% SDS-PAGE polyacrylamide gels (Bio-Rad) and included a positive control for the TrkA protein (H4 cell lysate; Santa Cruz, Santa Cruz, CA) with each gel. After electrophoretically transferring gels onto nitrocellulose membranes (Bio-Rad) in 48 mM Tris, pH 6.8, 39 mM glycine, 0.00375% SDS (v:v), and 20% methanol (v:v), we washed the membranes in Tris-buffered saline (TBS) (50 mM Tris, 0.9% NaCl (v:v), pH 7.5) for 10 minutes. We blocked membranes in TBS containing 5% non-fat dry milk and 0.03% Tween- 20 (v:v) for 1 hour prior to incubating membranes overnight with commercially available rabbit or mouse polyclonal antibodies recognizing the following epitopes: pMek1/2 (1:1000; Santa Cruz), pAkt (1:200; Santa Cruz), pPKC-α (1:5000; Promega, Madison, WI), and pTrkA (1:200; mouse-derived, Santa Cruz). After the membranes equilibrated to room

temperature, we washed the membranes in TBS and incubated them for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000; Santa Cruz). We used a chemiluminescent system for detection of the proteins (Pierce, Rockford, IL). We measured the presence of these proteins by the number of pixels present in scanned blots using NIH Image/Image J (v. 1.34, National Institutes of Health, Bethesda, MD).

RESULTS

Cell viability

Viability of cells in all test groups ranged from 90-100% at all timepoints. This included cells treated with 1 μM PSP + 1 ng/ml nerve growth factor (NGF), which were 100% viable at all timepoints.

Relative levels of the activated forms of Mek1/2, Akt, and PKC-α in SH-SY5Y neuroblastoma cells treated with 0.01 μM PSP, 0.1 μM PSP, 1.0 μM PSP, 1 μM PSP + 1 ng/ml NGF or 100 μM paraoxon

There was a significant effect on the level of phosphorylated Mek1/2 (pMek1/2) due to treatment ($p = 0.019$). This was indicated by the elevated levels of pMek1/2 seen in cells treated with 100 μM paraoxon and cells treated with 0.01 μM PSP, 0.1 μM PSP, and 1.0 μM PSP ($p = 0.037$, 0.001, and 0.002, respectively) or with 1.0 μM PSP + NGF ($p = 0.0003$) (Figures 6.5 and 6.8). The level of pMek1/2 in cells treated with 100 μM paraoxon was significantly different from control cells ($p = 0.0012$).

There was an effect on the level of phosphorylated Akt (pAkt) due to treatment ($p = 0.04$). Relative to control cells, there was a significant increase in the level of pAkt in cells treated with 0.1 μM PSP ($p = 0.03$) or cells treated with 100 μM paraoxon ($p = 0.048$) (Figures 6.6 and 6.8).

There was no significant effect on the level of phosphorylated PKC-α (pPKC-α) due to treatment ($p = 0.66$) (Figures 6.7 and 6.8).

Temporal levels of pMek1/2, pAkt, and pPKC-α in SH-SY5Y neuroblastoma cells treated with 0.01 μM PSP, 0.1 μM PSP, 1.0 μM PSP, 1 μM PSP + 1 ng/ml NGF or 100 μM paraoxon

There was a significant effect on pMek1/2 levels due to time ($p = 0.019$). In all treatment groups, there was a significant increase in the level of pMek 1/2 in cells harvested at 4 hours compared to cells harvested at 8 hours ($p = 0.017$) (Figures 6.5 and 6.8).

There was no significant effect on pAkt levels due to time ($p = 0.18$) (Figures 6.6 and 6.8).

There was a significant effect on pPKC-α levels due to time among all treatments ($p = 0.003$). In all treatment groups, the mean pPKC-α level in cells treated for 4 hours is significantly greater than cells treated for 48 hours ($p = 0.0021$); cells treated for 8 hours also expressed significantly higher levels of pPKC-α compared to cells treated for 48 hours ($p = 0.035$) (Figures 6.7 and 6.8).

DISCUSSION

This study reveals that exposure to a non-neuropathic OP compound (paraoxon) affects this intracellular cascade differently, and may relate to previously noted differences in cytotoxicity between cells exposed to a non-neuropathic OP compound (paraoxon) and cells exposed to a neuropathic OP compound (PSP) (Carlson *et al.*, 2000). The present findings also suggest that, following exposure to paraoxon, there were elevations in the level of phosphorylated Mek1/2 (pMek1/2) and phosphorylated Akt (pAkt). These proteins are central to the MAPK and PI-3K cascades, respectively. These cell signaling cascades are responsible for neurite outgrowth and cell survival (Kaplan 1995). Exposure to an intermediate concentration of PSP also caused increased pAkt levels. Elevated pMek1/2 levels after paraoxon, but not PSP, exposure suggests that neuropathic and non-neuropathic OP compounds affect this intracellular signaling cascade differently. These findings also indicate that both the MAPK and PI-3K pathways play an important role in neuronal response after OP exposure. Finally, our study indicates that the phosphorylated form of PKC-α, one isoform of the PKC family, is present and active after exposure to both neuropathic and non-neuropathic OP compounds.

Phosphorylation and rearrangement of cytoskeletal elements contribute to neurite outgrowth. Activation of the MAPK cascade causes phosphorylation of both the tau protein and neurofilaments, both of which are localized in axons (Holzer *et al.* 2001; Schoenfeld and Obar 1994; Veeranna *et al.* 2000; Weingarten *et al.* 1975). Like tau, rearrangement of microtubules and neurofilaments contributes to neurite outgrowth. An increase in tau expression and phosphorylation also protects neurons from apoptosis

(Holzer *et al.* 2001). The increase in pMek1/2 we observed after exposure to paraoxon may indicate that one or more of these cytoskeletal elements are upregulated by the MAPK pathway after exposure to a non-neuropathic OP compound. For example, cell survival after exposure to a non-neuropathic OP compound (Nostrandt *et al.* 1992) may be induced, in part, by an upregulation of tau in cells, and this upregulation may occur via the MAPK pathway. It is important to note that the dose of paraoxon we used was greater than the dose required for acetylcholinesterase inhibition, and just under the dose required for neurotoxic esterase inhibition (Ehrich *et al.* 1997), but still lower than cytotoxic doses.

The augmented presence of tau protein occurring as a result of MAPK upregulation may stimulate synthesis of other cytoskeletal proteins involved in neurite outgrowth, including tubulin (Holzer *et al.* 2001). Literature regarding mRNA transcripts of these cytoskeletal proteins, including neurofilaments and α-tubulin, reveals that these elements are regulated differently from each other after neuropathic OP compound exposure (Fox *et al.* 2003; Gupta *et al.* 1999). For example, Fox *et al.* (2003) found a transient increase at 12 hours followed by a decrease at 48 hours in 4.5 kb α-tubulin expression, but an overall decrease in 2.5 kb α-tubulin expression in chicken spinal cord after neuropathic OP compound exposure when compared to controls. Another study shows that all three types of neurofilament transcripts increase as early as 24 hours after neuropathic OP compound exposure (Gupta *et al.* 1999). In this study, we did not observe an increase in the level of pMek1/2 after neuropathic OP compound exposure. In fact, we observed a decrease in pMek1/2 level from 4 to 8 hours post-exposure to a neuropathic OP compound. This may suggest that the MAPK pathway is not directly

responsible for the initial increase in the transcripts of these neurofilament proteins and α-tubulin *in vivo*.

Together, these data suggest that exposure to a non-neuropathic OP compound causes increased activity of the MAPK pathway. Neuropathic OP compounds, on the other hand, may prevent upregulation of the MAPK pathway. Consequently, the neuron may use a redundant pathway to initiate transcription of certain cytoskeletal proteins. Without participation of the MAPK cascade as the primary neurite outgrowth pathway, neurite outgrowth cannot be sustained and therefore, decreased neurite outgrowth results.

The PI-3K cell signaling cascade, which is primarily responsible for cell survival (Kaplan 1995), is also affected by exposure to both neuropathic and non-neuropathic OP compounds. Exposure to a non-neuropathic OP compound or an intermediate dose of a neuropathic OP compound causes an increase in the level of pAkt compared to controls. The fact that an intermediate dose of a neuropathic OP compounds affects this cascade, as opposed to a low or high dose, is unexplained. There are, however, several possible reasons for this observation. For example, exposure to a low dose of a neuropathic OP compound may not be an insult great enough to warrant upregulation of the PI-3K pathway. Alternatively, upregulation of a redundant pathway, such as the MAPK pathway (Sanchez *et al.* 2001), may provide an adequate response to elicit cell survival after low-dose exposure. Conversely, exposure to a high dose of a neuropathic OP compound may overwhelm the capability of the neuron to upregulate the PI-3K pathway.

The Akt protein is central to the function of the PI-3K pathway. Because we saw an increase in the activated form of Akt after an intermediate concentration of a neuropathic OP compound, we propose that neurite retraction observed by Hong *et al.*

(2003) after neuropathic OP compound exposure is not due to inhibition of the PI-3K pathway. They observed neurite retraction between 4 and 8 days after OP compound exposure, which is considerably later than the timepoints used in this study. However, transcription, translation, and processing of a large protein only takes an hour at most (Lodish *et al.* 2000). Also, research has demonstrated that reductions in ATP levels that occur up to 12 hours after neuropathic OP compound exposure do not cause significant cell death until days after exposure (Massicotte *et al.* 2005; Nostrandt *et al.* 1992). This suggests that early intracellular changes do not manifest themselves morphologically for hours or days after exposure. Therefore, we propose that the changes observed by Hong *et al.* (2003) are subsequent effects of intracellular changes that occur within 48 hours of OP compound exposure.

Although the PI-3K cascade primarily results in cell survival, it can also contribute to neurite outgrowth (Nusser *et al.* 2002; Sanchez *et al.* 2001). In fact, some suggest that inhibition of the PI-3K pathway causes neurite retraction (Sanchez *et al.* 2001). We did not detect inhibition in the activation of Akt in the PI-3K pathway after exposure to a neuropathic OP compound compared to a non-neuropathic OP compound, demonstrating that Akt is activated at the same level after both neuropathic and non-neuropathic OP compound exposure. Because this protein is a mediator of the PI-3K survival pathway, this finding supports a previous study that found no difference in the occurrence of cell death in cells exposed to neuropathic and non-neuropathic OP compounds (Carlson *et al.* 2000). However, cells exposed to high concentrations (10^{-3} – 10^{-5} M) of a neuropathic OP compound induce early cell death and significantly decrease cell viability compared to non-neuropathic OP compounds (Carlson *et al.* 2000;

Nostrandt *et al.* 1992). Therefore, concentrations of a neuropathic OP compound higher than those used in this study may inhibit a protein downstream of Akt, such as Bad, resulting in decreased viability.

The PKC family, which is downstream of PLC-γ, also participates in the phosphorylation of cytoskeletal proteins. Members of the PKC family can help regulate the MAPK cascade activity (Roberson *et al.* 1999). Specifically, activated PKC-α is able to activate the MAPK pathway via the Mek1/2 protein (Choe *et al.* 2002). One cytoskeletal protein that is a PKC target, GAP-43, is a component in the growth cones of neurites and is, therefore, required for axonal growth (Meiri *et al.* 1986). Early levels of pPKC-α up to 8 hours after OP compound exposure observed in this study may contribute to MAPK cascade upregulation seen after non-neuropathic OP compound exposure. This could occur as PKC kinases are transiently activated shortly after exposure to an OP compound and phosphatases are activated between 8 and 48 hours after exposure.

With regards to exposure to neuropathic OP compounds, the level of pPKC-α progressively decreased from 8 to 48 hours. Therefore, it is unlikely that the PLC-γ pathway plays a major role in neurite outgrowth or cell survival after neuropathic OP compound exposure. Furthermore, because the time-dependent decrease in pPKC-α levels also occurred in control cells, it is unlikely that the changes in PKC-α levels can be implicated as an early contributing factor to cellular effects induced by neuropathy-inducing OP compounds. This observation is supported by a previous study that suggested PKC does not play a significant role in the pre-clinical stages of a delayed

neuropathy in animals exposed to certain OP compounds (OPIDN) (Gupta and Abou-Donia 2001).

There is little information on early molecular mechanisms of OPIDN. This study suggests that non-neuropathic OP compounds upregulate both the MAPK and PI-3K pathways to different degrees, as sources of neurite outgrowth and cell survival, respectively. This upregulation does not appear to occur uniquely after exposure to a neuropathic OP compound. Furthermore, the signaling cascades we examined may act in collaboration to promote cell survival and/or regeneration after insult. For example, while the PLC-γ pathway does not appear to be required for survival or regeneration after neuropathic OP compound exposure, it may contribute to upregulation of the MAPK pathway after exposure to a non-neuropathic OP compound by redundant mechanisms (Choe *et al.* 2002; Roberson *et al.* 1999).

Our data suggest that there is a difference in the regulation of the cell signaling cascades examined in this study after exposure to neuropathic versus non-neuropathic OP compounds. The consistent increase in pMek1/2 and pAkt levels after exposure to a non-neuropathic OP compound suggests that the MAPK and PI-3K pathways are integral to the molecular mechanisms of neuronal response after exposure to an OP compound. Finally, because this study examined only one neuropathic and non-neuropathic OP compound each, future studies using other OP compounds are needed to validate these results.

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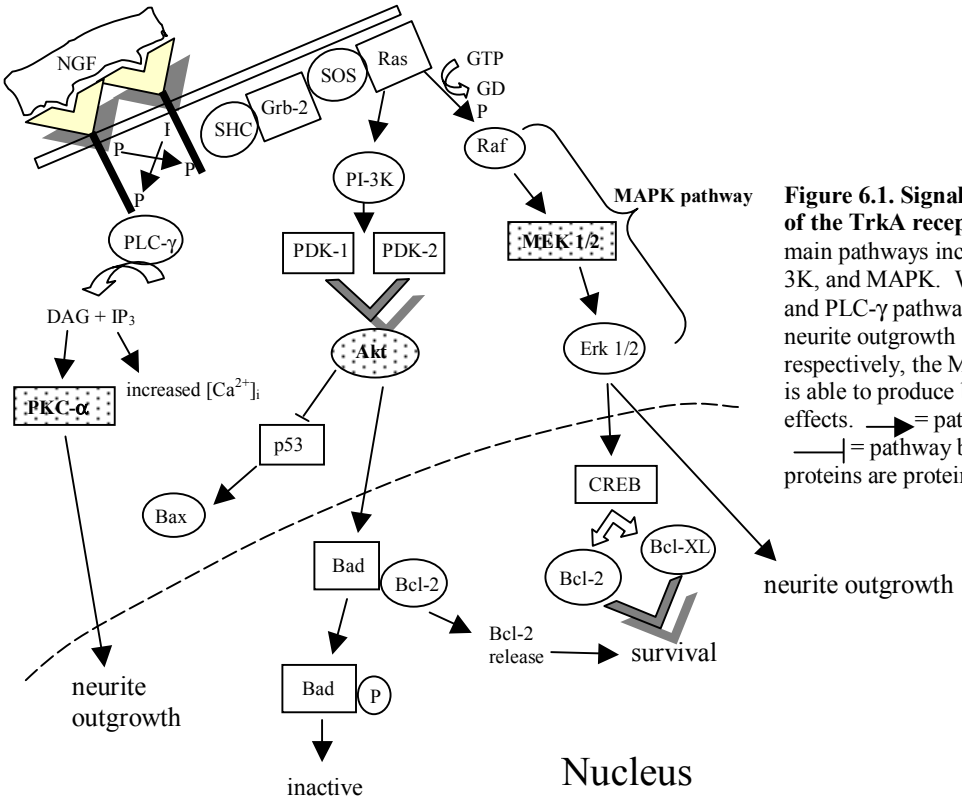

Figure 6.1. Signal transduction of the TrkA receptor. The 3 main pathways include PLC-γ, PI-3K, and MAPK. While the PI-3K and PLC-γ pathways result in neurite outgrowth and survival, respectively, the MAPK pathway is able to produce both these effects. → = pathway permitted; ⊣ = pathway blocked. Dotted proteins are proteins examined.

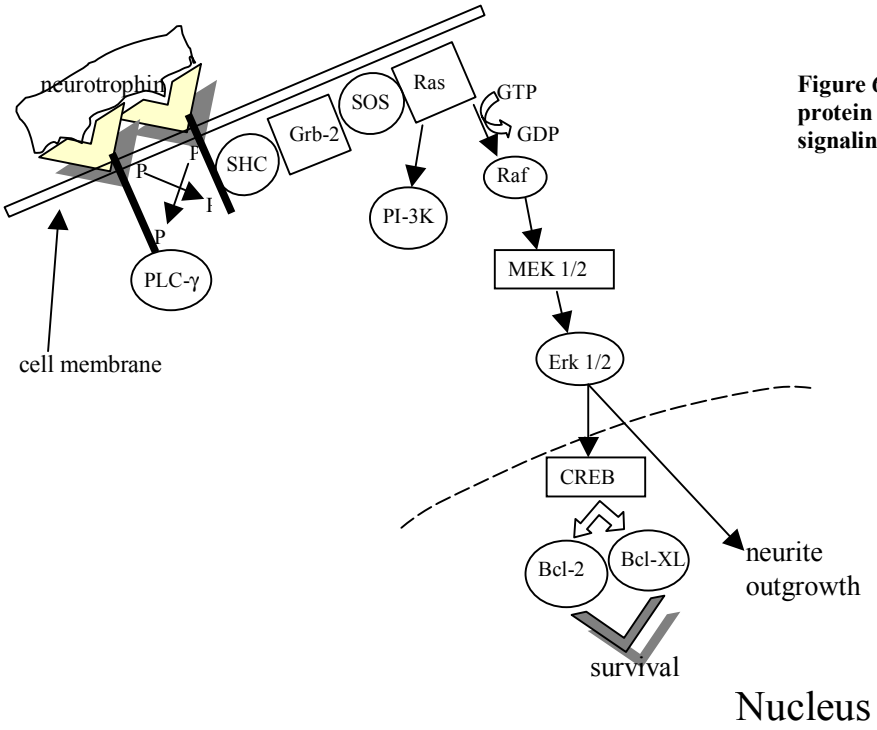

Figure 6.2. Mitogen activated protein kinase (MAPK) signaling pathway

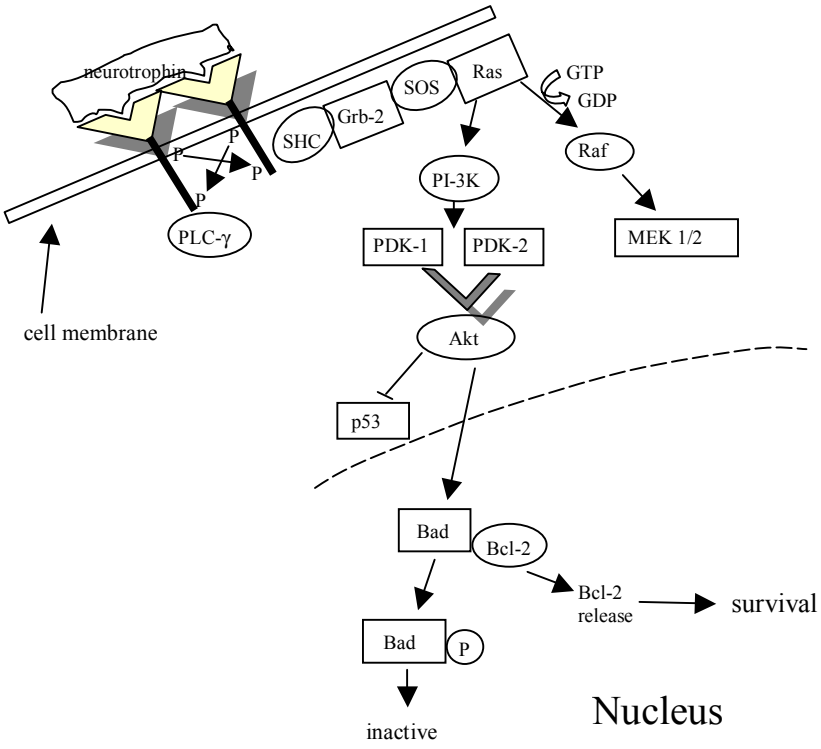

Figure 6.3. Phosphoinositol-3 kinase (PI-3K) signaling pathway

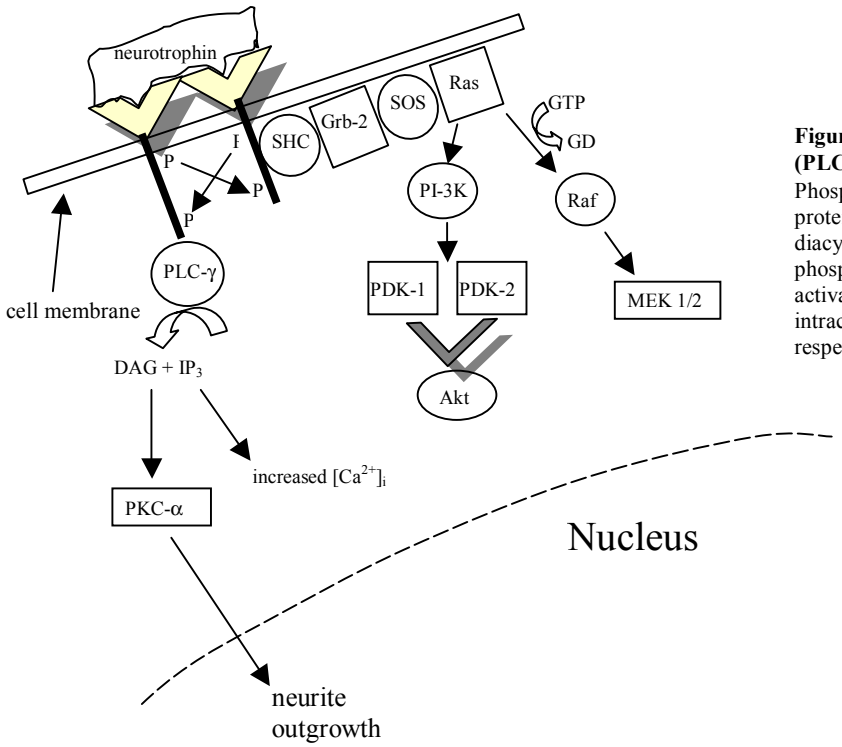

Figure 6.4. Phospholipase-C γ (PLC-γ) signaling pathway. Phosphorylation of the PLC-γ protein induces activation of diacylglycerol (DAG) and inositol-3 phosphate (IP_3). These two proteins activate PKC and increase intracellular Ca^{++} concentrations, respectively.

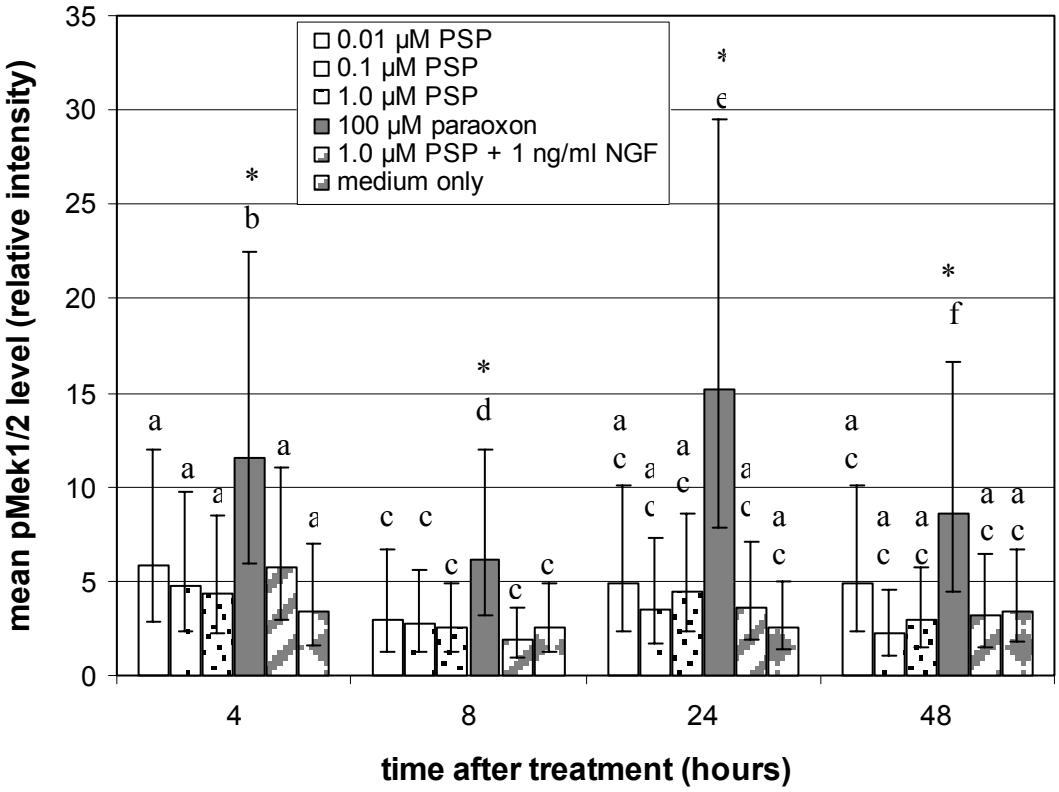

Figure 6.5. Least square means and standard error (represented by error bars) of phosphorylated Mek 1/2 (pMek1/2) in SH-SY5Y cells treated with a neuropathic esterase inhibitor (PSP; 0.01 µM, 0.1 µM, and 1.0 µM) (n = 6 per timepoint) and a non-neuropathic esterase inhibitor (paraoxon, 100 µM) (n = 6 per timepoint), a neuropathic esterase inhibitor plus NGF (1.0 µM PSP + 1 ng/ml NGF) (n = 6 per timepoint), and medium only (control) (n = 6 per timepoint). The level of pMek1/2 was significantly increased in cells treated with a non-neuropathic OP compound ($p < 0.05$), compared to controls, as indicated by an "*". Furthermore, cells exposed to a neuropathic or non-neuropathic OP compound for 4 hours had a greater level of pMek1/2 than did cells exposed for 8 hours. Controls had mean pMek1/2 levels of 3.36 (range 1.62 - 6.94) at 4 hours, 2.51 (range 1.3 - 4.9) at 8 hours, 2.57 (range 1.3 – 5) at 24 hours, and 3.43 (range

1.7 – 6.6) at 48 hours. Treatments with different letters were significantly different ($p < 0.05$).

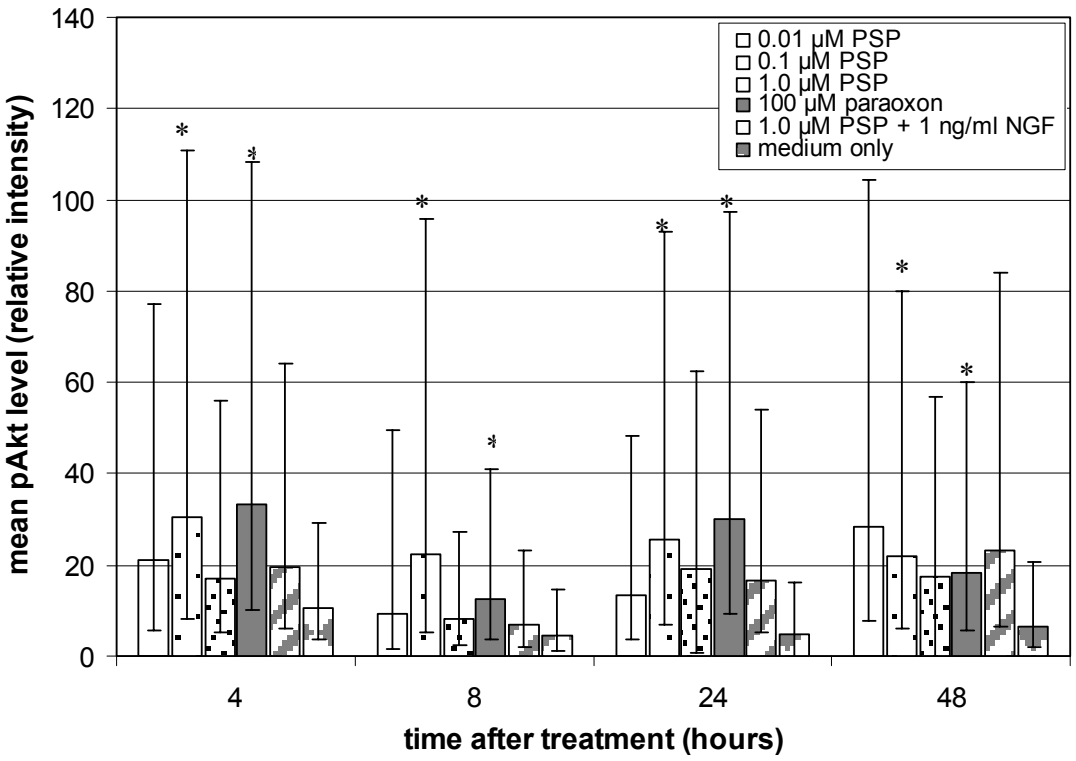

Figure 6.6. Least square means and standard error (represented by error bars) of phosphorylated Akt (pAkt) in SH-SY5Y cells treated with a neuropathic esterase inhibitor (PSP; 0.01 µM, 0.1 µM, and 1.0 µM) (n = 6 per timepoint) and a non-neuropathic esterase inhibitor (paraoxon, 100 µM) (n = 6 per timepoint), a neuropathic esterase inhibitor plus nerve growth factor (NGF) (1.0 µM PSP + 1 ng/ml NGF) (n = 6 per timepoint), and medium only (control) (n = 6 per timepoint). There was significantly more pAkt in cells treated with 0.1 µM PSP and 100 mM paraoxon compared to controls ($p < 0.05$), as indicated by an "*". There was no significant effect on pAkt levels due to time ($p = 0.18$). Controls had mean pAkt levels of 10.42 (range 3.7 – 29.3) at 4 hours, 4.52 (range 1.4 – 14.8) at 8 hours, 4.98 (range 1.5 – 16.2) at 24 hours, and 6.32 (range 1.9 – 20.6) at 48 hours.

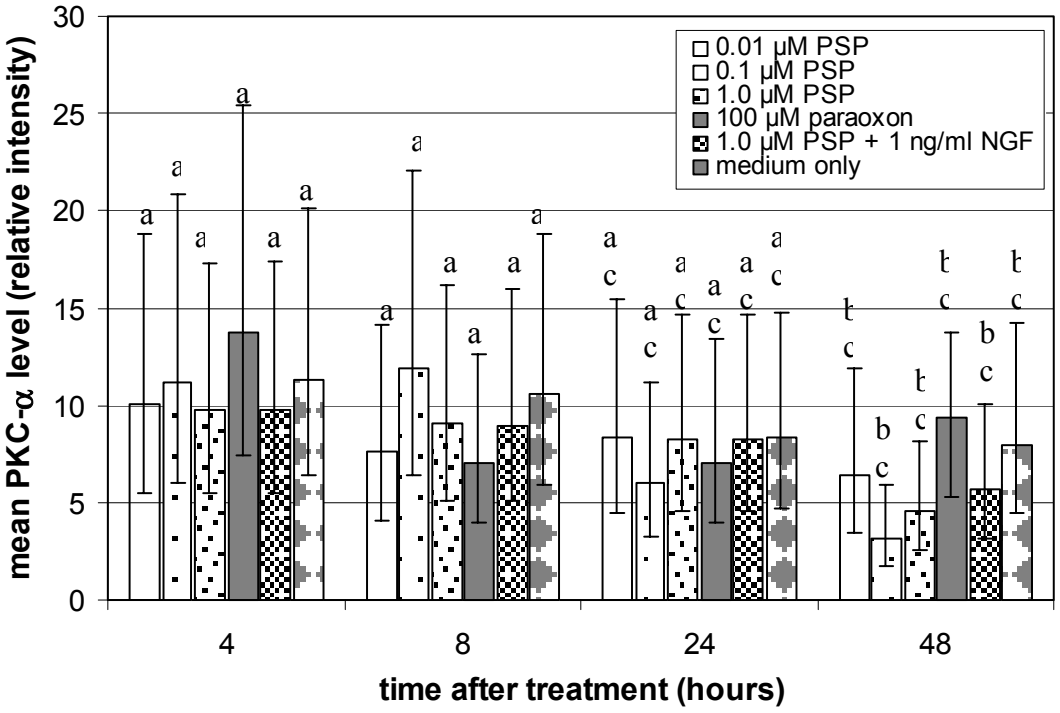

Figure 6.7. Least square means and standard error (represented by error bars) of phosphorylated PKC-α (pPKC-α) in SH-SY5Y cells treated with a neuropathic esterase inhibitor (PSP; 0.01 µM, 0.1 µM, and 1.0 µM) (n = 6 per timepoint) and a non-neuropathic esterase inhibitor (paraoxon, 100 µM) (n = 6 per timepoint), a neuropathic esterase inhibitor plus nerve growth factor (NGF) (1.0 µM PSP + 1 ng/ml NGF) (n = 6 per timepoint), and medium only (control) (n = 6 per timepoint). There was no significant effect on the level of pPKC-α due to treatment ($p = 0.66$). The mean pPKC-α level in cells exposed to a neuropathic or non-neuropathic OP compound for 4 hours or 8 hours is significantly more than that of cells exposed for 48 hours ($p < 0.05$). Controls had mean pPKC-α levels of 11.3 (range 6.4 – 20.1) at 4 hours, 10.6 (range 5.9 – 18.9) at 8 hours, 8.3 (range 4.7 – 14.8) at 24 hours, and 8.0 (range 4.5 – 14.2) at 48 hours. Treatments with different letters were significantly different ($p < 0.05$).

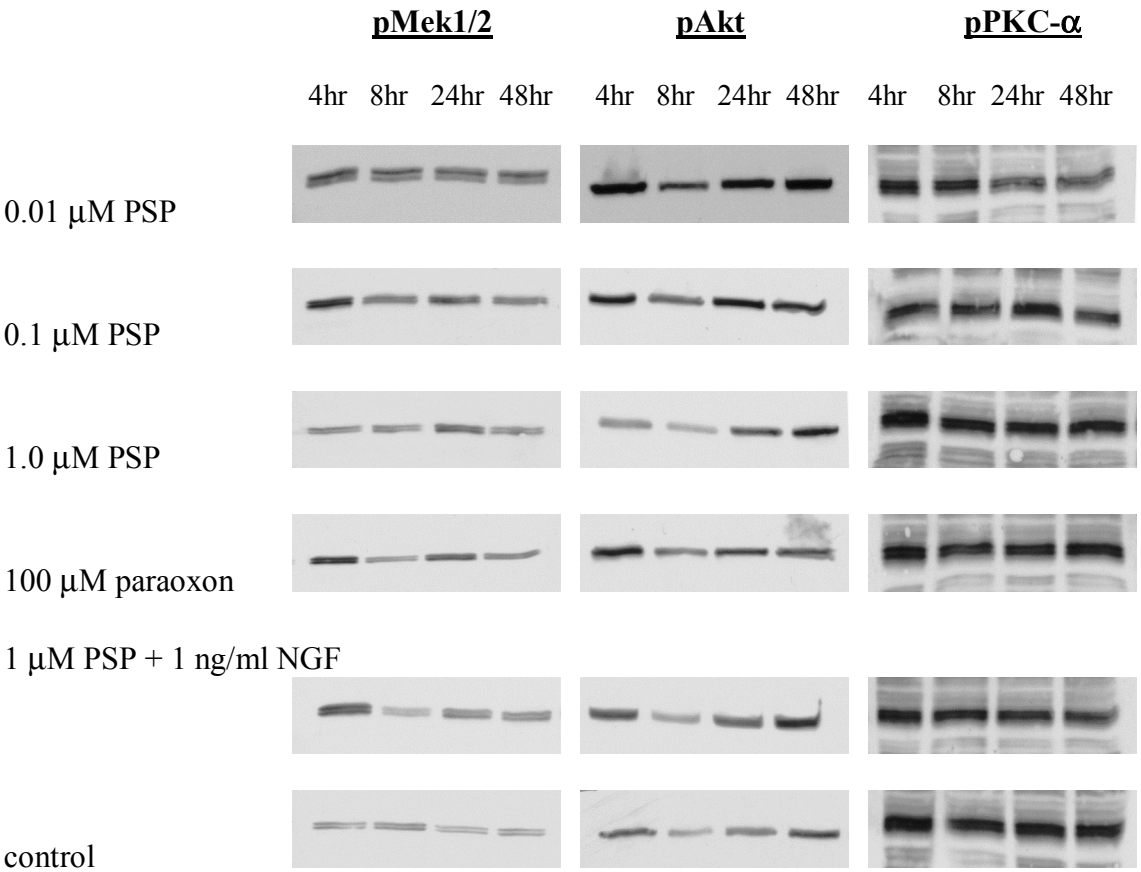

Figure 6.8. Representative total phosphorylated Mek1/2, phosphorylated Akt, and phosphorylated PKC-α Western blots of SH-SY5Y cells exposed to a neuropathic OP compound (0.01 μM, 0.1 μM, and 1.0 μM PSP), a non-neuropathic OP compound (100 μM paraoxon), a neuropathic OP compound supplemented with nerve growth factor (1 μM PSP + 1 ng/ml NGF) and medium only (control).

PART V.

GENERAL DISCUSSION AND CONCLUSION

Chapter 7.

GENERAL DISCUSSION AND CONCLUSION

This dissertation investigated the presence of neurotrophins and their receptors as well as intracellular changes in neurotrophin signaling cascades resulting from exposure to organophosphorus (OP) compounds. The hypothesis tested was that there is an immediate increase in growth factor release, specifically nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), upon exposure to neuropathy-inducing OP compounds *in vivo*, but that OP compounds block effects of these growth factors such that beneficial effects cannot be sustained. This study demonstrated that although neurotrophin concentrations did not increase after neuropathic OP compound exposure, OP compounds may interfere with intracellular effects of neurotrophins because they prevent upregulation of the primary signaling cascade responsible for neurite outgrowth and cell survival.

Organophosphorus compounds are found in household pest control products, plastics, and petroleum. The ubiquitous nature of OP compounds allows for regular human contact with them (Davis and Richardson 1980; Markowitz 1992). However, OP compounds are neurotoxic, and can cause both acute and delayed clinical signs (Davis and Richardson 1980).

One of these delayed effects is the development of organophosphate-induced delayed neuropathy (OPIDN), which is characterized by Wallerian-like degeneration of nerves in the central and peripheral nervous system (CNS and PNS, respectively) after exposure to neuropathic OP compounds (Davis and Richardson 1980; Ehrich and Jortner 2001). There are many questions surrounding the mechanisms of the onset of OPIDN.

Some proposed mechanisms include dysregulation of various cytoskeletal proteins (Cho *et al.* 2002; Fox *et al.* 2003; Gupta *et al.* 1999), induction of necrosis and/or apoptosis (Carlson *et al.* 2000; Fioroni *et al.* 1995), and depletion of ATP production (Massicotte *et al.* 2005).

Depending on the location of the injury (CNS or PNS), Wallerian-like degeneration after neuropathic OP compound injury is followed by different degrees of regeneration through axonal sprouting. Axonal sprouting is enhanced by the presence of neurotrophins, but the exact role of neurotrophins in regeneration and axonal sprouting is unclear. Production and release of neurotrophins protects neurons and restores impaired functions by binding coexpressed high- and low-affinity receptors (Blöchl *et al.* 1995; Hagg *et al.* 1993). Failure of sustained regeneration in the CNS may result from insufficient reception of neurotrophins, rather than unavailability of neurotrophic factors (Hagg *et al.* 1993; Raivich and Kreutzberg 1993). To test our hypothesis that neurotrophins have a role in OPIDN, we investigated their presence after OP exposure. Results demonstrated that, while neurotrophins are present in the lumbar spinal cord of chickens exposed to OP compounds, it is unlikely that high concentrations of neurotrophins alone are the essential factors that assist regenerative efforts of young animals' central nervous systems after exposure to neuropathic OP compounds. These neurotrophins did, however, activate receptors associated with a cell signaling pathway that can contribute to neurite outgrowth and cell survival.

Pope *et al.* (1995) suggested that a soluble factor contributing to regeneration after neuropathic OP compound exposure was a neurotrophin. Although our research suggests that this factor is not NGF, BDNF, or NT-3, remaining possibilities include

neurotrophin-4/5 or other factors found in the spinal cord, such as cytokines and proteins. The molecular weight of each of these neurotrophins (NGF, BDNF, and NT-3) falls into the molecular weight groups we isolated for soluble factor characterization. However, neither molecular weight group produced significant neurite outgrowth *in vitro*, supporting our suggestion that the soluble factor responsible for neurite outgrowth in the study of Pope *et al.* (1995) is not a neurotrophin working alone. If this factor were solely a neurotrophin, it is likely we would have seen an increase in the concentration of one or all of the neurotrophins we examined and/or neurite outgrowth from at least one of the isolated molecular weight groups. Other factors in the spinal cord, and perhaps in the cerebrospinal fluid, may be equally necessary for regenerative efforts after exposure to neuropathic-OP compounds. We suggest that rather than a single factor inducing neuronal regeneration, a combination of factors may collectively appear as a single "soluble factor." Therefore, while neurotrophins are not likely to be the primary cause of regenerative efforts, they certainly may contribute to it.

We propose that the cytokine family, including interleukins, tumor necrosis factors, interferons, and neuropoietins, plays a role in neuronal recovery after exposure to neuropathic OP compounds (Ebadi *et al.*, 1997). In the CNS, cytokines are produced by glial cells, which are integral in sustaining the CNS after injury or exposure to viruses, fungi, or bacteria (Cotran *et al.* 2005; Ebadi *et al.* 1997). Cytokines are produced after brain injury and in neurodegenerative disease, as well (Junier *et al.* 1998). We suggest that these cytokines work collaboratively with constitutively present neurotrophins in an attempt at regeneration. This is an area for future investigations.

This study also demonstrated that the specific high-affinity (Trk) and common low-affinity (p75) neurotrophin receptors for NGF are present in their phosphorylated state in cells exposed to OP compounds. This suggests that constituitively present neurotrophins bind and activate their respective receptors, initiating downstream signaling after exposure to OP compounds. Furthermore, the level of phosphorylated p75 (pp75) on the cell membrane was consistently greater than phosphorylated (pTrkA) levels after OP compound exposure. Binding of neurotrophins to p75 is more likely to contribute to cell death than binding to the TrkA receptor (Bredesen and Rabizadeh 1997; Chao and Hempstead 1995).

While we did not observe a change in the ratio of these two receptors to each other, the concentration of pp75 on the cell membrane differed significantly after exposure to a low concentration of a neuropathic OP compound (0.01 μM PSP) compared to a non-neuropathic OP compound. We suggest that the difference in p75 activation between a low dose of a neuropathic OP compound and a non-neuropathic OP compound contributes to the inherent difference in the method of cell death between these compounds. Apoptosis after non-neuropathic OP compound exposure (Carlson *et al.* 2000) may result from a greater level of pp75 on the cell membrane compared to pTrk receptors because it is activation of p75 that contributes to cell death (Kaplan and Miller 1997). Apoptosis is initially observed after neuropathic OP compound exposure as well, and it may also result from p75 activation (Bredesen and Rabizadeh 1997; Chao and Hempstead 1995). Interestingly, apoptosis can be switched to necrosis following adenosine tri-phosphate (ATP) depletion, and ATP depletion has been noted after exposure to a high dose neuropathic, but not non-neuropathic, OP compound (Leist *et al.*

1997; Massicotte *et al.* 2005). Therefore, we suggest that the greater level of pp75 on the cell membrane compared to pTrkA is a contributing factor to the Wallerian-like degeneration seen in OPIDN after neuropathic OP compound exposure.

During times of limited neurotrophins, pp75 augments cell response to neurotrophins (Rydén *et al.* 1997). Given that we observed greater levels of pp75 on the cell membrane compared to pTrkA after non-neuropathic OP compound exposure and that neurotrophins exist at a steady-state, and perhaps limited, concentration *in vivo*, we suggest that high levels of pp75 contribute to the ability of the cell to respond to available neurotrophins after non-neuropathic OP compound exposure. After initial apoptosis, pp75 may contribute to cell survival and recovery as the receptor switches from a pro-apoptotic pathway to a pro-survival pathway.

Intracellular signaling cascades initiated by high-affinity neurotrophin receptor activation are also affected by OP compound exposure. Exposure to a non-neuropathic OP compound, but not a neuropathic OP compound, elevated levels of phosphorylated Mek1/2 (pMek1/2) and phosphorylated Akt (pAkt). These two proteins are central to the MAPK and PI-3K cascades, which both contribute to neurite outgrowth and cell survival (Kaplan 1995). This observation suggests that neuropathic and non-neuropathic OP compounds may affect these signaling cascades differently and that the MAPK and PI-3K pathways play an important role in neuronal response after OP exposure.

Results of the experiments performed to test our hypothesis have led us to propose that exposure to a non-neuropathic OP compound causes increased activity of the MAPK pathway. Activation of the MAPK cascade causes phosphorylation of both neurofilaments and the tau protein, which is localized in axons (Holzer *et al.* 2001;

Schoenfeld and Obar 1994; Veeranna *et al.* 2000; Weingarten *et al.* 1975). The augmented presence of tau protein resulting from MAPK upregulation may stimulate synthesis of other cytoskeletal proteins involved in neurite outgrowth, including tubulin (Holzer *et al.* 2001). Therefore, the increase in pMek1/2 after exposure to a non-neuropathic OP compound may indicate that upregulation of one or more cytoskeletal elements involved in neurite outgrowth and regeneration occurs via the MAPK pathway. Neuropathic OP compounds, on the other hand, may prevent upregulation of the MAPK pathway.

After neuropathic OP compound exposure, the neuron may attempt to use a redundant pathway to initiate transcription of certain cytoskeletal proteins, such as the PI-3K pathway. Redundant pathways that are ineffective after neuropathic OP compound exposure may be effective after non-neuropathic OP compound exposure. For example, phosphorylated PKC-α (pPKC-α), one isoform of the PKC family, is able to activate the MAPK pathway via the Mek1/2 protein (Cho *et al.* 2002). We demonstrated the presence of pPKC-α in control and neuronal cells after exposure to OP compounds. Therefore, early high levels of pPKC-α after OP compound exposure may contribute to MAPK cascade upregulation after non-neuropathic OP compound exposure. Because we also observed the presence of pPKC-α after neuropathic OP compound exposure, we suggest that neurite retraction observed by Hong *et al.* (2003) is not due to inhibition of the PI-3K pathway. Rather, it is more likely that neurite retraction observed by Hong *et al.* (2003) is due to inhibition of the MAPK pathway occurring within 48 hours of OP compound exposure.

The signaling cascades we examined may act in collaboration to promote cell survival and/or regeneration after exposure to a non-neuropathic OP compound. For example, the PLC-γ pathway may contribute to survival or regeneration through MAPK pathway upregulation by redundant mechanisms (Cho *et al.* 2002; Roberson *et al.* 1999). We suggest that such cross-talk is effective after non-neuropathic OP exposure, but not after neuropathic OP exposure.

While progress was made in determining how nervous tissue responds to OP compounds, this project raised questions about induction of these changes and the role(s) of extracellular factors after OP compound exposure. Similar studies need to be conducted using other OP compounds than were used in this study in order to verify that the changes we observed are a common mechanism after exposure to neuropathic or non-neuropathic OP compounds. Future studies may also focus on the role of the combination of cytokines, or if cytokines even play a role, after OP compound exposure. Finally, neurotrophins that are present *in vivo* may or may not be interacting properly with their respective receptors. We investigated the role of receptors and intracellular changes using an *in vitro* model. Using an *in vivo* model to study the interactions of neurotrophins with their receptors after OP compound exposure may eventually lead to therapeutic interventions of OPIDN.

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PART VII.

APPENDICES AND VITA

Time (hours)	Mean (µg/mg protein) ± standard deviation		
	NGF	BDNF	NT-3
4	6.35 ± 0.99	7.13 ± 0.67	21.23 ± 0.77
8	4.09 ± 1.64	11.3 ± 2.79	28.73 ± 5.44
24	9.88 ± 1.14	9.4 ± 1.8	46.73 ± 5.67
48	7.5 ± 2.99	9.04 ± 0.53	33.98 ± 3.86
120	7.81 ± 3.35	8.05 ± 1.55	44.38 ± 3.22
240	9.08 ± 2.45	6.7 ± 1.32	20.62 ± 0.91

Appendix A. Means and standard deviations of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) concentrations in lumbar spinal cord of chickens treated with saline 4-48 hours and 5 and 10 days post-dosing.

Appendix B. Publication Submitted to *Neurotoxicity Research*

Early Effects of Neuropathy-Inducing Organophosphates on *In Vivo* Concentrations of Three Neurotrophins

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Keywords: Neurotrophins, Organophosphate-Induced Delayed Neuropathy, Organophosphate Compounds

ABSTRACT

Exposure to OP compounds that inhibit neurotoxic esterase (NTE) induces a delayed neuropathy (OPIDN) characterized by Wallerian-like degeneration of long axons in certain animals, including humans. Pope *et al*. (1995) found that neurite outgrowth occurred following the addition of spinal cord extracts from chickens with active OPIDN to neuroblastoma cells, suggesting growth factor expression during the neuropathy. We hypothesized that, shortly after exposure to a neuropathic OP compound, the central nervous system (CNS) attempts to recover from the toxic insult through upregulation of the neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) in susceptible regions of the nervous system. However, this upregulation is transient and cannot be sustained. To test this hypothesis, we exposed 10-week-old chickens to a neuropathic OP compound (PSP, 2.5 mg/kg), a non-neuropathic OP compound (paraoxon, 0.10 mg/kg), and vehicle (DMSO, 0.5ml/kg) intramuscularly. By day 8, all PSP-treated birds demonstrated clinical signs of OPIDN. We sacrificed chickens by pentobarbital overdose at 4, 8, 24, and 48 hours, and 5 and 10 days post-exposure and confirmed NTE inhibition in birds treated with PSP for 4 and 24 hours. Enzyme-linked immunosorbant assays indicate that NGF, BDNF, and NT-3 are found in chicken lumbar spinal cord after exposure to a neuropathic OP compound. However, exposure to the neuropathic OP compound, PSP, did not preferentially elevate levels of NGF, BDNF, and NTE compared to the non-neuropathic OP compound, paraoxon. This suggests that these neurotrophins alone do not contribute to a sustained regenerative effort in the CNS.

INTRODUCTION

Between days and weeks after exposure to neuropathy-inducing organophosphates (OP), certain species, including humans, cats and chickens, develop clinical signs that progress from abnormal foot placement and leg weakness to an unwillingness to walk and finally, flaccid paralysis (Ehrich and Jortner 2001; Lotti 1992). Histopathology reveals the primary lesion of organophosphate-induced delayed neuropathy (OPIDN) as bilateral degeneration that begins at distal levels of myelinated axons and spreads proximally toward the soma. This lesion is referred to as a dying-back axonopathy, or Wallerian-like degeneration (Jortner 2000). Wallerian-like degeneration may occur in greater than 10% of peripheral nerve fibers in moderate cases of OPIDN to greater than 30% of nerve fibers in severe cases (Jortner and Ehrich 1987). Large and/or long peripheral nerve fibers and fibers in the central nervous system (CNS) are the primary targets of OPIDN. Microscopically, these lesions appear as pale-staining, swollen axons with thin myelin sheaths (Ehrich and Jortner 2001; Lotti 1992). Clinical signs and lesions occur in chickens, the U.S. EPA approved animal model for OPIDN, as young as 8 weeks, although of a lesser severity than that seen in adult hens (Funk *et al.* 1994; Harp *et al.* 1997; Peraica *et al.* 1993; U.S.EPA 1996).

Neurotoxic esterase (NTE) is an enzyme that is inhibited in the brain, spinal cord, and peripheral nerves in all species of animals within hours of exposure to certain OP esters (Ehrich and Jortner 2001; Levi-Montalcini 1987; Lotti 1992). The development of a strong bond between NTE and the OP compound ("aging") is an essential factor in the initiation of OPIDN (Ehrich and Jortner 2001). Sufficient doses of neuropathic OP compounds inhibit and age 70-80% of available NTE within hours of exposure (Ehrich and Jortner 2001; Moretto *et al.* 1991). Young animals appear relatively resistant to the clinical effects of neuropathic OP exposure although they do exhibit NTE inhibition (Ehrich and Jortner 2001; Funk *et al.* 1994; Harp *et al.* 1997; Moretto *et al.* 1991; Peraica *et al.* 1993).

Published information identifies a role for neurotrophins after traumatic injury to the nervous system (Hagg *et al.* 1993; Perez-Polo and Werrbach-Perez 1987). In both OPIDN and traumatic nervous system injury, axonal sprouting is the primary means for

regeneration. Increasing evidence suggests that neurotrophins play a role in the synaptic plasticity that allows the growth of axonal sprouts (Blöchl *et al.* 1995; Brunello *et al.* 1990; Grill *et al.* 1997; Perez-Polo and Werrbach-Perez 1987). The exact role of neurotrophins in this process is unclear, but it is known that neurotrophin concentrations rapidly change in the spinal cord under pathological conditions (Raineteau and Schwab 2001). Several studies demonstrate that axonal sprouts do not elongate without appropriate growth factors (Schnell *et al.* 1994; Schwab and Bartholdi 1996). Initial axonal regeneration occurs as a result of localized, transient increase in neurotrophic factors at the lesion site (Hagg *et al.* 1993). Upregulation of growth factors and chemoattractants directs new sprouts to the appropriate target area (Thallmair *et al.* 1998). Consequently, production, release, and reception of neurotrophins are part of the response to injury in neural tissues (Hagg *et al.* 1993).

There are 4 recognized proteins that compose the neurotrophin family: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (Barker and Shooter 1994; Meakin and Shooter 1992). The best-characterized neurotrophin is NGF, a 26 kilodalton protein. This neurotrophin was recognized by its specific ability to induce neurite stimulation *in vitro* (Hagg *et al.* 1993; Levi-Montalcini 1987). Following peripheral nerve injury, neuronal and nonneuronal cells distal and immediately proximal to the lesion site produce NGF (Brunello *et al.* 1990; Hagg *et al.* 1993; Heumann *et al.* 1987). Upregulation of NGF induces Schwann cell migration. These migrating cells form bands of Bünger around the distal and proximal ends of the injured nerve, which guide the regenerating axon back to its target (Anton *et al.* 1994).

Both BDNF and NT-3 share just over 50% sequence homology with NGF (Hagg *et al.* 1993; Yin *et al.* 1998). These neurotrophins also overlap in biological activity and target cells with NGF (Hagg *et al.* 1993). In the intact nervous system, BDNF and NT-3 serve as maintenance factors, supporting the survival and axonal outgrowth of certain populations of neurons (Cai *et al.* 1998; Landreth 1999). Oligodendrocytes and their progenitors may serve as a primary and continuing source of increased BDNF and NT-3 after injury (McTigue *et al.* 1998). Evidence indicates that the roles and concentrations

of BDNF and NT-3 differ from that of NGF during degeneration and regeneration of peripheral nerves (Grill *et al.* 1997; McTigue *et al.* 1998).

Time-related concentrations of specific neurotrophins have not been examined after exposure to neuropathy-inducing OP compounds. We investigated NGF, BDNF, and NT-3 concentrations early after OP exposure prior to the appearance of clinical signs and after the onset of clinical signs. We hypothesized that the level of NGF, BDNF, and NT-3 would increase shortly after exposure to a neuropathy-inducing OP compound as the nervous system attempts to recover from the toxic insult, but this release would not be sustained. Consequently, Wallerian degeneration continues to affect axons and eventually clinical signs of OPIDN emerge.

MATERIALS AND METHODS

Chemicals and Animals

We obtained phenyl saligenin cyclic phosphate (PSP) from Oryza Laboratories, Inc., Chelmsford, MA, paraoxon from ChemService Inc., West Chester, PA, and DMSO from Sigma, St. Louis, MO. The sample studied consisted of 140 mixed-sex 10-week-old White Leghorn (*Gallus gallus*) chickens supplied by the Department of Animal and Poultry Science, Virginia Polytechnic Institute and State University, Blacksburg, VA. We used juvenile chickens based on a previous study that suggested neurotrophin-related effects after neuropathic OP compound exposure (Pope *et al.* 1995). Importantly, chickens of this age are old enough to demonstrate clinical signs of OPIDN, although not to the extent seen in older hens. This suggests that younger hens are more successful at repair because of the developing state of their nervous system (Harp *et al.* 1997). All chickens were housed together in a temperature-controlled environment at 23^{o}C with free access to water and commercial chicken feed through 10 weeks of age. Three days before treatment, we moved chickens to wire-bottom cages for acclimation. We randomly allocated chickens to compound*time combinations. The study was divided into 3 blocks to overcome logistical problems of sacrificing numerous birds.

Treatment

On day 0, chickens received one of three treatments (PSP, paraoxon, or DMSO) by intramuscular injection. We dissolved PSP in DMSO to yield a 5 mg/ml solution and administered a dose of 2.5 mg/kg PSP. The dose of 2 mg/kg PSP is sufficient to cause OPIDN in adult hens (Jortner and Ehrich 1987); 2.5 mg/kg caused ataxia in 10-week-old chickens in a previous study (Pope *et al.* 1995). Immediately after diluting a stock solution of paraoxon (13.76 mg/ml) in saline to yield a 0.20 mg/ml solution, we injected a second group of chickens with 0.10 mg/kg paraoxon. Fifteen minutes prior to and immediately after administration of paraoxon, we administered atropine (1 ml/kg and 0.61-1.4 ml/kg, respectively, from a solution of 0.5 mg/ml) to prevent and then alleviate cholinergic poisoning. We administered 0.5 ml/kg DMSO to control chickens.

Using an established procedure, we observed chickens daily for clinical signs from days 1-4, and days 1-10 for chickens sacrificed at 5 and 10 days post-exposure, respectively (Ehrich *et al.* 1993; Jortner and Ehrich 1987; Pope *et al.* 1995). Signs of ataxia were recorded as: 0 = no clinical signs; 1-2 = slight, infrequent hindlimb incoordination; 3-4 = moderate but definite incoordination; and, 5-6 = frequent difficulties in standing erect and walking.

Brain esterase measurements

We determined the activity of whole brain NTE and acetylcholinesterase (AChE) in chickens euthanized at 4 and 24 hours post-exposure to PSP and paraoxon using spectrophotometric assays (Correll and Ehrich 1991). After placing the brain in a labeled tube, we froze the brain samples on dry ice. Upon finishing all sacrifices for each timepoint, we stored the samples at –80^{o}C for up to one month prior to running enzyme assays.

Enzyme-linked immunosorbant assays

We euthanized chickens at 4, 8, 24, 48 hours, and 5 and 10 days post-exposure by intravenous pentobarbital overdose and immediately removed the lumbar spinal cord. After removing the glycogen body, we wrapped the lumbar spinal cord in aluminum foil, placed it in a labeled scintillation tube, and froze it on dry ice. After completing all sacrifices for a timepoint, we froze the spinal cords at –80^{o}C.

Within 2 weeks of sample collection, we weighed and suspended lumbar spinal cord 1:10 (w:v) in cold lysis buffer [150 mM NaCl , 20 mM Tris-HCl, 10% glycerol (v:v), 1% Triton X-100 (v:v), 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1:200 dilution of Protease Inhibitor Cocktail Set III (Calbiochem, La Jolla, CA)]. The wet weight of the cord in BDNF ELISAs ranged from 50-70 mg in all blocks (Table 1). The wet weight of spinal cord used in NGF and NT-3 ELISAs ranged from 110-140 mg in block 1, 110-130 mg in block 2, and 90-120 mg in block 3 (Table 1).

After homogenizing lumbar spinal cord in lysis buffer with an ultrasonic homogenizer (W-225R model, Heat Systems-Ultrasonics, Inc., Farmingdale, NY), we

centrifuged the homogenate at 4°C at 14000 x *g* for 30 minutes. We removed the supernatants, placing each respective supernatant in a pre-labeled 2 ml microcentrifuge tube. All supernatants were maintained at –20°C until assayed.

We determined the protein concentration of each supernatant using the Bio-Rad *DC* Protein Assay (Bio-Rad, Hercules, CA) following the manufacturer's directions. Prior to each assay, we diluted each sample 1:5 with lysis buffer. We prepared standard dilutions in the range of 0 mg/ml – 2.0 mg/ml and read the absorbance at 750 nm using a SPECTRAmax Plus^{384} microplate spectrophotometer and Softmax Pro software (Molecular Devices, Sunnyvale, CA).

We measured BDNF concentrations using the ChemiKine™ BDNF kit (Chemicon, Temecula, CA) following the manufacturer's directions. For each assay, we performed serial dilutions for a standard curve in the range of 0 pg/ml – 500 pg/ml, added samples in triplicate, and read absorbance at 450 nm. We calculated the concentration of BDNF and expressed it as µg BDNF per mg protein.

We measured NGF and NT-3 levels using the NGF and NT-3 E_{max} ImmunoAssay System (Promega, Madison, WI) following the manufacturer's directions. For each assay, we performed serial dilutions for a standard curve in the range of 0 pg/ml – 250 pg/ml for NGF and 0 pg/ml – 300 pg/ml for NT-3. After diluting samples to be used in the NT-3 ELISAs (1:1) in saline, we added samples in triplicate and read absorbance at 450 nm. We did not dilute samples used in NGF ELISAs. We calculated the concentrations of NGF and NT-3 and expressed the concentrations as µg NGF or NT-3 per mg protein.

Statistical Analysis

For analysis of neurotrophin data, we used a two-way factorial treatment structure with a generalized randomized block design. Treatment factors were compound (PSP, paraoxon, and DMSO) and time (4, 8, 24, 48 hours, 5 and 10 days post-exposure). There were 2-3 chickens within each block*compound*time combination with a total of 8-9 chickens per treatment group. We performed an analysis of variance (ANOVA) for time and compound main effects and their interactions using the MIXED procedure in SAS

(version. 8.2, SAS Institute, Cary, NC). Additionally, we performed pre-planned comparisons of the interactions using Bonferroni corrected comparisons of each compound at each timepoint. For NGF analysis, we log-transformed data to stabilize variances. Esterase data were collected at only two timepoints and were compared using a t-test (GraphPad Prism, v. 1.0, GraphPad Software, Inc., San Diego, CA).

RESULTS

Clinical signs of delayed neuropathy did not appear in chickens given paraoxon and were not detected before day 8 in any PSP-treated chickens. We did not see signs of acute cholinergic poisoning in chickens given PSP, and such signs were ameliorated by administration of atropine in chickens given paraoxon. Chickens treated with PSP and sacrificed at 5 days had a clinical score of 1.0 +/- 0.58 (mean +/- SD) prior to sacrifice. Chickens treated with PSP and sacrificed at 10 days had a clinical score of 3 +/- 1.5 (mean +/- SD) prior to sacrifice.

Brain NTE and AChE assays confirmed the inhibition of NTE and AChE by PSP and paraoxon, respectively. The lowest values for NTE occurred 4 hours post-dosing with PSP (1.62 +/- 1.1 nmole/min/mg protein, mean +/- SD, n = 8) with some recovery at 24 hours to 3.31 +/- 3.3 nmole/min/mg protein (mean +/- SD, n = 8). Paraoxon-treated chickens had NTE values of 17.8 +/- 4.6 nmole/min/mg protein and 14.6 +/- 3.4 nmole/min/mg protein at 4 and 24 hours post-exposure, respectively. The lowest values for AChE activity occurred at 4 hours post-dosing with paraoxon (34.4 +/- 9.3 nmole/min/mg protein, mean +/- SD, n = 7-8) with significant recovery at 24 hours to 48.8 +/- 9.7 nmole/min/mg protein (mean +/- SD, n = 7-8). PSP-treated chickens had AChE values of 111.2 +-/ 20.4 nmole/min/mg protein and 99.64 +/-19.4 nmole/min/mg protein at 4 and 24 hours post-exposure, respectively.

Neurotrophin response to a neuropathic or non-neuropathic OP compound in the lumbar spinal cord of chickens

Relative concentrations of NGF ($p = 0.81$), BDNF, ($p = 0.70$), and NT-3 ($p = 0.86$) were not affected by the compound administered. Temporal concentrations of the neurotrophins depended on the neurotrophin being examined. There was no effect on NGF concentration due to time ($p = 0.16$). There was not a significant effect due to the interaction of compound administered and time of sacrifice ($p = 0.74$). We found a trend toward increase in NGF concentration between 8 hours and 24 hours post-exposure among all groups of chickens, such that NGF concentration is greater at 24 hours than at 8 hours post-exposure ($p = 0.063$) (Figure 1a).

There was a trend toward decrease in BDNF concentration from 4 hours to 10 days post-exposure ($p = 0.09$). We found no significant effect from the interaction of compound administered and time of sacrifice on BDNF concentration in the spinal cord ($p = 0.19$) (Figure 1b).

We found no significant effect from the interaction of compound administered and time of sacrifice on NT-3 concentration in the spinal cord ($p = 0.60$). The concentration of NT-3 increased from 4 hours to 10 days post-exposure among all compounds administered ($p = 0.045$) (Figure 1c).

DISCUSSION

These results established the presence of NGF, BDNF, and NT-3 in the spinal cord of 10-week-old chickens. This included control chickens and chickens treated with PSP, a neuropathy-inducing compound, and paraoxon, a non-neuropathic OP compound. Concentration of each of these neurotrophins did not change significantly from levels in control chickens after exposure to a neuropathy-inducing OP compound or to a non-neuropathy-inducing OP compound. Previous studies report an increase in NGF after trauma, suggesting that NGF contributed to recovery efforts after trauma (Brunello *et al.* 1990; Hagg *et al.* 1993; Heumann *et al.* 1987). Because there was no upregulation of these neurotrophins, our data suggest that NGF, BDNF, and NT-3 do not play a significant role in recovery efforts of the CNS or peripheral nervous system (PNS) after exposure to neuropathic OP compounds. Alternatively, the relatively small percentage of nerve fibers affected in OPIDN (Jortner and Ehrich 1987) may be making an insufficient contribution to the total neurotrophin concentration in the lumbar spinal cord.

The neurotrophins NGF, BDNF, and NT-3 are the three members of the neurotrophin family that historically have been the most studied in relation to neuronal injury. Another less studied member of the neurotrophin family, NT-4/5, promotes survival of spinal ganglion neuronal cultures equivalent to the survival seen after BDNF administration and greater than that post-NT-3 administration. Interestingly, NGF showed no detectable effect on survival in that same study (Ebadi *et al.* 1997). We did not include NT-4/5 in our study.

We are interested in the concentrations of neurotrophins after exposure to neuropathic OP compounds because Pope *et al.* (1995) discovered a soluble factor in the supernatant of homogenized cervical spinal cords of birds exposed to a neuropathy-inducing compound that elicited neurite outgrowth similar to that induced by NGF in SH-SY5Y neuroblastoma cells. These results indirectly suggested an increase in the presence of neurotrophic agents in the spinal cord of neuropathic OP-treated chickens. Experiments in our laboratory confirm that this effect is not seen at 4 or 8 hours post-dosing, but is seen at 24 hours post-dosing (M. Pomeroy-Black, unpublished observations). The present study directly measured specific neurotrophic factors in the

supernatant of homogenized chicken spinal cords. We did not see increases in BDNF or NT-3 between 8 and 24 hours post-dosing. Our results suggest that neither BDNF nor NT-3 is the soluble factor responsible for neurite outgrowth noted by Pope *et al.* (1995).

This study demonstrates no significant differences in the concentrations of NGF, BDNF, and NT-3 in birds exposed to vehicle alone (DMSO) versus a neuropathy-inducing OP compound. In Pope's study (1995), homogenates of birds exposed to vehicle alone or a non-neuropathy-inducing compound did not elicit neurite outgrowth (Pope *et al.* 1995). If Pope's proposed soluble factor were one of the three neurotrophins we examined, it is likely we would have seen significant increased concentration of one or all of the neurotrophins in birds treated with PSP compared to vehicle. Therefore, the soluble factor eliciting neurite outgrowth in the Pope *et al*. (1995) study is not likely to be NGF, BDNF, or NT-3.

Trends toward temporal differences were noted for NGF, BDNF, and NT-3. These trends could be developmental effects or a response to dosing. Future studies using adult birds, which are more susceptible to OPIDN (Moretto *et al.* 1991), may confirm that these trends are effects of treatment and not age.

These data support findings of other studies that suggest other factors in the spinal cord, and perhaps in the cerebrospinal fluid, are necessary for regeneration efforts after exposure to neuropathic-OP compounds. Although our study suggests that neurotrophins are not the essential factors that assist regenerative efforts of young animals' central nervous systems, a combination of factors may collectively appear as a single "soluble factor" that induces neuronal regeneration, seen as neurite outgrowth in cell culture. For example, glial cells serve as the immune system of the CNS, playing an integral role in sustaining the CNS after injury or exposure to infectious agents (Cotran *et al.* 2005). These cells give the CNS its own cytokine network, such as interleukins, tumor necrosis factors, and neuropoietins (Ebadi *et al.* 1997). Studies have detected the production of various cytokines after brain injury, in neurodegenerative disease, and in viral and bacterial infections (Junier *et al.* 1998).

While the neurotrophins we examined are constitutively produced, cytokines are induced upon injury. For example, ciliary neurotrophic factor and leukemia inhibitory factor (LIF) are induced upon peripheral nerve injury and possess similar activity to

NGF, BDNF, and NT-3 (Ebadi *et al.* 1997; Landreth 1999). Other studies implicate fibroblast growth factor (FGF) as a role-player in the promotion of axonal regeneration (Landreth 1999). Exogenous basic FGF applied to sensory neurons supports neurite outgrowth after axotomy (Grothe and Nikkhah 2001). Finally, research demonstrates that treating neuronal cells with interleukins such as IL-6 and –12 enhances neurite outgrowth *in vitro* (Lee *et al.* 2000; Lin *et al.* 2000). It is likely that, in response to neuropathic-OP compound exposure, non-neuronal cells release cytokines and that these cytokines work collaboratively with constitutively present neurotrophins.

Most of the cytokines noted above have similar signaling pathways as neurotrophins, particularly NGF, BDNF, and NT-3, because they bind receptor tyrosine kinases (RTKs) (Landreth 1999). A common pathway that is activated by proteins that bind RTKs is the mitogen-activated protein kinase (MAPK) pathway. One result of activation of the MAPK pathway is neurite outgrowth and cell survival (Kaplan and Miller 2000). Consequently, it is not unreasonable to suggest that any of the above mentioned factors could contribute to the neurite outgrowth seen by Pope *et al.* (1995).

The presence of the neurotrophins NGF, BDNF, and NT-3 in the avian model of OPIDN is clearly established with this study. However, it appears unlikely that any of these three neurotrophins contribute to a sustained regenerative effort within the CNS after exposure to a neuropathy-inducing OP compound. It is likely that NGF collaborates with other factors in the CNS to attempt regeneration.

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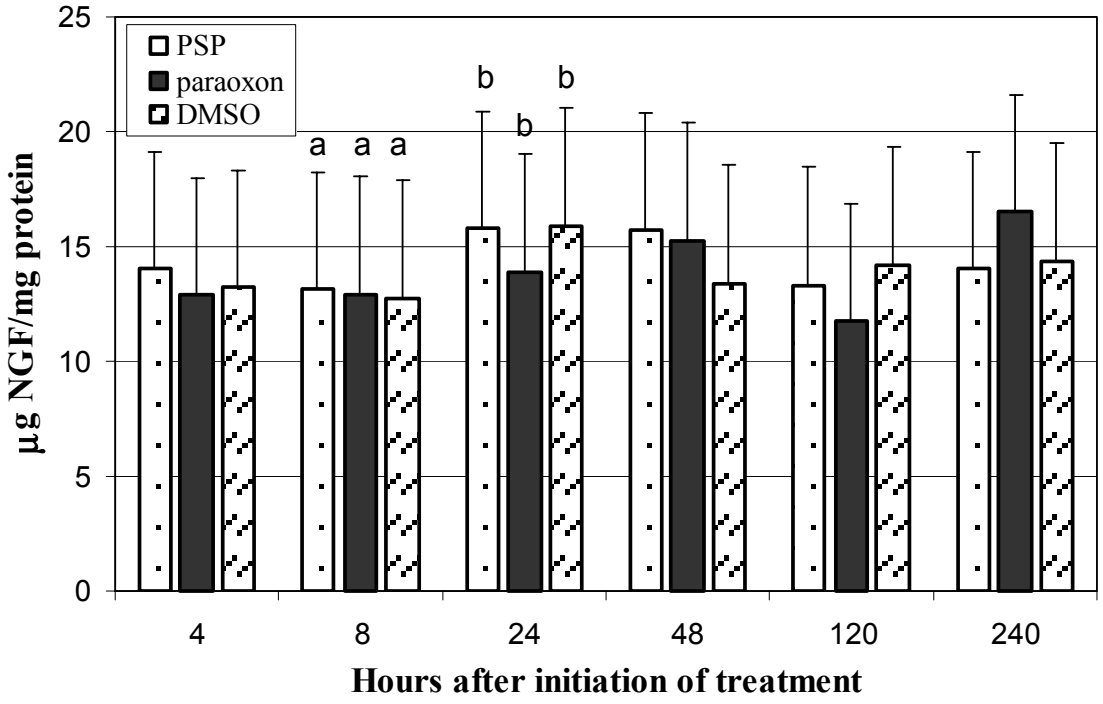
Neurotrophin	Block	N	Mean (mg)	SD (mg)
BDNF	1	51	62.4	6.5
	2	46	63.3	6.3
	3	37	57.8	7.1
NGF/ NT-3	1	51	123.1	9.3
	2	46	120.7	7.1
	3	37	112.2	6.3

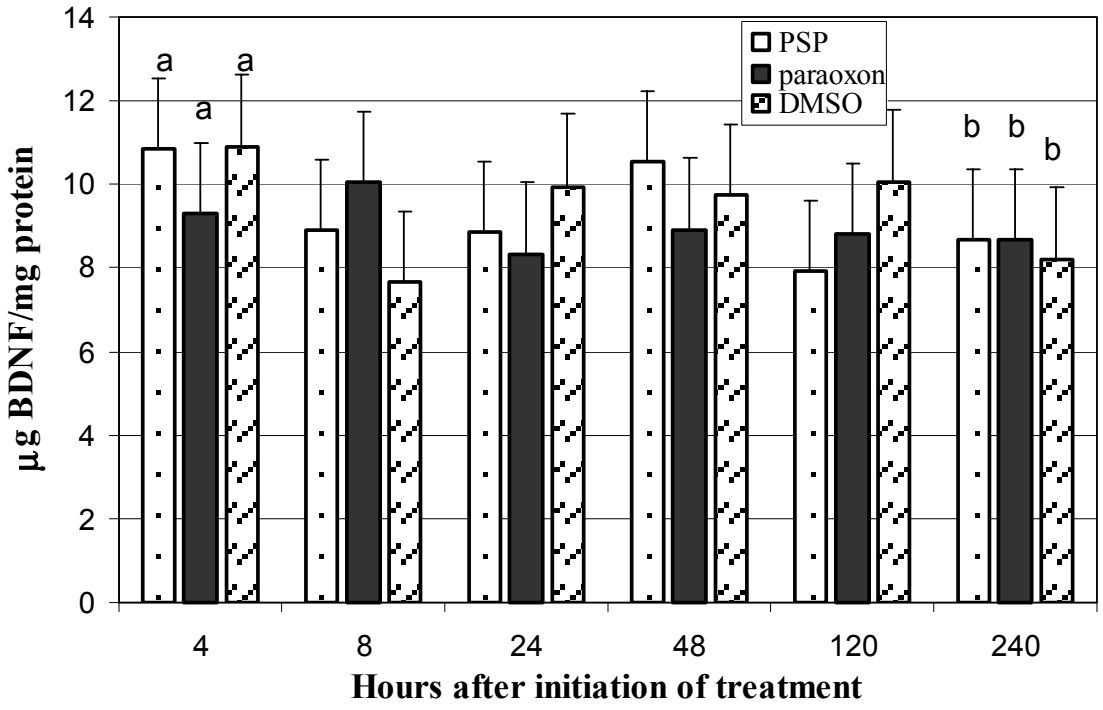
Table I. Spinal cord weights for NGF/NT-3 ELISAs are approximately double that used in BDNF ELISAs due to logistics of ELISA kits. Means and standard deviations of spinal cord weights are given.

Figure 1. Least square means and standard error (represented by error bars) of NGF (a), BDNF (b), and NT-3 (c) concentrations in lumbar spinal cord of chickens treated with a neurotoxic esterase inhibitor (PSP, 2.5 mg/kg) (n = 5-9 per timepoint), a non-neurotoxic esterase inhibitor (paraoxon, 0.10 mg/kg) (n = 4-8 per timepoint), and vehicle (DMSO, 0.5 ml/kg) (n = 4-7 per timepoint) 4-48 hours and 5 (120 hours) and 10 (240 hours) days post-dosing. Treatments with similar letters were not significantly different ($p < 0.05$). Treatments with different letters were different ($p < 0.1$ for NGF and BDNF, $p < 0.05$ for NT-3). Concentrations of NGF were not affected by compound administered ($p = 0.81$) or by time ($p = 0.16$). There was no significant compound*time interaction ($p = 0.74$). Concentrations of neither BDNF nor NT-3 were not affected by compound administered ($p = 0.7$ and 0.86, respectively). There was no significant compound*time interaction on BDNF concentration ($p = 0.19$) or NT-3 concentration ($p = 0.6$).

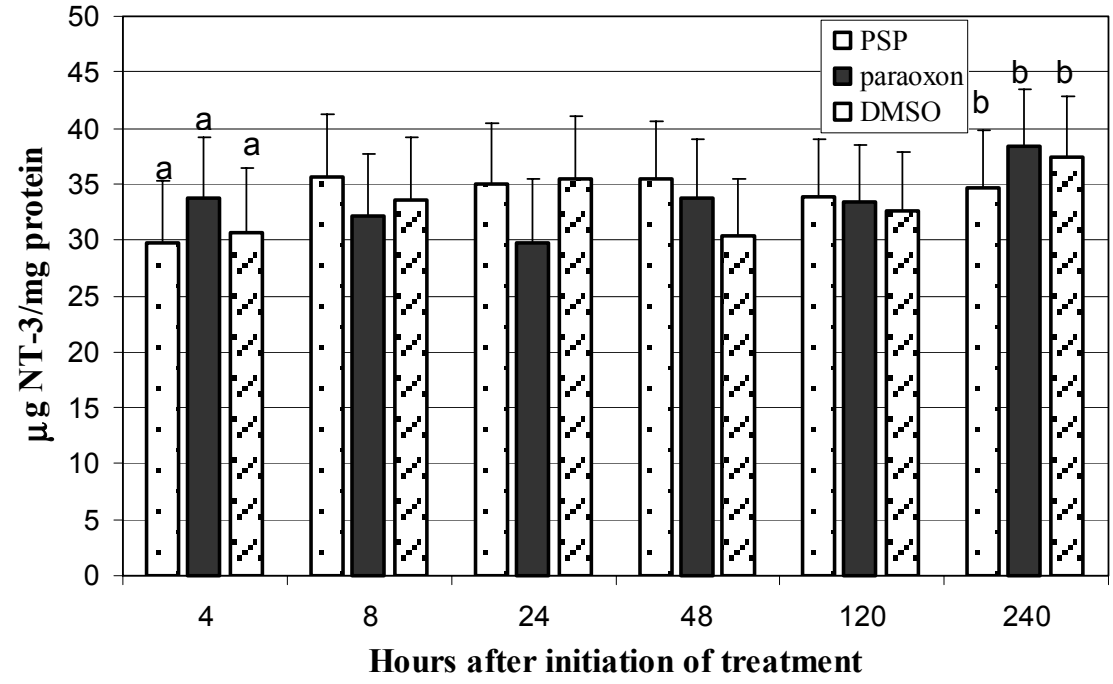
a.



b.


PSP
paraoxon
DMSO
μg BDNF/mg protein
Hours after initiation of treatment
0
2
4
6
8
10
12
14
4
8
24
48
120
240
a
a
a
b
b
b

c.


PSP
paraoxon
DMSO
μg NT-3/mg protein
Hours after initiation of treatment
0
5
10
15
20
25
30
35
40
45
50
4
8
24
48
120
240
a
a
a
b
b
b

Appendix C. Characterization of a Soluble Factor Released in the Lumbar Spinal Cord of Chickens Exposed to Neuropathy-Inducing Organophosphates

ABSTRACT

Organophosphorous (OP) esters are used as insecticides, petroleum additives and modifiers of plastics (Davis and Richardson, 1980), and have also been employed as weapons of terrorism. Exposure to OP compounds that inhibit neurotoxic esterase (NTE) induces a delayed neuropathy (OPIDN) characterized by Wallerian-like degeneration of long axons in certain animals, including humans. Pope *et al*. (1995) found that neurite outgrowth occurred after the addition of spinal cord extracts from chickens with active OPIDN to SH-SY5Y cells. We exposed juvenile chickens to a neuropathy-inducing OP (PSP, 2.5 mg/kg) or an OP compound that does not induce neuropathy (paraoxon, 0.10 mg/kg) intramuscularly. By day 8, all PSP-treated birds demonstrated clinical signs of OPIDN. We sacrificed chickens by pentobarbital overdose at 24 hours, and confirmed NTE inhibition in birds treated with PSP. We separated lumbar spinal cord supernatant into 2 molecular weight groups (<100 kDa, >100 kDa) using ultrafiltration and applied these fractions to SH-SY5Y human neuroblastoma cells. Using digital morphometry, we measured neurite length in order to classify the soluble factor causing the effect of neurite outgrowth on the basis of molecular weight. Our data indicated that neurite length was not affected by the type of OP compound administered or by the molecular weight of a protein fraction. We suggest that ultrafiltration renders the soluble factor ineffective because this factor is actually two or more proteins working in concert to produce the observable effect of neurite outgrowth. Furthermore, it is likely that myelin-associated inhibitory proteins remain intact during ultrafiltration to inhibit neurite outgrowth both *in vitro* and *in vivo*.

INTRODUCTION

Regeneration in the central nervous system (CNS) requires that axons migrate long distances in order to reconnect with an appropriate target (Horner and Gage 2000). Axonal sprouting, observed *in vitro* as neurite outgrowth, is the initial step in the reestablishment of neural pathways (Goldberger *et al.* 1993). Sprouting *in vivo* appears to be a regulated process rather than a random event (Goldberger *et al.* 1993).

After injury to a nerve fiber, Schwann cells in the distal stump of the fiber migrate to form bands of Bünger around distal and proximal ends of the injured fiber (Anton *et al.* 1994). Neurite extension begins at the proximal stump of the nerve (Hagg *et al.* 1993). The axon growth cone contains the machinery necessary for elongation of the axonal sprout (Horner and Gage 2000). The bands of Bünger go on to guide the regenerating axon back to its target (Anton *et al.* 1994).

In organophosphate induced delayed neuropathy (OPIDN), axonal sprouting is the primary means for regeneration. One working hypothesis proposes that a release of growth factors and chemoattractants results in neurite outgrowth and synaptogenesis (Goldberger *et al.* 1993). Research demonstrates that an upregulation of growth factors and chemoattractants directs new sprouts to the appropriate target area. These sprouts do not elongate without the appropriate growth factors present (Schnell *et al.* 1994; Schwab and Bartholdi 1996). However, the exact nature of such factors participating in guiding axonal sprouts remains to be determined (Thallmair *et al.* 1998).

If an axonal sprout encounters inhibitory factors to elongation during reestablishment of a neural path, the sprout loses the ability to respond to growth factors (Cai *et al.* 1999). These factors, which can be soluble or diffusible, are most commonly

found in proteins associated with myelin (McKerracher *et al.* 1994; Schwab and Caroni 1988), but are also found in glia, as well (Li *et al.* 1996; Nieto-Sampedro 1999). Inhibitory proteins likely bind a specific receptor, thereby activating a signaling cascade within the cell that results in retraction, or dying back, of the sprout (Tang *et al.* 1997). Histologically, this appears as a bulb at the end of a neurite (Schwab and Bartholdi 1996). Although inhibitory proteins play an important role in determining the success of the axonal sprouting response, their activity is often balanced by neurite-promoting proteins.

Several studies have demonstrated neurite promoting properties of different proteins in the CNS and determined the molecular weight of the activity. Proteins with neurite-promoting activity demonstrate a broad range of molecular weights (10 kDa to greater than 200 kDa) (Finklestein *et al.* 1988; Goncharova *et al.* 1987; Le and Esquenazi 2002; Needels *et al.* 1986), suggesting that neurite-promoting activity occurs in more than one protein. In fact, well-characterized proteins such as b-FGF and GAP-43 have both been shown to play an integral role in eliciting axonal sprouting *in vivo* (Soto *et al.* 2003).

Pope *et al.* (1995) discovered that treating cultured SH-SY5Y human neuroblastoma cells with soluble extracts from the cervical spinal cord of young chickens exposed to the neuropathy-inducing OP compounds diisopropyl phosphorofluoride (DFP) and PSP resulted in neurite outgrowth similar to that seen when SH-SY5Y cells are treated with nerve growth factor (NGF) (1 μg/ml). They proposed the existence of a soluble factor that induces the neurite outgrowth response. The appearance of this factor suggests that there is an initial mechanism in the exposed animal which attempts to counteract the neurodegenerative potential of the toxicant. We have confirmed that this

effect occurs 24 hours after PSP administration to juvenile chickens and did a crude characterization of the factor causing this effect on the basis of molecular weight as a preliminary experiment that will allow further classification into a family of proteins with known neurotrophic properties.

MATERIALS AND METHODS

Experimental Design and Analysis

We used a complete randomized block design. The treatment factor was compound (supernatant with proteins greater than 100 kDa, supernatant with proteins less than 100 kDa, 10 ng/ml NGF, and medium). The study was divided into 2 whole blocks and 5 sub-blocks—block A was compound administered to birds in a series of 3 blocks (PSP, paraoxon), and block B was protein molecular weight applied to cells (>100 kDa, <100 kDa). We acquired 5-6 images within a block A*block B combination with a total of 20-35 neurite measurements per combination. Using the MIXED procedure in SAS (version 8.2, SAS Institute, Cary, NC), we performed an analysis of variance (ANOVA) for time and compound main effects and their interactions.

In Vivo Treatments

The sample studied consisted of 140 mixed-sex 10-week-old White Leghorn (*Gallus gallus*) chickens. We selected young chickens based on a previous study that suggested the soluble factor is present in birds of this age (Pope *et al.* 1995). All chickens were housed together in a temperature-controlled environment at 23^{o}C with free access to water and feed through 10 weeks of age. Three days before treatment, we moved chickens to cages for acclimation. On day 0, we administered 2.5 mg/kg PSP (Oryza Laboratories, Inc., Chelmsford, MA) to chickens by intramuscular injection. The dose of 2 mg/kg is sufficient to cause OPIDN in adult hens (Jortner and Ehrich 1987).

Using an established procedure, we observed chickens daily for clinical signs from days 1-10 for chickens sacrificed 10 days post-exposure (Dyer *et al.* 1991; Ehrich *et*

al. 1993; Jortner and Ehrich 1987; Pope *et al.* 1995). This confirmed the onset of OPIDN. Signs of ataxia were recorded as: 0 = no clinical signs; 1-2 = slight, infrequent hindlimb incoordination; 3-4 = moderate but definite incoordination; and, 5-6 = frequent difficulties in standing erect and walking.

We euthanized chickens at 24 hours post-exposure by intra-venous pentobarbital overdose and immediately removed the lumbar spinal cord. After removing the glycogen body, we wrapped the lumbar spinal cord in aluminum foil, placed it in a labeled scintillation tube, and froze it on dry ice. After all sacrifices for a particular timepoint, we froze the spinal cords at –80^{o}C.

Within 2 weeks of extraction, we weighed the entire spinal cord and suspended one-third of the cord 1:10 (w:v) in cold 0.03 M PBS. The wet weight of spinal ranged from 30-340 mg in block 1, 50-250 mg in block 2, and 70-3100 mg in block 3 (Table I). After homogenizing lumbar spinal cord using an ultrasonic homogenizer (W-225R model, Heat Systems-Ultrasonics, Inc., Farmingdale, NY), we centrifuged the homogenate at 4^{o}C at 48000 x g for 10 minutes. We removed the supernatants, placed each respective supernatant in a labeled 2 ml microcentrifuge tube, and maintained them at –80^{o}C until assayed.

Cell Culture and Differentiation

We maintained SH-SY5Y human neuroblastoma cells (passages 16-18, ATCC, Rockville, MD) at 37^{o}C in a humidified atmosphere with 5% CO_2. We cultured cells with Ham F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 1% (v/v) antibiotic/antimycotic solution

(Sigma, St. Louis, MO), and 1% (v/v) L-glutamine (Mediatech, Herndon, VA). We cultured cells in 75 cm^2 flasks (Corning, Acton, MA) containing 15 ml medium, replacing the medium every 2-3 days. We passaged cells every 7-10 days after using 0.25% trypsin-EDTA (Invitrogen) to detach them from the flask. At 80% confluency, we treated the cells with 10^{-7} M retinoic acid (RA; Sigma) in fresh medium for 3 days. After placing three 13 mm collagen-coated discs (VWR International, West Chester, PA) in each well of 6-well plates (Fisher Scientific, Pittsburgh, PA), we plated cells at a concentration of 1 x 10^4 cells/ml and incubated overnight, prior to treating with supernatants the following day.

In Vitro Treatments

In order to separate each supernatant into two groups based on molecular weight, greater than 100 kDa and less than 100 kDa, we ultrafiltered 500 µl of the supernatants of birds exposed to PSP and paraoxon using Microcon YM-100 filters (Millipore, Bedford, MA). After cell differentiation, we sterile filtered each molecular weight group of the supernatants with a 0.2 µm filter and treated cells with 3 ml of a 1:250 dilution. For a final concentration of 10 ng/ml NGF, we diluted a stock solution of 10 µg/ml NGF in medium. After treating, we incubated the cells for 5 days at 37°C in 5% CO_2. Because of the low cell density, we did not need to change the medium over the course of the 5 days.

Cell Fixation

After 5 days, we washed each well with 1X PBS prior to fixing the cells in 10% buffered formalin for 30 minutes. We washed each well with 1X PBS again and then stained cells with toluidine blue for 4 minutes. After rinsing the wells with distilled H_2O, we mounted each coverslip to a slide using Fluoromount G and dried the slides overnight.

Neurite Outgrowth Measurement

We randomly selected 2 areas on each disc that contained at least 2 cells and photographed the image with a Nikon 990 digital camera (Nikon, Inc., Melville, NY) mounted on a Nikon E600 microscope (Nikon Instruments, Inc., Melville, NY). For each image, we selected 1-2 cells, and traced and measured the neurites using the Image Processing Tool Kit (v.5, Reindeer Graphics, Asheville, NC). We classified a neurite as an extension from the cell body that was less than half the width of the cell body. Using the MIXED procedure in SAS, we performed an ANOVA for time and compound main effects and their interactions.

RESULTS

Neurite length was not affected by the compound administered ($p = 0.31$) or by the molecular weight of proteins applied to the cells ($p = 0.69$). There was not a significant effect due to the interaction of compound administered and molecular weight ($p = 0.98$) (Figure 7.1).

Preliminary qualitative experiments using unseparated lumbar spinal cord supernatent applied to SH-SY5Y cells demonstrated that supernatent of birds treated with PSP produced neurites approximately twice as long as supernatent of birds treated with DMSO. Lumbar spinal cord supernatent from birds exposed to 2.5 mg/kg PSP for 24 hours demonstrated neurite outgrowth more similar in length to the positive control cells (1 μg/ml NGF) than to negative control cells (medium only). However, the length of neurite outgrowth in cells exposed to lumbar spinal cord supernatents of DMSO-treated birds was more similar to negative control cells than to positive control cells. These data compare favorably to data from the Pope *et al.* (1995) study.

DISCUSSION

This study demonstrates that a proposed soluble factor responsible for neurite outgrowth observed in SH-SY5Y cells after exposure to lumbar cord supernatent of chickens treated with a neuropathic OP compound (Pope *et al.* 1995) cannot be separated by ultrafiltration on the basis of molecular weight at the 100 kDa range and still produce neurite outgrowth. Therefore, we propose that the molecular weight of the soluble factor is greater than 100 kDa and that this factor is likely two or more proteins acting in concert to produce neurite outgrowth. Separation of the lumbar spinal cord supernatent into 2 groups (greater than and less than 100 kDa) may denature the soluble factor such that the neurite outgrowth effect observed by Pope *et al.* (1995) and this effect does not occur. Methods of denaturation may include disassembly from a polymeric to a monomeric form, dephosphorylation of essential bonds, and/or breaking of hydrogen bonds within the soluble factor (Lodish *et al.* 2000). Protein aggregation has also been shown to occur during the ultrafiltration process through protein adsorption onto the membrane surface or through intermolecular exchange of disulfide bonds (Maruyama *et al.* 2001). Finally, pathology slides revealed that very few nerves were damaged after PSP exposure. Consequently, healthy nerves could mask the effect induced by damaged nerves.

It is possible that while the soluble factor was denatured during ultrafiltration, some or all inhibitory factors of myelin were not. Homogenization of the lumbar spinal cord almost certainly disrupts myelin, causing a release of inhibitory factors associated with myelin. Consequently, these myelin-associated proteins may have contributed to the inhibition of neurite outgrowth. For example, research demonstrates that two unnamed

proteins (35 kDa and 250 kDa), as well as myelin-associated glycoprotein (MAG, ~100 kDa) are primary components of myelin and account for most of the inhibitory activity of myelin (Caroni and Schwab 1988; McKerracher *et al.* 1994; Tang *et al.* 1997). The molecular weights of these three proteins fall within the two molecular weight groups isolated in this study.

Inhibitory neurite outgrowth properties of myelin may override the ability of the soluble factor to elicit neurite outgrowth, especially if the neurite-promoting soluble factor is denatured during the isolation process. Some suggest that there is a balance between positive (neurite-promoting) and negative (inhibitory) influences on a cell. Inhibition of neurite outgrowth likely occurs when negative signals are stronger than postive signals (Tang *et al.* 1997). When the soluble factor is separated according to molecular weight, inhibitory signals may become stronger than neurite-promoting signals. Consequently, the effect that we and Pope *et al.* (1995) observed may be abolished.

Finally, we suggest that the soluble factor proposed by Pope *et al.* (1995) may not be one factor, but two or more that work in concert to produce neurite outgrowth. Separating the proteins on the basis of molecular weight may separate these factors, eliminating the effect of neurite outgrowth that they cause when working together. Other neurite-promoting proteins have been found to work together, including bFGF and GAP-43. After nerve injury, bFGF upregulates sythesis and axonal distribution of GAP-43 (Soto *et al.* 2003). There is no research investigating the effect these two proteins have on promoting neurite outgrowth if they are separated from each other. We suggest that there is a similar feedback mechanism that occurs with bFGF and GAP-43 between two

or more proteins, resulting in neurite outgrowth *in vitro* after neuropathic OP exposure *in vivo*.

We suggest that the molecular weight of the soluble factor proposed by Pope *et al.* (1995) that causes neurite outgrowth after neuropathic OP exposure is greater than 100 kDa. Furthermore, this soluble factor is likely two or more proteins acting in concert, that together have a molecular weight greater than 100 kDa. Because this protein(s) may be separated into monomeric form after ultrafiltration, myelin-associated proteins with neurite-inhibitory signals may then be stronger than neurite-promoting signals that would otherwise be induced by the soluble factor. Consequently, we observed a hindrance of neurite outgrowth after ultrafiltration.

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Table A. Average wet lumbar spinal cord weight used in neurite outgrowth assay.

Compound	N	Mean (mg)	SD (mg)
PSP	8	172.5	53.9
Paraoxon	7	137.1	55.6

Means and standard deviations of cord weights are given.

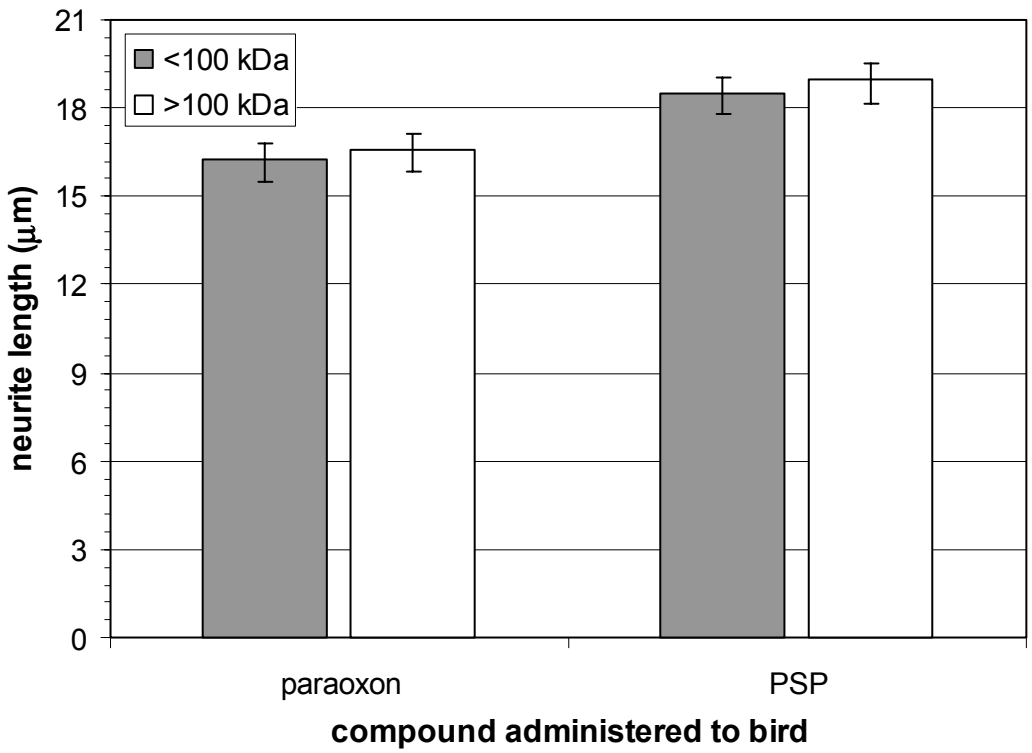

Figure A. Least square means and standard error (represented by error bars) of neurite length in SH-SY5Y cells treated with lumbar spinal cord supernatant from PSP or paraoxon pre-treated birds after cells were exposed to supernatant proteins greater than 100 kDa in size and less than 100 kDa in size (n = 392 for paraoxon-treated birds with SH-SY5Y cells exposed to proteins less than 100 kDa; n = 368 for paraoxon-treated birds with SH-SY5Y cells exposed to proteins greater than 100 kDa; n = 449 for PSP-treated birds with SH-SY5Y cells exposed to proteins less than 100 kDa; n = 438 for PSP-treated birds with SH-SY5Y cells exposed to proteins greater than 100 kDa). There was no significant effect on neurite length due to compound administered ($p = 0.31$), molecular weight of proteins ($p = 0.69$), or an interaction of compound and molecular weight ($p = 0.98$).

VITA

Melinda Pomeroy-Black was born on September 3rd, 1973 in Knoxville, Tennessee. After completing high school in Nashville, she attended Rhodes College in Memphis, Tennessee. She completed her B.A. in Biology in 1995 and, not knowing what she would do for a career, spent four years working in animal hospitals as a veterinary technician in Chattanooga, Tennessee. In 1999, she came to Virginia-Maryland Regional College of Veterinary Medicine, where she completed a M.S. in Veterinary Medical Sciences in 2001. The time spent working on her M.S. was important in her realization to pursue a faculty position at a small liberal arts school. Consequently, she enrolled in the doctoral program and investigated the early mechanisms of organophosphate neurotoxicity. She currently lives in LaGrange, Georgia with her husband and serves as an Assistant Professor of Biology at LaGrange College.