

**SPINAL CORD GENE EXPRESSION CHANGES IN THE  
CHICKEN (*GALLUS GALLUS*) MODEL OF PHENYL SALIGENIN  
PHOSPHATE INDUCED DELAYED NEUROTOXICITY**

By Jonathan Fox

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Veterinary Medical Sciences

Approved

---

Marion Ehrich (Co-chairperson)

---

Bernard Jortner (Co-chairperson)

---

Bradley Klein

---

Cornelis van der Schyf

---

Peter Kennelly

---

William Huckle

April, 2002

Blacksburg, Virginia

Keywords: phenyl saligenin phosphate, neurotoxicity, gene expression, axon, neurodegeneration, organophosphorus.

## **ABSTRACT**

# **SPINAL CORD GENE EXPRESSION CHANGES IN THE CHICKEN (*GALLUS GALLUS*) MODEL OF PHENYL SALIGENIN PHOSPHATE INDUCED DELAYED NEUROTOXICITY**

Jonathan Fox

Some organophosphorus (OP) esters induce a central-peripheral distal axonopathy called organophosphorus ester-induced delayed neurotoxicity (OPIDN). In the chicken model neurological deficits and microscopic lesions develop 7-21 days after exposure. Neurotoxic esterase (NTE) is thought to be the initial target in OPIDN. Evidence indicates that neuropathic OP esters have to bind NTE and chemically ‘age’ for OPIDN induction. It was hypothesized that phenyl saligenin phosphate (PSP), a neuropathic OP ester that essentially irreversibly inhibits NTE as it undergoes the chemical aging process, results in changes in spinal cord gene expression that do not occur with phenylmethylsulfonyl fluoride (PMSF), a non-neuropathic compound that inhibits NTE without aging. This hypothesis was tested in *Gallus gallus* in experiments designed to detect differences in spinal cord gene expression between PSP, PMSF and vehicle-treated birds 24 hours after exposure. Two approaches were used. Targeted display was developed and used to screen approximately 15000 gel bands. Three candidate genes were identified by targeted display. One, designated P1 has 100% homology with expressed sequence tag pgp1n.pk010.m23, another, P2, is homologous to human KIAA1307, and a third,

P3, is unidentified. Northern blotting was used to measure spinal cord expression of  $\alpha$ -tubulin and other genes previously reported to be differentially expressed following exposure to di-isopropyl phosphorofluoridate, another agent causing OPIDN. Only expression of  $\alpha$ -tubulin was altered in PSP-treated hens. Time course experiments were undertaken to determine spinal cord expression changes of P1, P2, P3 and  $\alpha$ -tubulin transcripts at 12, 24, 36 and 48 hours post-exposure. Findings indicated decreases and increases, respectively, of P1 (22%, p=0.0011) and P2 (26%, p=0.0055) transcripts at 12 hours in PSP treated hen spinal cord compared to DMSO controls. A ~2.5 kb  $\alpha$ -tubulin transcript was decreased across most time points with maximum change at 48 hours (33%, p=0.0479); an ~4.5 kb  $\alpha$ -tubulin transcript was upregulated at 12 hours (38%, p=0.0125) and down regulated at 48 hours (28%, p=0.0576). Responses to PMSF were different than responses to PSP. Spinal cord *in-situ* hybridization experiments revealed, 1.) mainly neuronal expression of P1, P2 and  $\alpha$ -tubulin transcripts, and, 2.) decreased expression of neuronal P1 and  $\alpha$ -tubulin transcripts at 12 and 48 hours, respectively. Results indicate that PSP can induce changes in gene expression distinct from those induced with the non-neuropathic NTE inhibitor, PMSF. However, expression changes were low in frequency and magnitude, and their mechanistic importance remains to be fully established.

## **ACKNOWLEDGEMENTS**

I would like to acknowledge the following people without which I would not have been able to complete this work. Firstly, members of my committee: Drs. Jortner, Ehrich, Huckle, Kennelly, Klein, and van der Schyf for their excellent advise and input in various ways throughout this work. Special mentions to Dr. Jortner for his continued help and financial support; to Dr. Ehrich for her encouragement and drive in helping this whole dissertation project through to completion; and, to Dr. Huckle for his many useful suggestions. Thanks to Daniel Ward (DW) and Dr. David Barber (DB) for the many helpful discussions; more specifically to DW for help in experimental design, and DB for assistance in overcoming numerous technical problems. Special thanks also to Sandy Perkins for assistance with the *in-situ* hybridization, and to Linda Correll and Lisa Flory for their assistance with bird sacrifices, and for performing esterase assays. Additionally, thanks to Ka Lee Farris, Margaret Telsch and Jonathan Hinckley who also helped with sacrifices. Thanks to Steven Salmon and Dr. Calvin Larsen for managing the birds used in this study. Finally, I would like to thank the Virginia-Maryland Regional College of Veterinary Medicine for stipend support.

## **DECLARATION OF WORK PERFORMED**

I declare that I, Jonathan H. Fox, performed all the work described herein, except for the brain esterase assays, DNA sequencing, tissue sectioning for *in-situ* hybridization and tissue processing for electron microscopy.

## TABLE OF CONTENTS

<b>ABSTRACT.....</b>	<b>ii</b>
<b>ACKNOWLEDGEMENTS.....</b>	<b>iv</b>
<b>DECLARATION OF WORK PERFORMED.....</b>	<b>v</b>
<b>TABLE OF CONTENTS.....</b>	<b>vi</b>
<b>TABLE OF FIGURES.....</b>	<b>viii</b>
<b>TABLE OF TABLES.....</b>	<b>x</b>
<b>LIST OF ABBREVIATIONS.....</b>	<b>xi</b>
<b>CHAPTER 1: Introduction.....</b>	<b>1</b>
HYPOTHESIS AND SPECIFIC AIMS .....	1
REVIEW OF LITERATURE .....	5
<i>Introduction and history</i> .....	5
<i>Clinical findings</i> .....	5
<i>Structural abnormalities</i> .....	6
<i>Mechanistic investigations</i> .....	7
<b>CHAPTER 2: Identification of Differentially Expressed mRNA Transcripts in the Spinal Cord of <i>Gallus Gallus</i> 24 Hours Post-Exposure to Phenyl Saligenin Phosphate.....</b>	<b>24</b>
INTRODUCTION .....	24
SPECIFIC QUESTIONS AND RATIONALE .....	28
EXPERIMENTAL DESIGN AND ANALYSIS .....	35
MATERIAL AND METHODS .....	37
<i>Targeted display method overview and development</i> .....	37
<i>Treatments</i> .....	42
<i>Brain esterase measurements</i> .....	42
<i>Targeted display</i> .....	42
<i>Northern blotting</i> .....	51
RESULTS .....	54
<i>Clinical signs</i> .....	54
<i>Brain esterase activities</i> .....	54
<i>Targeted display</i> .....	54
<i>Northern blotting</i> .....	60
DISCUSSION .....	64
<b>CHAPTER 3: Temporal Changes in Phenyl Saligenin Phosphate-Induced Spinal Cord Expression of <math>\alpha</math>-Tubulin, P1, P2 (KIAA1307) and P3 Transcripts.....</b>	<b>69</b>
INTRODUCTION .....	69
SPECIFIC QUESTION AND RATIONALE.....	72
EXPERIMENTAL DESIGN AND ANALYSIS .....	75
MATERIALS AND METHODS.....	76
RESULTS .....	77
DISCUSSION .....	85
<b>CHAPTER 4: Cellular and Differential Cellular Spinal Cord Expression of <math>\alpha</math>-Tubulin, P1 and P2 (KIAA1307) in Phenyl Saligenin Phosphate Treated <i>Gallus Gallus</i> .....</b>	<b>90</b>
INTRODUCTION .....	90

<b>SPECIFIC QUESTIONS AND RATIONALE.....</b>	91
<b>EXPERIMENTAL DESIGN .....</b>	94
<b>MATERIALS AND METHODS.....</b>	95
<i>Treatments and perfusion.....</i>	95
<i>In-situ hybridization .....</i>	95
<b>RESULTS .....</b>	100
<b>DISCUSSION .....</b>	115
<b>CHAPTER 5: General Discussion, Future Directions and Conclusion.....</b>	<b>118</b>
<b>GENERAL DISCUSSION .....</b>	118
<b>FUTURE DIRECTIONS .....</b>	126
<b>CONCLUSION.....</b>	129
<b>LITERATURE CITED.....</b>	<b>130</b>
<b>APPENDIX A: TARGETED DISPLAY PRIMER DESIGN.....</b>	<b>157</b>
<b>PRIMER DESIGN .....</b>	157
<b>GENE DETECTION RATES USING TARGETED DISPLAY PRIMERS.....</b>	160
<b>APPENDIX B: DNA SEQUENCE OF P1, P2 AND P3.....</b>	<b>163</b>
<b>APPENDIX C: MORPHOLOGIC ANALYSIS OF ADULT WHITE LEGHORN SPINAL MOTOR NEURONS .....</b>	<b>165</b>
<b>VITA .....</b>	<b>170</b>

## TABLE OF FIGURES

FIGURE 1. DISTAL DEGENERATION OF SPINAL TRACT AXONS IN OPIDN .....	9
FIGURE 2. ANATOMY OF <i>GALLUS GALLUS</i> LUMBO-SACRAL PLEXUS AND SPINAL CORD.....	33
FIGURE 3. STRUCTURES OF PHENYL SALIGENIN PHOSPHATE (PSP), PHENYLMETHYLSULFONYL FLUORIDE (PMSF), AND DIMETHYL SULFOXIDE (DMSO) .....	34
FIGURE 4. <i>GALLUS GALLUS</i> BRAIN ESTERASE ACTIVITIES 24 HOURS POST DOSING WITH PSP, PMSF AND DMSO .....	56
FIGURE 5. TARGETED DISPLAY AUTORADIOGRAPH.....	57
FIGURE 6. PUTATIVE DIFFERENTIALLY EXPRESSED GENES.....	58
FIGURE 7. PROCESSING DIFFERENTIALLY EXPRESSED GENES .....	59
FIGURE 8. EXPRESSION OF SPINAL CORD $\alpha$ -TUBULIN mRNA (2.5 KB) 24 HOURS POST EXPOSURE TO PSP, PMSF, AND DMSO.....	62
FIGURE 9. EXPRESSION OF SPINAL CORD P1 mRNA 24 HOURS POST EXPOSURE TO PSP, PMSF, AND DMSO.....	63
FIGURE 10. RESPONSE OF $\alpha$ -TUBULIN (~2.5 KB) TRANSCRIPT LEVELS IN SPINAL CORD TO TREATMENT WITH NEUROPATHIC ESTERASE INHIBITOR (PSP), NON- NEUROPATHIC ESTERASE INHIBITOR (PMSF), AND VEHICLE (DMSO).....	79
FIGURE 11. RESPONSE OF $\alpha$ -TUBULIN (~4.5 KB) TRANSCRIPT LEVELS IN SPINAL CORD TO TREATMENT WITH NEUROPATHIC ESTERASE INHIBITOR (PSP), NON- NEUROPATHIC ESTERASE INHIBITOR (PMSF), AND VEHICLE (DMSO).....	80
FIGURE 12. RESPONSE OF P1 TRANSCRIPT LEVELS IN SPINAL CORD TO TREATMENT WITH NEUROPATHIC ESTERASE INHIBITOR (PSP), NON-NEUROPATHIC ESTERASE INHIBITOR (PMSF), AND VEHICLE (DMSO) .....	81
FIGURE 13. RESPONSE OF P2 TRANSCRIPT LEVELS IN SPINAL CORD TO TREATMENT WITH NEUROPATHIC ESTERASE INHIBITOR (PSP), NON-NEUROPATHIC ESTERASE INHIBITOR (PMSF), AND VEHICLE (DMSO) .....	82

FIGURE 14. RESPONSE OF P3 TRANSCRIPT LEVELS IN SPINAL CORD TO TREATMENT WITH NEUROPATHIC ESTERASE INHIBITOR (PSP), NON-NEUROPATHIC ESTERASE INHIBITOR (PMSF), AND VEHICLE (DMSO) .....	83
FIGURE 15. LUMBOSACRAL SPINAL CORD $\alpha$ -TUBULIN EXPRESSION.....	104
FIGURE 16. NEURAL TISSUE P1 TRANSCRIPT EXPRESSION.....	105
FIGURE 17. EXPRESSION OF P2 TRANSCRIPT IN SPINAL CORD ANTERIOR HORN .....	106
FIGURE 18. ALPHA-TUBULIN TRANSCRIPT EXPRESSION IN LUMBOSACRAL LATERAL ANTERIOR HORN 48 HOURS AFTER EXPOSURE TO PSP OR DMSO.....	107
FIGURE 19. P1 TRANSCRIPT EXPRESSION IN LUMBOSACRAL LATERAL ANTERIOR HORN 12 HOURS AFTER EXPOSURE TO PSP OR DMSO.....	108
FIGURE 20. NEURAL TISSUE P1 TRANSCRIPT EXPRESSION CONTINUED.....	109
FIGURE 21. EXTRANEURAL P2 TRANSCRIPT EXPRESSION .....	110
FIGURE 22. EXTRANEURAL P2 TRANSCRIPT EXPRESSION CONTINUED.....	112
FIGURE 23. COMPARISON OF EXPECTED GENE DETECTION RATES BY 30 HUMAN STATISTICALLY DESIGNED PRIMER PAIRS AND 30 RANDOMLY SELECTED OCTA-NUCLEOTIDE PRIMER PAIRS IN DIFFERENT SPECIES.....	162
FIGURE 24. SPINAL CORD MOTOR NEURON CYTOPLASMIC GRANULES .....	167
FIGURE 25. ULTRA-STRUCTURAL FEATURES OF CYTOPLASM OF MOTOR NEURON .....	168
FIGURE 26. ULTRA-STRUCTURAL FEATURES OF CYTOPLASMIC GRANULES .....	169

## **TABLE OF TABLES**

TABLE 1. TARGETED DISPLAY PRIMERS USED IN THIS STUDY .....	40
TABLE 2. SUMMARY OF GENE EXPRESSION DATA.....	84
TABLE 3. EXPRESSION OF P1 TRANSCRIPT OUTSIDE CENTRAL NERVOUS SYSTEM....	113
TABLE 4. EXPRESSION OF P2 TRANSCRIPT OUTSIDE CENTRAL NERVOUS SYSTEM....	114
TABLE 5. FULL SET OF TARGETED DISPLAY PRIMERS.....	158

## LIST OF ABBREVIATIONS

- A or a = adenosine
- ACHE = acetylcholinesterase
- ATP = adenosine tri-phosphate
- bp = base pair
- C or c = cytosine
- $^{\circ}\text{C}$  = degrees centigrade
- CaM-Kin II = calcium-calmodulin protein kinase II
- CBE = carboxylesterase
- CNS = central nervous system
- $\Delta\psi_m$  = mitochondrial membrane potential
- DD = differential display
- D E P C = diethyl pyrocarbonate
- DFP = di-isopropyl-phosphorofluoridate
- DMSO = dimethyl sulfoxide
- DRG = dorsal root ganglion
- DBDCVP = di-n-butyl-2, 2-dichlorovinyl phosphate
- EPA = Environmental Protection Agency
- EST = expressed sequence tag
- G or g = guanosine
- x g = multiples of unit of gravitational force on earth
- GFAP = glial fibrillary acidic protein
- GG = *Gallus gallus* (domestic fowl, often simply called ‘chicken’)
- GLM = general linear model
- H I V = human immunodeficiency virus
- IP3 = inositol tri-phosphate
- ISH = *in-situ* hybridization
- iv = intravenous
- im = intramuscular
- kb = kilobases

- LB = Luria-bertani
- LS = least square
- MPST = medial pontine spinal tract
- NGS = normal goat serum
- NTE = neurotoxic esterase, or neuropathy target esterase
- ODC = ornithine decarboxylase
- OP = organophosphorus
- OPIDN = organophosphorus induced delayed neurotoxicity
- ORF = open reading frame
- PCR = polymerase chain reaction
- P M S F = phenylmethylsulfonyl fluoride
- PNS = peripheral nervous system
- PSP = phenyl saligenin phosphate
- SE = standard error of the mean
- SSC = sodium chloride / sodium citrate buffer
- *Sws* = *Drosophila melanogaster* homologue of NTE
- T or t = thymidine
- TD = targeted display
- TOTP = tri-*ortho*-tolyl phosphate
- TSP = tolyl saligenin phosphate
- WLH = White Leghorn
- UTR = untranslated region of mRNA

## **CHAPTER 1: Introduction**

### **Hypothesis and Specific Aims**

Some organophosphorus (OP) esters, for example, phenyl saligenin phosphate (PSP), induce nervous system injury characterized predominantly by degeneration of distal regions of long large diameter axons within the central and peripheral nervous system in susceptible mammals and birds. This is called organophosphorus-induced delayed neurotoxicity (OPIDN). In the adult hen (*Gallus gallus*) clinical and morphologic alterations consistent with axon injury occur 7-21 days post-exposure. Several weeks later nerve lesions are virtually resolved, however, there is persistent axon loss and gliosis in the spinal cord.<sup>1, 2</sup>

Inhibition of neurotoxic esterase (NTE, also known as neuropathy target esterase), a serine carboxylesterase, occurs 2-48 hours after OP exposure. This enzyme is thought to be the primary molecular target in OPIDN. NTE inhibition alone, however, is not sufficient to induce OPIDN. Neurotoxic esterase inhibition by covalent binding of the OP ester to its active site serine followed by chemical aging of the bound OP compound is necessary before OPIDN can occur.<sup>3, 4</sup> Aging is characterized by loss of a side group of the covalently bound OP ester, leaving behind a negative charge. Once this occurs, the OP ester cannot be removed from the enzyme. This makes the covalent binding essentially irreversible. Substances that inhibit NTE but do not age, such as phenylmethylsulfonyl fluoride (PMSF), do not induce OPIDN even under high or repeated dosing regimes.<sup>4-6</sup>

Very little is known concerning the presumed cascade of events that occurs between NTE inhibition and aging, and the development of neurological deficits and morphological lesions. Evidence has been provided from one laboratory to indicate that in spinal cord gene expression changes occur during this clinically silent phase of OPIDN. In chickens, exposure to di-isopropyl-phosphorofluoridate (DFP) has been reported to induce alterations in the expression of spinal cord transcripts for the  $\alpha$ -subunit of calcium-calmodulin kinase II,<sup>7</sup> neurofilaments,<sup>8</sup> vimentin,<sup>9</sup> glial fibrillary acidic protein,<sup>9</sup>  $\alpha$  tubulin,<sup>10</sup> *c-fos*<sup>11</sup> and *c-jun*.<sup>12</sup> Studies *in-vitro* in our laboratory demonstrated that spinal cord extracts taken from chickens 24 hours after PSP exposure were able to promote neuritic outgrowth in human SH-SY5Y neuroblastoma cells,<sup>13</sup> providing indirect evidence for upregulation of a neurotrophic factor(s). Glucocorticoids act through altering gene expression<sup>14</sup> and are also able to affect responses to neuropathy inducing OP esters. For example, corticosterone pre-treatment of hens ameliorated the adverse effect of PSP on A $\beta$  compound action potentials,<sup>15</sup> and modulated the clinical response to tri-*ortho*-cresyl phosphate.<sup>16</sup> Preliminary work in this laboratory has shown that there is down regulation of spinal mRNA for both  $\mu$  and m calpain on day 1 and upregulation on day 2 after PSP exposure in hens (personal communication, Dr. D. Barber). Evidence for gene expression changes in OPIDN have, therefore, been developed using a variety of approaches. However, whether these expression changes are of

mechanistic significance, epiphenomena, or protective remains to be determined. Neuropathic OP esters bind to a number of esterases and proteases in addition to NTE. Therefore, dissecting gene expression changes that may be related to neuropathic effects of OP ester on NTE, rather than non-neuropathic effects on NTE, or other hydrolases, may be necessary to help determine mechanistic relevance of specific gene expression changes.

The following hypotheses were proposed and form the basis for this dissertation.

- 1. Changes in gene expression occur in adult hen (*Gallus gallus*) lumbosacral spinal cord in the pre-clinical phase of OPIDN following PSP-induced NTE inhibition/aging.**
- 2. None, or a limited subset of the above changes do not occur following exposure to equivalent doses of the non-neuropathic NTE inhibitor, PMSF.**

These hypotheses are based on the assumption that NTE is the primary molecular target in OPIDN. There is considerable evidence that this is the case (see literature review). Secondly, this hypothesis suggests that there may be gene expression changes resulting from NTE inhibition/aging that do not occur following NTE inhibition alone. This hypothesis does not address the issue of whether gene expression changes are of mechanistic significance, and lead to axonal degeneration. This possibility is discussed but is not addressed experimentally. The following Specific Aims were proposed to test the hypotheses.

**Specific Aim #1 (Chapter 2):** To identify differentially expressed mRNA transcripts in lumbo-sacral spinal cord of adult hens (*Gallus gallus*) 24 hours after exposure to equivalent NTE inhibiting doses of PSP, PMSF, or DMSO vehicle.

**Specific Aim #2 (Chapter 3):** To identify temporal changes in mRNA expression of genes identified in Specific Aim #1 through the clinically silent phase of OPIDN in PSP and PMSF, or DMSO vehicle exposed adult birds.

**Specific Aim #3 (Chapter 4):** To determine the spinal cord cell types that express and differentially express the gene transcripts identified in Specific Aim #1.

## **REVIEW OF LITERATURE**

### **Introduction and history**

Degeneration of the distal extremities of axons within the central and peripheral nervous system is a feature of exposure of susceptible mammals and hens to a variety of toxicants including hexane, acrylamide, some organophosphorus (OP) esters, *p*-bromophenylacetylurea, and carbon disulfide.<sup>17-22</sup> Compared to other neurodegenerative disorders these central-peripheral distal axonopathies have received comparatively little attention, and their mechanisms are generally poorly understood.

Organophosphorus induced delayed neurotoxicity (OPIDN) was first reported in 1899 in six tuberculosis patients treated with phosphocreosote contained 15% (v/v) tri-*ortho*-tolyl phosphate (TOTP).<sup>23</sup> Since then, there have been several outbreaks in man and animals resulting mainly from contamination of food or drink.<sup>24-28</sup> The largest of these occurred in the 1930's in the USA where approximately 50000 people were affected as a result of ingestion of an extract of ginger called "Ginger Jake" that had been contaminated with TOTP.<sup>24</sup> The most recent outbreak occurred in England in 1990 where over 700 pigs were affected as a result of ingestion of feed contaminated with isofenphos.<sup>28</sup>

### **Clinical findings**

In man motor and sensory deficits start about 7-21 days after a single exposure. Patients typically report aching and pain in the calf muscles; this is

followed by paresthesiae and anesthesia, affecting the hands and feet. One-two days later, signs of motor weakness develop, generally leg muscles of dorsiflexion are affected before calf muscles, and hands are only affected in the more severely affected patients.<sup>26</sup> There is muscular atrophy, hypotonia, and hyporeflexia. Patients that survive acute toxicosis have a persistent upper motor neuron syndrome characterized by distal muscular hypertonia.<sup>29</sup>

### **Structural abnormalities**

Morphological lesions have been characterized in experimental animals, especially the domestic fowl (*Gallus gallus*) and domestic cat. Swelling and subsequent degeneration (lysis) of the distal regions of long, large diameter axons within the peripheral and central nervous system is first noted around the time of onset of neurological deficits. Regions of axonal swellings in nerves consist of intra-axonal and/or intra-myelinic vacuoles, and accumulations of axonal agranular reticulum are sometimes observed.<sup>30</sup> Axonal lesions in peripheral nerves occur distally, but not terminally. These changes progress to chemical transection with subsequent somatofugal axonal lysis. Retrograde axonal degeneration starting at axonal termini is not thought to occur.<sup>31</sup> In the spinal cord long ascending (dorsal columns and spinocerebellar) and descending (mammalian corticospinal and tectospinal, avian medial pontine spinal) tracts are primarily affected (Figure 1). Large diameter, heavily myelinated axons are most susceptible to degeneration. Axonal degeneration is followed by secondary

myelin degeneration (Wallerian degeneration) in central and peripheral systems.

Axonal regeneration is detectable in nerves (but not in the spinal cord) starting around day 14 after a single dose exposure in one experimental paradigm.<sup>1</sup> Silver impregnation studies in the chicken have revealed axonal degeneration in additional regions of the nervous system including the cerebellum (nuclei and internal granule layer) and medulla oblongata (lateral vestibular, gracile, external cuneate and lateral cervical nuclei).<sup>32</sup>

### **Mechanistic investigations**

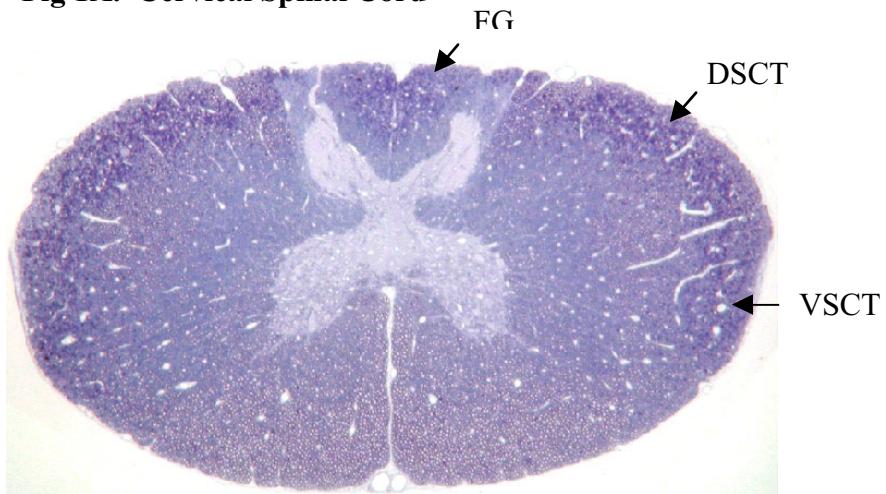
Work by Johnson starting in the 1960's resulted in the identification of an esterase activity, named neurotoxic esterase (NTE), that appeared to be the primary molecular target in OPIDN. Neurotoxic esterase was initially identified by using [<sup>32</sup>P]-labelled DFP to non-specifically label hen brain esterases and proteases *ex-vivo* with and without prior incubation with non-radioactive non-OPIDN inducing OP esters.<sup>33</sup> Neurotoxic esterase was shown to have esterase activity based upon the ability of N, N'-diisopropyl phosphorodiamidofluoridate (mipafox) to inhibit phenyl phenylacetate hydrolysis in brain homogenates.<sup>34</sup> More than 70% NTE inhibition by a neuropathic OP ester is considered necessary to reliably induce severe neurological deficits in hens, and inhibition 40-60% results in an absence of, or mild deficits.<sup>35, 36</sup>

Sulfonyl, carbamyl and phosphinyl esters also inhibit NTE.<sup>4-6</sup> Carbamate inhibition of NTE is transient. Activity returns quickly, and repeated

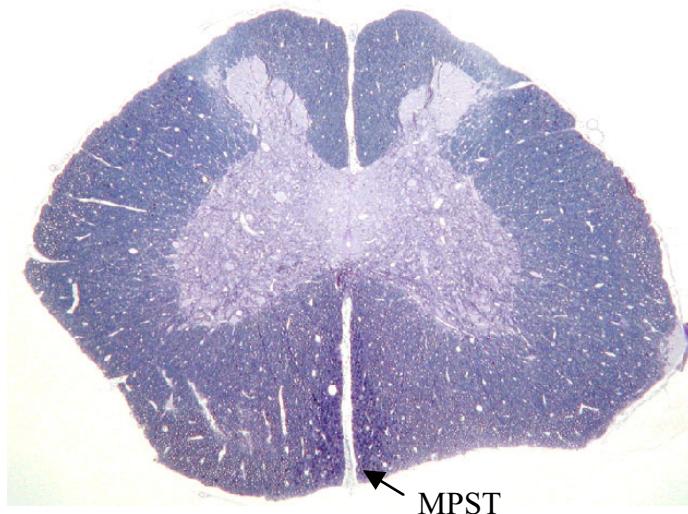
administration fails to produce OPIDN. Exposure to phenylmethylsulfonyl fluoride (PMSF) results in prolonged reversible NTE inhibition, but does not induce OPIDN.<sup>5</sup> Phosphinyl esters (phosphinates) are also non-neurotoxic. These findings suggest that NTE inhibition may be irrelevant to OPIDN initiation. However, sulfonyl, carbamyl and phosphinyl esters, when given before a neurotoxic OP ester, are protective towards the development of OPIDN.<sup>6</sup> This indicates that NTE inhibition by OP ester via active site serine phosphorylation in and of itself is insufficient to induce OPIDN; an additional biochemical event is required. It also suggests that initiation of OPIDN depends upon the nature of the side groups attached to the phosphorus of the OP ester. When neuropathic OP esters bind to the active site serine a molecular aging process takes place that is characterized by loss of one of the side groups of the OP ester. One or two mechanisms may be involved in this process. Loss of a side group from a phosphate or phosphonate could leave behind a negative charge that may disrupt protein structure, and unmask a latent non-esterase activity of NTE. With at least some neuropathic OP compounds the leaving side group is thought to bind to another site on NTE. This is a feature of NTE aging that does not occur with acetylcholinesterase.<sup>3, 4, 37, 38</sup> Recent work has shown that the additional site includes an aspartate residue on NTE.<sup>39</sup> The chemical aging process occurs rapidly after covalent binding; the half time is on the order of a few minutes.<sup>39</sup> (Continued on page 12).

**Figure 1. Distal Degeneration of Spinal Tract Axons in OPIDN**

**Fig 1A. Cervical Spinal Cord**



**Figure 1B. Lumbo-sacral Spinal Cord**



Hens were treated with 2.5 mg / kg PSP and sacrificed 21 days later.

Cervical level cord (1A) is characterized by degeneration of long ascending tracts (arrows). Lumbo-sacral cord (1B) is characterized by degeneration of a long descending tract (arrow). Abbreviations: FG = fasciculus gracilis, DSCT = dorsal spinocerebellar tract, VSCT = ventral spinocerebellar tract, and, MPST = medial pontine spinal tract. Slides are courtesy of Dr. B. Jortner.

The requirements of NTE inhibition and aging for OPIDN induction can be explained if it is assumed that the chemical aging process results in a toxic gain or loss of function within NTE or an adjoining molecule. Lack of delayed neurotoxicity of phosphinyl esters is explained by the lack of a leaving group remaining once these OP esters are covalently bound to NTE.<sup>2, 40</sup>

Purification and cloning of NTE proved difficult, mainly because NTE is a membrane associated protein that loses activity readily during purification. However, the development of biotinylated saligenin cyclic phosphates enabled labeling and affinity purification of NTE.<sup>41</sup> Neuropathy target esterase has now been cloned and sequenced, and is thought to represent a member of a new family of esterases.<sup>42</sup> Human NTE gene has 36 exons. It is located on chromosome 19p13.3-p13.2, a region that has been linked to or associated with several human disorders including vacuolar neuromyopathy, autosomal dominant muscular dystrophy with rimmed vacuoles, and familial febrile convulsions.<sup>2, 43</sup> Human NTE has 41% homology with swiss cheese (Sws) protein of *Drosophila melanogaster*, a neuronal protein that when mutagenically altered leads to brain lesions including glial hyperwrapping, vacuolization, and neuronal apoptosis.<sup>44, 45</sup> Neurotoxic esterase and Sws also exhibit homology to the type I regulatory sub-unit (RI $\alpha$ ) of protein kinase A.<sup>44, 45</sup> This sub-unit forms part of a protein complex suggested to be involved in neuron-glia interactions<sup>46</sup> including the induction of neuron-glia adhesion molecules in neurons.<sup>47</sup> Immunohistochemical

studies in the chicken revealed that NTE was expressed in all neurons, but at higher levels in large neurons such as those present in the spinal anterior gray matter horns, dorsal root ganglia, cerebellar nuclei, optic tectum, and cerebellar Purkinje cells.<sup>48</sup> Murine swiss cheese / NTE gene has also been cloned.<sup>49</sup> Developmental expression studies revealed widespread expression in brain areas of young mice, with increasingly restrictive expression with the approach of maturity. Adult mice expressed sws / NTE transcripts in sub-populations of large neurons in a few brain areas such as the pons, medulla oblongata, mesencephalon and thalamus. Expression also occurred in the choroid plexi, ependymal epithelium and various non-nervous system epithelial tissues.<sup>49</sup>

While this important information provides clues to NTE structure and function, the exact function(s), molecular interactions and physiological substrates of NTE remain unknown. Recent work provided evidence that NTE may mediate that portion of the ionic conductance across liposome membranes that is inhibited by neuropathic OP esters, but not by non-neuropathic NTE inhibitors.<sup>50</sup> However, until the function(s) of NTE are better defined, linking downstream biochemical effects following OP-induced NTE inhibition / aging to later events, such as axon degeneration is problematic.

Non-neuropathic NTE inhibitors are able to promote as well as protect against OPIDN when given after or before, respectively, a single dose of a neuropathic OP ester.<sup>51</sup> Protection has been shown to be the result of occupancy

of the NTE active site by a non-neurotoxic compound, thus preventing covalent binding of the neurotoxic OP ester.<sup>37</sup> By the time the non-neurotoxic compound has been removed from NTE the neurotoxic compound has presumably been metabolized and excreted in sufficient quantity that it can no longer result in sufficient NTE inhibition and aging to induce OPIDN. The mechanism of promotion of OPIDN is not known, but is not thought to involve interaction with NTE. Glucocorticoids and calcium channel blockers modulate the clinical and morphologic manifestations of OPIDN. Dietary corticosterone was able to modulate the expression of neurotoxic response to TOTP and DFP in a dose dependent manner.<sup>16</sup> Verapamil, an L-type calcium channel blocker, was protective against PSP induced OPIDN in hens.<sup>2, 52</sup>

Neurotoxic esterase is present within axons as well as cell bodies and has been shown to undergo fast anterograde, as well as retrograde, axonal transport.<sup>48, 53</sup> It is not known whether mechanistically important interactions of neuropathic OP esters are exerted via direct effects on axons, their cell bodies, or both. Unilateral femoral artery injection of di-isopropyl-phosphorofluoridate (DFP) in domestic cats resulted in clinical and electrophysiologic evidence of mononeuropathy in the injected hind limb.<sup>54</sup> The electrophysiologic changes indicated injury to the axonal terminal arborization rather than to the distal main axon, suggesting that this model may not replicate classical OPIDN. Further work in this experimental paradigm using labeled DFP demonstrated that

distribution is primarily to the affected limb, and not adjoining spinal cord, supporting the hypothesis that neuropathic OP esters can act directly on axons.<sup>55</sup> Confirmation that neuropathic OP esters induce OPIDN only through direct effects on axons would require the converse experiment; exposure of spinal cord, but not peripheral nerves. This experiment has not been performed presumably for technical reasons.

Very little is known concerning the processes that occur between NTE inhibition and aging, and the onset of neurological deficits and morphological lesions of OPIDN. Studies performed to date have focused on effects on axonal transport, cytoskeletal protein phosphorylation, calcium homeostasis, and gene expression.

Axonal transport is necessary for axonal structural maintenance, function, and neuronal survival. There are several transport mechanisms that have been identified. Fast axonal transport occurs at a rate of approximately 400 mm and 200-270 mm / day for anterograde and retrograde directions, respectively. It is concerned with movement of membranous organelles such as mitochondria, smooth endoplasmic reticulum, endosomes and synaptic vesicle precursor membrane. The mechanism involves ATP dependent molecular motors that move along microtubule ‘tracks’.<sup>56</sup> Slow axonal transport occurs in the anterograde direction only and has two kinetic components. The slower slow component travels at 0.25-2.5 mm / day and carries neurofilaments and tubulin proteins. The faster component travels at about 0.5-5.0 mm / day and carries actin, actin-

binding proteins and cytosolic enzymes.<sup>56</sup> Several studies have examined the effects of neuropathic OP esters on axonal transport. Results have been conflicting. Fast anterograde axonal transport rate was decreased 40-50% three hours after intra-vitreal dosing in a rat optic ‘nerve’ model using a variety of neuropathic OP esters, but not with the non-neuropathic OP ester, parathion.<sup>57</sup> Evidence for increased fast anterograde axonal transport was reported in cat ischiatic nerve following treatment with DFP. However, it was concluded that the effect was small and probably secondary.<sup>58</sup> The OPIDN inducing compound di-*n*-butyl-2,2-dichlorovinyl phosphate (DBDCVP) resulted in progressive, up to ~70%, inhibition of retrograde axonal transport in hens. Furthermore, an effect was not noted with the non-neuropathic NTE inhibitor, PMSF, indicating that the response may be of mechanistic relevance to OPIDN.<sup>59</sup> Gupta<sup>60</sup> has observed mildly decreased tubulin polymerization in hen brain 18-21 days after DFP treatment. Tubulin heterodimers are a major component of microtubules. Therefore, alterations in microtubule stability could affect axonal transport. Other work in the DFP-hen ischiatic nerve model<sup>61</sup> or cat-TOTP model<sup>62, 63</sup> reported an absence of significant change in axonal transport rates. An *in vitro* assay of hen brain kinesin-driven microtubule motility failed to identify significant effects of 10 mM (each) DFP, PSP, or the non-neuropathic compound, paraoxon.<sup>64</sup> These discrepancies may be due to differences in animal model, the nervous system region studied, the neuropathic OP ester used, or other factors. Little is

known about causes of altered axonal transport rates. Possibilities include altered expression of transport proteins, dysregulation of post-translational modification(s), or axonal energy deprivation.

Changes in calcium homeostasis and calcium activated pathways have been hypothesized to play a role in OPIDN. Calcium-calmodulin protein kinase II (CaM-Kin II) activation was observed during DFP induced OPIDN, and it was postulated that this may lead to increased phosphorylation of axonal cytoskeletal proteins and their subsequent dysfunction.<sup>65</sup> *Ex-vivo* experiments using DFP and TOTP have shown increased phosphorylation of  $\alpha$  and  $\beta$ -tubulin, microtubule-associated protein-2 (MAP-2), and three neurofilament proteins, as early as day 1 post-exposure continuing up to day 21 in chicken ischiatic nerve and spinal cord.<sup>66-68</sup> However, decreased neurofilament phosphorylation has been observed 9-20 days after exposure of hens to another neuropathy inducing compound, PSP, possibly suggesting a lack of mechanistic significance of these alterations.<sup>69</sup> Calcium-calmodulin kinase II is distributed throughout the nervous system; in neurons it is found within the soma and cell processes.<sup>70</sup> Structurally it is a complex protein comprising 8-10 sub-units with five known sub-unit genes ( $\alpha$ ,  $\beta$ ,  $\beta'$ ,  $\chi$ ,  $\delta$ ) that are expressed in different proportions in different brain regions.<sup>70</sup> Activation of CaM-Kin II involves binding of calcium/calmodulin to the kinase complex leading to dis-inhibition and subsequent autophosphorylation to produce a state active in the absence of CaM.<sup>70</sup> The mechanism of CaM-Kin

II activation in OPIDN has not been studied. However, the protective effect of the L-type calcium channel blocker, verapamil, in the development of chicken OPIDN provides indirect support for a possible role of increased intra-cellular calcium in activating this enzyme.<sup>52</sup> Effects of CaM-Kin II activation will depend on the substrate proteins phosphorylated, and the effect of phosphorylation on their function. Numerous likely neuronal substrates of CaM-Kin II have been identified and include: the pre-synaptic protein tau<sup>71</sup> and synapsin I<sup>72</sup>; the post-synaptic proteins calcineurin<sup>73</sup> and the IP3 receptor;<sup>74</sup> and, proteins involved in the regulation of gene expression such as the translation factor S6,<sup>75</sup> and the transcription factors CREB<sup>76, 77</sup> and C/EBP $\beta$ .<sup>78</sup> Activation of this enzyme could therefore lead to changes in neuronal gene expression. A comprehensive understanding of all the functions of CaM-Kin II in neurons has yet to be achieved, and it is likely that many neuronal substrates remain to be identified.<sup>79</sup> While work done in the laboratory of Abou-Donia provides evidence for phosphorylation of cytoskeletal proteins by CaM-Kin II,<sup>65</sup> the possible role played by phosphorylation of non-cytoskeletal substrates has not been determined. Increased neuronal cytosolic calcium concentration may also play a role in activation of the calcium sensitive protease, calpain, which may then mediate cytoskeletal degradation.<sup>80</sup> Assuming that there is calcium dysregulation, the possibility that calcium may be disturbing other signaling pathways such as those involving protein kinase C, and phospholipase A<sub>2</sub>, should

be considered. Inhibition of hen brain P2 synaptosomal fraction calcium-magnesium activated ATPase occurs for PSP and TOTP, but not DFP. Since inhibition is not related to neuropathic OP ester exposure, this effect is not thought relevant to OPIDN.<sup>81</sup>

Proper patterns of gene expression are critical to cellular differentiation, maintenance, survival, and development. Given the large number of genes expressed by a given cell type (estimated between 12000-15000),<sup>82</sup> regulation is a hugely complex process. Expression of eukaryotic genes is a multistep process involving transcription of pre-mRNA, splicing, cytoplasmic export, RNA stability, translation, post-translational modifications, and transport to site of action. The process can potentially be regulated at one or more levels, and at each level there are many possible regulatory mechanisms.<sup>83</sup> Transcription is thought to be an important step in regulating expression of many genes. However, it cannot be assumed that a gene altered at the mRNA level exhibits the same change at the protein level. For example, perturbation of the galactose utilization pathway in *Saccharomyces cerevisiae* leads to changes in the expression of many genes. Some changes occur at the mRNA, but not protein level, while others occur at the protein, but not mRNA level.<sup>84</sup> Transcript levels are often measured instead of protein in gene expression studies because antibodies are not available for many proteins, especially in less commonly used research species. Additionally, the phenomenon of nucleic acid hybridization has resulted in

development of a variety of approaches for measuring mRNA expression. While these approaches are of great value, results should be interpreted carefully until protein expression is determined.

Alterations to gene expression in the nervous system have been studied in numerous nervous system toxicity and repair models. Cell bodies of transected hypoglossal nerve axons have been shown to upregulate kinesin light chain isoenzymes and dynein mRNA transcripts, the gene products of which are involved in fast anterograde and retrograde axonal transport, respectively.<sup>85</sup> Neurofilament-L and M mRNA are down regulated 1-3 weeks after axotomy in rat facial and rubrospinal neurons. In the same system, following axotomy, tubulin and actin mRNA amounts were both increased for one and three weeks, respectively, in the red and facial nuclei.<sup>86</sup> Another study using the hypoglossal nerve transection model identified upregulation of glutamine synthetase mRNA levels, suggesting a possible function in the prevention of excitotoxicity.<sup>87</sup> A study of axonal regeneration identified differential expression of LA12.2 and LC12 mRNA in spinal dorsal root ganglia.<sup>88</sup> The sequence of LC12 corresponds to a secreted protein, called Reg-2 that is known to act as a Schwann cell mitogen.<sup>89</sup> Several studies have looked at the effects of drugs or toxicants on central nervous system gene expression. Upregulation of calcineurin A mRNA occurred in the striatum of amphetamine treated rats,<sup>90</sup> and a novel gene is upregulated in the cerebral cortex of lithium treated rats.<sup>91</sup> A combination of

corticosteroid administration and adrenalectomy in rats has been found to result in the upregulation of a number of mRNA transcripts, one of which is thought to be the homologue of the mouse transcription factor, KROX-20.<sup>92</sup> One study in alcoholic human patients identified upregulation of brain mitochondrial 12S rRNA; subsequent blotting experiments in rats chronically exposed to alcohol identified upregulation of mitochondrial 12S and 16S rRNA.<sup>93</sup> A study of a mouse model of the fetal alcohol syndrome resulted in the identification of three genes that were upregulated, heat shock protein 47, alpha-tropomyosin and an unknown gene.<sup>94</sup>

Little is known about OP-induced nervous system gene expression. In a non-OPIDN model Balduini<sup>95</sup> found that post-natal rats treated with the delayed organophosphorus neurotoxicant, DFP showed decreased expression of the mRNA encoding the muscarinic, sub-type 1, receptor. This and other evidence suggests that changes in nervous system gene expression occur during the clinically silent phase of OPIDN. For example, cervical cord extracts prepared from eight week old chickens 24 hours after treatment with PSP and DFP were able to promote neuritic outgrowth in the human neuroblastoma cell line, SH-SY5Y. Neuritic outgrowth was less marked in spinal cord extracts from birds protected by pre-treatment with PMSF. The interpretation was that there may be upregulation of a neurotrophic factor(s).<sup>13</sup> A number of publications have resulted from studies with DFP using the hen model. These studies have shown

that DFP induced increases in transcripts for calcium-calmodulin protein kinase II  $\alpha$ -subunit,<sup>7</sup> and all three neurofilament in brain and spinal cord 24 hours post-exposure.<sup>8</sup> Spinal cord vimentin and GFAP mRNA transcripts have been demonstrated to reach their minimum levels at 24 hours (50%) and 48 hours (45%) after dosing with DFP, respectively.<sup>9</sup> An  $\alpha$ -tubulin transcript was down regulated ~31% on day 2 and upregulated about ~100% on day 10 while alterations were not found for  $\beta$ -tubulin.<sup>10</sup> Spinal cord *c-fos* and *c-jun* transcripts were increased 2 hours after exposure to DFP.<sup>11, 12</sup> The relevance of these DFP induced changes in gene expression is not known. DFP inhibits acetylcholinesterase as well as NTE. In addition, controls using non-neuropathic esterase inhibitors were not included in these experiments. Therefore, few clues exist as to its possible mechanistic relevance. However, other investigators found that *c-fos* was upregulated in the brains of rats treated with the neurotoxicant  $\gamma$ -hexachlorocyclohexane.<sup>96</sup> Therefore, alterations in the expression of this gene may be part of a general response to injury rather than being specific to OPIDN. It has yet to be determined whether DFP induced gene expression changes in the hen contribute, protect or are epiphenomenal to OPIDN. Gene expression change as a mechanism of xenobiotic induced toxicity is precedented. It was found to occur following activation of the aryl hydrocarbon<sup>97</sup> and peroxisome proliferator receptors.<sup>98</sup> In addition to the effects cited above, spinal cord ornithine decarboxylase (ODC) activity reportedly decreased >90% 24 hours after DFP

exposure in 8 week old chickens.<sup>13</sup> Ornithine decarboxylase catalyzes the first step in the synthesis of polyamines. The significance of ODC inhibition in this context is not known. Although NTE is a recognized target of neuropathy-inducing OP esters, an immunohistochemical study failed to identify altered neuronal NTE protein expression in mono-*o*-cresyl diphenyl phosphate treated chickens one and three days after dosing.<sup>48</sup> Therefore, given that only a few genes have been studied at 24 hours after administration of neuropathy inducing OP esters, it is possible other genes show differential expression, and that these could be mechanistically important. Genes coding for proteins involved in the neuronal cytoskeleton, neurotrophic factor pathways and transcription factors could be involved.

Gene expression is affected by rates of protein degradation in addition to synthesis. In this regard it is interesting that alterations of calcium activated neutral protease (calpain) have been reported in OPIDN. Calpains are known to hydrolyse cytoskeletal proteins,<sup>99</sup> so their dysregulation could lead to altered cytoskeletal homeostasis. Calpain was activated in brain and ischiatic nerve of PSP treated hens before onset of clinical signs.<sup>80</sup> However, another study reported DFP decreased calpain activity in the hen ischiatic nerve even though there was increased cytoskeletal degradation.<sup>100</sup>

Recent *in-vitro* work indicates that neuropathic OP esters can induce mitochondrial injury. One mM doses of tri-phenylphosphite and TOCP induce

collapse of the mitochondrial membrane potential ( $\Delta\psi_m$ ),<sup>101</sup> activation of caspase 3 and apoptosis<sup>102</sup> in human SH-SY5Y neuroblastoma cells. In chicken embryo dorsal root ganglion cultures neuropathy inducing PSP and mipafox, but not paraoxon, induced opening of the mitochondrial permeability transition pore.<sup>103</sup> The *in-vivo* significance of these mitochondrial changes is not known. If mitochondrial injury does occur in OPIDN it certainly does not initiate apoptosis in spinal anterior horn neurons to any significant extent as this would result in irreversible lower motor neuron failure and reduced clinical recovery. However, it is possible that mitochondrial injury is sub-lethal to neurons, or that the mitochondria within axons are more susceptible to injury. This latter possibility has a precedent in neuropathy. Inhibition of mitochondrial  $\gamma$ -DNA polymerase was demonstrated to lead to mitochondrial deficiency and energy failure. This was thought to be the basis of nucleoside analogue induced sensory neuropathy (axonopathy) in HIV patients.<sup>104</sup>

Understanding of OPIDN will be improved greatly if the function(s) of NTE can be identified and related to known or newly identified downstream events. The evidence described above for a possible role of gene expression changes as an important mechanism in OPIDN suggests that there could be a link between events occurring on NTE and down stream gene expression changes. A major goal of this dissertation was to probe for possible links between the events

of NTE inhibition and aging, and downstream spinal cord gene expression changes.

**CHAPTER 2: Identification of Differentially Expressed mRNA Transcripts  
in the Spinal Cord of *Gallus Gallus* 24 Hours Post-Exposure to Phenyl  
Salignenin Phosphate**

**Introduction**

Phenyl saligenin phosphate (PSP) and other neuropathy-inducing organophosphorus (OP) esters induce degeneration of long, large diameter axons within the central and peripheral nervous system of susceptible species, including domestic fowl (*Gallus gallus*). Symptoms appear 7-21 days after a single dose exposure. The exact mechanism(s) involved are not understood. However, there is considerable evidence that development of this OP ester induced delayed neurotoxicity, termed OPIDN, requires prior inhibition of the neuronal enzyme, neurotoxic esterase (NTE), and subsequent chemical change of the bound OP ester in a process called aging.<sup>105, 106</sup> Compounds such as phenylmethylsulfonyl fluoride (PMSF), that only inhibit NTE but do not age on NTE, are not neurotoxic. Based on these findings it has been suggested that disruption of some unidentified non-esterase function of NTE by OP aging leads to induction of OPIDN.<sup>105</sup> The normal function(s) of NTE remain unknown. Neurotoxic esterase is a carboxylesterase, but physiologic substrates have not been identified. Recent evidence suggests that the non-esterase function of NTE may involve facilitation of ionic conductance within intra-cellular membranes.<sup>50</sup>

Down stream events immediately following the early neurotoxic OP interaction with NTE are not known. However, numerous biochemical alterations

have been detected in OPIDN. These include changes in spinal cord expression of cytoskeletal genes and transcription factors (see below), cytoskeletal protein phosphorylation,<sup>69, 107-109</sup> protease activation,<sup>80, 100, 110</sup> fast axonal transport,<sup>57, 58, 61, 111</sup> and the dynamics of tubulin polymerization.<sup>60</sup> Additionally, *in vitro* work has provided evidence for mitochondrial injury leading to changes in mitochondrial membrane potential, mitochondrial permeability transition, caspase 3 activation, and apoptosis.<sup>101-103</sup>

Changes in spinal cord gene expression occur following exposure to DFP (an OPIDN inducing agent that also causes acute cholinergic poisoning), but before onset of clinical neurotoxicity or structural alterations. Expression changes have been identified for a number of genes including those encoding the neurofilaments,<sup>112</sup>  $\alpha$ -tubulin,<sup>10</sup> vimentin,<sup>9</sup> GFAP,<sup>9</sup> c-fos,<sup>11</sup> c-jun,<sup>12</sup> and CaM-Kin II.<sup>7</sup> There is also indirect evidence for upregulation of a neurotrophic factor in the spinal cord of PSP treated hens.<sup>13</sup>

The mechanistic relevance of gene expression and other changes to OPIDN is not known. Whether these changes occur with other neuropathic OP esters such as phenyl saligenin phosphate (PSP) is not known. Similarities between OP esters such as DFP and PSP are important because PSP can induce OPIDN without causing cholinergic poisoning. Links between early effects of neuropathic OP esters on NTE, and downstream biochemical/cellular alterations have not been identified.

If NTE inhibition and aging is sufficient to induce OPIDN, then this offers a possible approach for distinguishing changes in gene expression that are of no mechanistic significance from those that might be related to the crucial processes on NTE. In this study, patterns of spinal cord gene expression were compared and contrasted among hens (*Gallus gallus*) exposed to a neuropathy inducing OP ester (PSP), a non-neurotoxic NTE inhibitor (PMSF), and vehicle (DMSO). Use of PMSF is considered important. It was included to help distinguish expression changes that may be related to NTE inhibition alone, or non-specific inhibition of esterases and proteases, from changes that are hypothesized to occur only in OPIDN. While considered important, PMSF treated experimental controls have rarely been used in previous studies of gene regulation in OPIDN.<sup>7, 8</sup> Studies were performed in hens because they constitute the US-EPA approved animal model for studies of OPIDN.<sup>113</sup>

In this study, some gene transcripts or proteins reported altered following exposure to DFP were investigated to determine if similar alterations occurred following exposure to PSP. Northern blotting was used to evaluate vimentin, neurofilament-M, and calcium-calmodulin protein kinase II. It was expected that DFP and PSP would induce similar alterations in expression if changes were necessary for manifestations of OPIDN. Given the evidence for the involvement of neurotrophic factors in OPIDN,<sup>13</sup> attempts were also made to evaluate transcript expression of neurotrophic receptors *trk A*, *trk B* and *trk C*, and NGF- $\beta$  by Northern blotting. Additionally, a gene screening strategy, termed targeted

display (TD), was developed and used to screen for spinal cord expression changes 24 hours after PSP exposure in hens.

Targeted display (TD)<sup>114</sup> is a cDNA screening approach related to differential display (DD).<sup>115</sup> Both techniques amplify multiple cDNA's by polymerase chain reaction. However, TD has the theoretical advantage that primers should amplify regions of cDNA coding for protein, while DD primers amplify 3'-untranslated regions (UTR). This can enable ready identification of differentially expressed genes with TD. This advantage has been demonstrated in a rat pheochromocytoma cell line (PC12),<sup>114</sup> but not in *Gallus gallus*, which are phylogenetically more distant than rats from humans, the species for which the primers were designed.<sup>114, 116</sup> The technique can identify both known and unidentified genes. Complementary DNA screening is most commonly used for the systematic search for differentially expressed genes in specific experimental systems when comparing responses to two<sup>117</sup> or more<sup>92</sup> treatments. The approach has been used throughout the neurosciences.<sup>92, 118-122</sup>

It was expected that genes found to be differentially expressed could be those involved in, 1.) the axonal cytoskeleton (such as structural proteins, kinases, phosphatases, or proteases), 2.) neurotrophic factor signaling, and, 3.) cell-cell, or cell-matrix interactions.

## **Specific Questions and Rationale**

### **1. What lumbo-sacral spinal cord gene transcripts are differentially expressed in PSP treated compared to DMSO treated hens (*Gallus gallus*) 24 hours post-exposure?**

The goal was to identify spinal cord transcripts that are up or down regulated in PSP compared to DMSO exposed birds. With some exceptions, proteins are the functional end product of gene expression. They are therefore of potentially greater interest than mRNA. This study however focuses on mRNA transcript expression because high throughput methods to study multiple genes at mRNA level are readily available. A disadvantage of this approach is that knowledge of changes in mRNA levels cannot be extrapolated to changes in protein expression. Some genes are regulated post-transcriptionally.<sup>84</sup>

Domestic fowl (*Gallus gallus*) are the animal model accepted by the Environmental Protection Agency for testing OP compounds for delayed neurotoxicity.<sup>113</sup> They have a similar level of susceptibility to OPIDN induction as humans and develop very similar lesions. When studying gene expression, results from an *in vivo* model should be of more relevance to the human neurotoxicity than those from *in vitro* work. Performing the same approach using a cell culture model may lead to results that do not reflect those that take place *in vivo*. For example, it is possible that cell-cell interactions are important in OPIDN. The use of an *in vivo* model also has the potential to detect altered gene

expression in non-neuronal cells such as astrocytes, oligodendrocytes, microglial and endothelial cells.

A 24 hour time point was chosen to examine spinal cord gene expression by TD. The number of differentially expressed genes may increase with time through the pre-clinical phase of OPIDN. However, many later changes in gene expression changes may be part of a regenerative response. Knowledge of regeneration responses is unlikely to provide additional understanding of OPIDN because mechanisms of regeneration may be similar regardless of the initial insult to the axon. It is important to examine gene expression sufficiently early that changes found might be linked to the mechanism of PSP action, and not a response to PSP induced lesions. Choosing too early a time point could result in finding few or no gene expression changes. The 24 hour time point was chosen for this study because DFP induced changes in gene expression were detectable in chicken spinal cord at this or earlier times<sup>9, 10-12, 112</sup>. In addition, preliminary work in this laboratory identified expression changes in mRNA encoding  $\mu$  and m calpain in chicken spinal cord 24 hours after dosing with PSP (personal communication, Dr. D. Barber). By 96 hours after exposure in hens, PSP induced lesions are present in some nerves.<sup>123</sup>

The long axons of motor neurons that innervate the hind-legs, biventer cervis muscle, ascending fasciculus gracilis and spinocerebellar tracts, and descending medial pontine spinal tract (MPST) of the chicken are most susceptible to degeneration in OPIDN.<sup>2</sup> Experiments were performed using the

portion of the lumbo-sacral spinal cord corresponding to spinal nerves 25-30 (Figure 2). This region contains cell bodies of motor neurons whose axons degenerate in OPIDN, and the distal end of the medial pontine spinal tract, but not cell bodies of spinocerebellar or fasciculus gracilis axons. Targeted display type methods have been used on chicken tissue previously.<sup>124-127</sup>

There are many OP esters capable of inducing OPIDN.<sup>40</sup> Phenyl saligenin phosphate (Figure 3) was used in this study mainly because it results in only slight acetylcholinesterase inhibition, insufficient to result in clinical manifestations of acute cholinergic toxicity (see results). It is a stable congener of tolyl saligenin phosphate, the active metabolite of TOTP. Additionally, PSP has been used extensively in OPIDN research<sup>13, 128, 129</sup>. Use of PSP also avoids the confounding factor of debilitation from acute cholinergic toxicity produced by DFP.

Several methods are available that could be used to address the above question. These include, 1.) some form of array for screening large numbers of genes simultaneously, 2.) a cDNA screening strategy, or, 3.) methods that examine genes on an individual genes basis such as Northern blotting, or *in-situ* hybridization. Two approaches were chosen in this study. Firstly, a cDNA screening approach, targeted display (TD), was developed for screening large numbers of transcripts for differential expression. It was considered that using an approach that randomly screens cDNA for expression changes would avoid potential bias as to what cellular systems might be affected in OPIDN (see

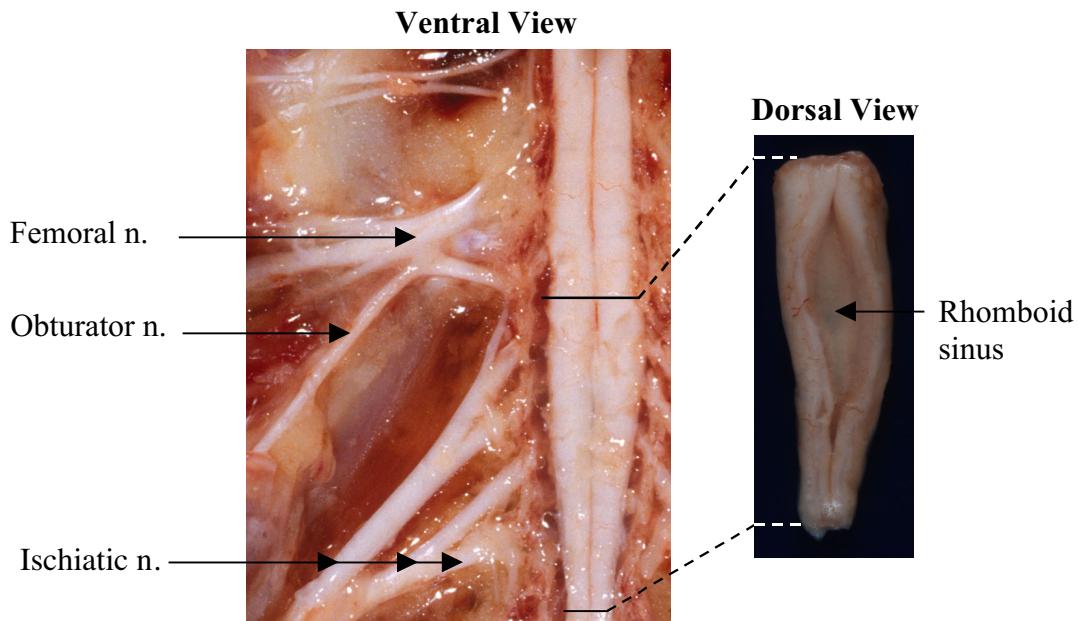
methods for details). This type of approach has been used to address a number of biological questions that range from the identification of genes involved in mammalian development<sup>130</sup> to the genes associated with strawberry ripening.<sup>131</sup> It has also been used in the neurosciences, including neurotoxicology,<sup>118</sup> pharmacology,<sup>92</sup> aging,<sup>119</sup> psychiatry<sup>120</sup> and studies of axonal regeneration.<sup>121, 122</sup> Secondly, Northern blotting was used on selected genes. This enabled evaluation of specific genes of interest, namely, those reported to be affected by DFP, and those involved in neurotrophic signaling pathways. An array approach was not used because many genes of interest were not available for array production at the time this study was initiated.

**2. Are spinal cord genes differentially expressed following PSP exposure also differentially expressed following PMSF exposure?**

The goal was to determine whether genes that are differentially expressed with PSP have altered expression following exposure doses of PMSF sufficient to produce an equivalent inhibition of NTE activity. Gene transcripts that show altered expression with PSP, but not PMSF, are of more interest than expression changes that occur with both these compounds. Induction of OPIDN requires inhibition and aging of NTE; inhibition alone is not sufficient. Use of a compound that inhibits NTE without aging represents a crucial control, beyond the requisite DMSO vehicle control.

Numerous compounds inhibit, but do not age NTE. Examples include sulfonyl, carbamyl and phosphinyl esters. Carbamate inhibited NTE is rapidly reactivated by loss of the carbamate. Phenylmethylsulfonyl fluoride was chosen for use in this study (Figure 3). Although PMSF is not an OP ester it is considered the compound of choice for this purpose (rather than a non-neuropathic OP ester) because, 1.) it is a potent and prolonged inhibitor of NTE, with recovery of activity in a manner temporally similar to that of PSP (see results), 2.) unlike neuropathic OP esters, it does not undergo chemical “aging” after covalent binding to NTE, and does not induce OPIDN, 3.) it is a non-specific inhibitor of serine esterases and proteases like most OP esters, 4.) it does not produce clinical toxicity at doses that result in >70 % NTE inhibition, and, 5.) it does not induce significant acetylcholinesterase inhibition.<sup>4-6, 105</sup> The use of a non-neuropathic OP ester such as paraoxon would result in acute neurotoxicity as a result of acetylcholinesterase inhibition; this would constitute a confounding factor.

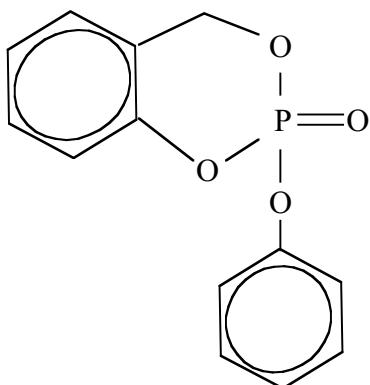
**Figure 2. Anatomy of *Gallus gallus* lumbo-sacral plexus and spinal cord**



Two important macroscopic anatomic differences exist between avian and mammalian spinal cords.<sup>132</sup> Firstly, birds lack a cauda equina; consequently, the spinal cord is almost the same length as the neural canal. Spinal nerves therefore pass laterally to their intervertebral foramina. Secondly, avian lumbosacral spinal cord contains a dorsal midline sinus (rhomboid sinus) that is filled with glycogen rich glial cells innervated by unmyelinated axons (gelatinous or glycogen body). The function of this structure is unknown. These anatomic differences are thought by the author to have little / no effect on the validity of *Gallus gallus* as a model for studying human OPIDN. The region of spinal cord shown in dorsal view was used in this study. It gives rise to spinal nerves 25-30.

**Figure 3. Structures of phenyl saligenin phosphate (PSP), phenylmethylsulfonyl fluoride (PMSF), and dimethyl sulfoxide (DMSO)**

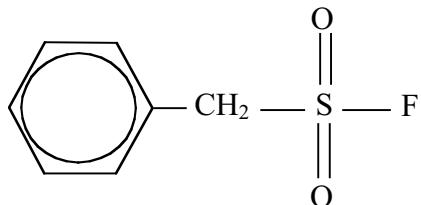
**PSP**



PSP is an active congener

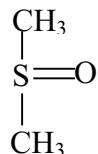
of TOTP. It was chosen for use in this study mainly due to its minimal ability to cause inhibition of acetylcholinesterase.

**PMSF**



PMSF is a sulfonyl fluoride. It results in prolonged inhibition of NTE, but is not neurotoxic.

**DMSO**



In this study DMSO was used as

a vehicle for PSP and PMSF.

## **Experimental Design and Analysis**

For the targeted display, a completely randomized design,<sup>133</sup> with two treatments (PSP and DMSO) and two birds per treatment group was used. A PMSF treatment group was not incorporated at this point. It was considered that large numbers of cDNA species could be screened more rapidly if the number of hens and treatments could be minimized during the developmental stage of the project. Phenylmethylsulfonyl fluoride treated hens were incorporated in subsequent experiments so that Specific Question 2 dealing with the relevance of NTE inhibition could be addressed.

For later Northern blotting experiments on candidate differentially expressed genes, and on chosen known genes, a randomized complete block design<sup>133</sup> with three treatments (PSP, PMSF and DMSO), and six hens per treatment group was used. For logistical reasons, the experiment was split into two blocks with three hens per treatment\*block combination. It was considered that performing confirmatory Northern blotting experiments for putative differentially expressed genes using different hens would increase the statistical validity of findings. It was possible to use the same hens for TD and Northern blotting experiments, but findings would have had less external validity.

An additional PSP treated hen was used to confirm that PSP administration would produce clinical evidence for OPIDN; this hen was kept until the onset of neurological deficits consistent with OPIDN. Brain NTE measurements on all hens also confirmed adequacy of treatments.

Results from Northern blotting experiments were analyzed using the general linear model (GLM) procedure in SAS (version 6.12, SAS Institute, Cary, NC). Pre-planned two sided contrasts were made when the global ANOVA hypothesis was rejected ( $p<0.05$ ).

## **Material and Methods**

### **Targeted display method overview and development**

Targeted display (TD)<sup>114</sup> is a methodologic approach that shares similarities with differential display (DD)<sup>134</sup> and arbitrarily primed RNA fingerprinting.<sup>135</sup> These three approaches use reverse transcriptase polymerase chain reaction (rtPCR) with multiple combinations of primers under low stringency conditions such that many cDNA molecules are amplified simultaneously. During PCR, a labeled nucleotide is incorporated. Products are then resolved on a gel and visualized by autoradiography or fluorescence. The major difference between TD and DD resides in the area of primer design. In TD, primers are designed to target sequences coding for proteins in cDNA's. Classical DD uses an anchored 3' primer (oligo-dT) and an arbitrary primer; this essentially anchors nearly all the PCR products to the 3' end of the mRNA transcripts. This approach has the disadvantage that only 3'-UTR tend to be amplified. Therefore, other methods such as cDNA library screening may be necessary before a gene transcript of interest can be identified. This problem may not be as significant when working with species for which there is abundant sequence data. However, it was considered important in this project because sequence data for the chicken is less abundant than for the rat and mouse. This is why TD was used in this study. Targeted display primers were designed based on octa-nucleotides targeted for protein coding regions of genes. These regions

contain many octamers that are conserved across protein families.<sup>116</sup> These octamers were extended by six nucleotides at five prime ends to produce fourteen-mers with a 50% GC ratio.<sup>114</sup> Previous studies have shown that the 3' end of the primer is mainly responsible for determining specificity.<sup>136</sup> A total of 30 sense primers and their reverse complement anti-sense primers have been designed. Each sense primer can be used with all of the anti-sense primers except its own reverse complement. Table 1 lists all the primers used in this study. Appendix A gives a full list of TD primers, and probabilistic argument regarding detection of differentially expressed genes.

Before this study was initiated different methods of DNA resolution and visualization were evaluated. Use of high resolution agarose and gel staining as described previously<sup>114</sup> lacked sufficient resolution and sensitivity for a tissue as complex as the CNS. Fluorescently labeled nucleotides incorporated by PCR were resolved on an automated DNA sequencing gel, but this led to problems with sensitivity and DNA band extraction.<sup>a</sup> Incorporation of [ $\alpha$ -P<sup>33</sup>]-dATP with DNA sequencing gel resolution gave the best combination of sensitivity and resolution. Dried gels were exposed to film. Bands of interest were excised, re-amplified and cloned. Bands are often heterogenous,<sup>137</sup> so clones may contain different inserts. Screening of different clones from one cloning reaction is therefore necessary. There are several ways in which this can be done including

---

<sup>a</sup> Primers labeled with FAM, TAMRA and HEX-6 were utilized.

single stranded conformational polymorphism,<sup>138</sup> reverse Northern blotting,<sup>139</sup> and use of base specific DNA ligands to separate equally sized fragments differing in base composition.<sup>140, 141</sup> The later approach was used in this study.

This type of technique is subject to a high rate of false positives.<sup>142, 143</sup> Consequently, it is necessary to confirm putative differentially expressed genes as being actually differentially expressed by an alternative technique, such as Northern blotting, quantitative PCR or *in-situ* hybridization. Northern blotting was used in this study.

**Table 1. Targeted Display Primers Used in this Study**

Sense primers		Anti-sense primers	
1s	t g g t g a a a g c a g g a	6a	t g a t g a g g c c t t c t
2s	t g a t g a c a t c g t g g	8a	t g g t g a t t c c a g g
3s	t g a t g a c c t g a g g a	10a	t g a t g a g t c c a g g a
4s	t g g t g a g a a g c t g a	11a	t g a t g a c a g g g t c t
5s	t g g t g a g t g g a g a a	14a	t g a t g a t c c a g g t c
6s	t g a t g a a g a a g g c c	15a	t g a t g a g c t c a g c a
7s	t g g t g a c a t g a a g g	17a	t g g t g a t c a t c t g g
11s	t g a t g a a g a c c c t g	18a	t g a t g a c c a g g t a g
12s	t g g t g a c c a a g a a g	19a	t g g t g a t t c a g c t c
13s	t g a t g a c c t g g a g a	20a	t g g t g a c t t g t c c a
15s	t g a t g a t g c t g a g c	25a	t g a t g a c a g g t c c a
16s	t g a t g a a g a g g a g c	26a	t g g t g a t c t c c t t g
17s	t g g t g a c c a g a t g a	27a	t g a t g a c c t t c a g g
		28a	t g a t g a c t t g g c a g
		29a	t g g t g a c t t t c t c c
		30a	t g g t g a t c t c t c c a

All sense and anti-sense primer combinations were used except for those having the same number. Theoretically sense primers have sequence that is the reverse complement of mRNA; and anti-sense primers have sequence that is

reverse complement to the equivalent regions of cDNA. Based on the three genes studied, sense and anti-sense primers worked in the correct orientation (appendix B).

## **Treatments**

Adult female White Leghorn (*Gallus gallus*), age and strain matched were used in this study. Hens were administered 2.0 mg/kg PSP (Lark Enterprises, Webster, MA), 90 mg/kg PMSF (Sigma, St. Louis, MO), or DMSO (Sigma) vehicle by intra-muscular (im) injection such that total volumes administered were 0.5 ml/kg. These dosages of PSP and PMSF result in similar levels of NTE inhibition (figure 4). Hens were evaluated for neurological deficits by visual examination of movement and behavior while in cages.

## **Brain esterase measurements**

Euthanasia 24 hours post dosing was by intra-venous pentobarbital overdose. Whole brains were removed immediately, snap frozen on dry ice and stored at  $-70^{\circ}\text{C}$ . Brain total carboxylesterase, neurotoxic esterase and acetylcholinesterase activities were determined as described.<sup>144</sup>

## **Targeted display**

*RNA isolation and DNase I digestion:* Lumbo-sacral spinal cords from spinal nerves 25-30 were immediately removed (Figure 1). From this segment of spinal cord arise the spinal nerves that form the ischiatic nerve, terminal axons of which degeneration in OPIDN. Dura mater and glycogen body were removed (Figure 1). The cord was weighed and homogenized in (>1 ml / 100 mg) TRIzol<sup>TM</sup> Reagent (Life Technologies, Gaithersburg, MD) for 30 seconds using a Polytron PT-7EC homogenizer (Fisher, Pittsburgh, PA). Homogenates were incubated at  $25^{\circ}\text{C}$  for 5 minutes to ensure dissociation of nucleoproteins then

snap frozen in an isopropanol-dry ice bath prior to storage at -70<sup>0</sup> C. Samples of RNA were isolated the next day, according to the manufacturer's instructions. In brief, after thawing at 25<sup>0</sup> C, 0.2 volumes of chloroform was added, the samples were shaken briefly, incubated for a further 3 minutes and then centrifuged at 12000 x g for 10 minutes at 4<sup>0</sup> C. Precipitation of RNA out of the isolated aqueous phase was induced by the addition of an equal volume of isopropanol, incubation for 10 minutes at 25<sup>0</sup> C, then centrifugation at 12000 x g for 10 minutes at 4<sup>0</sup> C. The supernatant liquid was removed taking care not to disturb the gel-like pellet. The RNA containing pellet was washed in 1 ml 75% (v/v) cold ethanol to remove salts, then centrifuged at 7500 x g for 5 minutes at 4<sup>0</sup> C. The pellet was partially air dried at 25<sup>0</sup> C, then re-suspended in a minimal volume of de-ionized formamide by gentle pipetting at 25<sup>0</sup> C. The RNA concentration was determined by measuring absorption at 260 nm. As it is desirable to keep RNA concentration high (>1 µg / µl formamide), the minimum amount of formamide added was based on an estimated total RNA yield of 0.8 µg of RNA per mg wet spinal cord. Aliquots, containing 50 and 10 µg RNA each, were stored at -70<sup>0</sup>C.

Removal of DNA from RNA was performed to reduce the incidence of false positives due to amplification of genomic DNA. RNA aliquots, 50 µg, were thawed and four volumes of ethanol added. The samples were incubated at -70<sup>0</sup> C for 30 minutes and then centrifuged at 12000 x g for 15 minutes at 4<sup>0</sup> C. The supernatant was discarded and the pellets partially air-dried at room temperature, re-suspended in 40 µl of 50 mM Tris.HCl pH 7.5, then RNA concentration was

determined by measuring absorption at 260 nm. Immediately thereafter, contaminating DNA was digested by combining 10 units of RQI RNase free DNase I (Promega, Madison, WI), 10 units of ribonuclease inhibitor (Life Technologies, Gaithersburg, MD), 1 mM MnCl<sub>2</sub>, 4 mM dithiothreitol and 40 µg of the prepared total RNA in a total reaction volume of 100 µl.<sup>b</sup> The mixture was then incubated for 30 minutes at 37<sup>0</sup> C. The reaction was stopped in an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v). The aqueous fraction was re-extracted in an equal volume of chloroform to remove residual phenol, RNA was then precipitated by adding one ninth volumes of 3 M sodium acetate, pH 7.5, followed by 2.5 volumes of ethanol. The solution was mixed then cooled to -70<sup>0</sup> C for at least 30 minutes. RNA was collected by centrifugation at 12000 x g for 15 minutes at 4<sup>0</sup> C. The pellet was washed in 1 ml cold 80% ethanol to remove salts, partially dried at 25<sup>0</sup> C, then re-suspended in a minimum volume of 50 mM Tris.HCl, pH 7.5, prior to determination of concentration by absorption at 260 nm. One µg aliquots were frozen at -70<sup>0</sup> C in PCR tubes. Integrity of ribosomal RNA was evaluated by resolution of 1 µg total RNA from pre- and post- DNase I digests on a 1.5% denaturing formaldehyde-agarose gel. Subsequent steps were only performed if 28S and 18S ribosomal

---

<sup>b</sup> The use of Mn<sup>2+</sup> instead of Mg<sup>2+</sup> has been shown to result in digestion of DNA to produce blunt ends or protruding termini only 1-2 nucleotides in length. In the presence of Mg<sup>2+</sup> DNase I attacks each strand of DNA independently and the sites of cleavage are distributed randomly; this may lead to recombination during PCR.<sup>145</sup>

RNA bands were well defined, and if the staining of the 28S bands was more intense than the 18S bands.

*cDNA synthesis:* First strand cDNA was prepared using 1 µg aliquots of the DNase-treated total RNA and Superscript II reverse transcriptase (Life Technologies) in a total volume of 20 µl, according to the manufacturer's instructions. In brief, to 1 µg of total RNA was added 500 ng of oligo (dT<sub>17</sub>V), where V is A, G, or C (random mixture) in a total volume of 12 µl. The mixture was heated to 70° C for 10 minutes to denature secondary structure then chilled on ice prior to the addition of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 500 µM of each deoxy-nucleoside triphosphate, 2 units of ribonuclease inhibitor (Life Technologies) and 10 mM dithiothreitol in a total volume of 7 µl. This mixture was heated at 42° C for 2 minutes prior to the addition of 200 units of superscript II reverse transcriptase, followed by incubation for 50 minutes at 42° C. Superscript II was inactivated by incubation at 70° C for 15 minutes. Following generation of first strand cDNA, remaining RNA was removed by the addition of 2 units of RNase H (Life Technologies), followed by incubation at 37° C for 20 minutes. This step is not essential but was found to improve the clarity of the gel banding patterns. Complementary DNA was diluted to a volume of 150 µl in nuclease free water and 20 ng equivalents of starting RNA (3 µl) were aliquoted into 500 µl thin walled PCR tubes (Fisher) and stored at -20° C.

*Targeted differential display PCR:* Polymerase chain reaction was performed in a Hybaid Omn-E thermal cycler using 500 µl thin walled tubes and

a 25  $\mu$ l mineral oil (Sigma) overlay. The PCR mixture was prepared such that there were 1.5 mM MgCl<sub>2</sub>, 12  $\mu$ M of each deoxynucleoside tri-phosphate, 1 unit of ampli-taq gold (Perkin Elmer), 1  $\mu$ M each of sense and anti-sense primers, 15 mM Tris-HCl, pH 8.0, 50 mM KCl and 1.5  $\mu$ Ci [<sup>33</sup>P]- $\alpha$ -dATP (>2500 Ci/mmol, 10 mCi/ml) (Amersham, Piscataway, NJ) in a total volume of 20  $\mu$ l. To improve [<sup>33</sup>P]- $\alpha$ -dATP incorporation, nucleotide concentrations are much lower than compared to standard PCR. The PCR reaction mixture was prepared as a “super” master-mix containing all components except primers and cDNA; this mixture was then aliquoted into tubes containing sets of primer pairs. Seventeen  $\mu$ l of this mixture was added to PCR tubes already containing 3  $\mu$ l of cDNA. All primers were obtained de-salted at “standard” purity (Life Technologies). They were diluted to 100 ng /  $\mu$ l in nuclease free water and stored at -20<sup>0</sup> C. Cycle parameters were 95<sup>0</sup> C for 9 minutes 30 seconds (hot start); 35 cycles of 95<sup>0</sup> C for 30 seconds, 50<sup>0</sup> C for 45 seconds and 72<sup>0</sup> C for 45 seconds; with a final extension step of 10 minutes at 72<sup>0</sup> C. Primers used in this study are shown in Table 1.

*Acrylamide gel electrophoresis and autoradiography:* Labeled PCR products were resolved on a 0.4 mm thick, 6% (w/v) polyacrylamide gel containing 8 M urea at 1600 volts. It was found important to resolve PCR products the same day as the PCR reaction, otherwise radioactive decay of <sup>33</sup>P would lead to increased background due to DNA strand breaks. One gel plate

was treated with glue<sup>c</sup> and the other with chlorinated organopolysiloxane in heptane (Sigmacote®, Sigma). The gel was pre-run for about 30 minutes to warm the buffer, gel and plates. Six  $\mu$ l of PCR reaction was mixed with 8  $\mu$ l of sequencing gel loading buffer. Samples were boiled for 5 minutes, quick cooled on ice, and then loaded. The gel was run until the xylene cyanol dye was about to enter the lower buffer chamber (~4 hours). Based on the position of a  $^{33}\text{P}$ -labelled PCR generated product it was found that 100 bp DNA fragments were very close to the bottom of the gel. Following electrophoresis, the gel was washed in 10% (v/v) acetic acid with gentle agitation for 15 minutes to remove urea, briefly rinsed in water, and dried overnight at  $37^0\text{ C}$ . Corners of the dried gel were marked with a radioactive marker comprising 1 volume of labeled PCR mixture to 20 volumes of a xylene cyanol solution. The gel was then exposed to Kodak BioMax MS film (Amersham) for 24 to 72 hours at  $-70^0\text{ C}$ . An intensifying screen designed for use with low energy beta particle emissions (BioMax Transcreen LE, Amersham) was sometimes used; this reduced exposure times to 24 hours but resulted in decreased resolution. Candidate differentially expressed genes were defined as those that showed the same pattern of altered expression, when the particular PCR reaction was repeated on the same or different cDNA.

*Cloning PCR products into plasmid vector pCRII-TOPO:* Gel bands representing reproducible changes were excised by aligning the film over the

---

<sup>c</sup> Glue recipe: 19 mls ethanol, 1 ml water, 100  $\mu$ l  $\gamma$ -methacryloxypropyl-trimethoxysilane (Sigma), 60  $\mu$ l acetic acid.

marked spots, then cutting the acrylamide corresponding to the position of the band of interest using a sterile scalpel blade and forceps. A new scalpel blade was used for each band excised. The gels were re-exposed to film to verify that band excision was accurate. Dried acrylamide was placed in a sterile tube containing 20  $\mu$ l of a solution of 10 mM Tris.HCl, pH 8.3, and 50 mM KCl. The mixture was incubated at 25 $^{\circ}$  C for 10 minutes and then placed in boiling water for 20 minutes. The tube was then centrifuged to sediment debris and 2  $\mu$ l of supernatant liquid was used for subsequent PCR. This was performed the same day. The PCR mixture contained 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each deoxy-nucleoside triphosphate, 1  $\mu$ M of each primer, 15 mM Tris-HCl pH 8.0, 50 mM KCl and 0.5 units of ampli-taq gold in a final volume of 20  $\mu$ l. Cycle parameters were 94 $^{\circ}$  C for 9 minutes 30 seconds; and 40 cycles of 95 $^{\circ}$  C for 30 seconds, 50 $^{\circ}$  C for 30 seconds and 72 $^{\circ}$  C for 30 seconds; with a final extension step of 10 minutes at 72 $^{\circ}$  C. It was necessary to estimate approximate band molecular weights and determine band purity. PCR products were resolved in parallel with a 100 base pair DNA ladder on a 2 % (w/v) metaphor agarose (FMC Bioproducts, Rockland, ME) gel, stained with SYBR gold<sup>146</sup> (FMC Bioproducts) and visualized with 300 nm transillumination using an appropriate filter. If the band was considered sufficiently pure the PCR mixture was used directly in the cloning reaction, otherwise the band of interest was excised, and the DNA allowed to diffuse into nuclease free water overnight. Polymerase chain reaction products were cloned into the plasmid vector pCRII-TOPO (Invitrogen, Carlsbad, CA)

essentially according to the manufacturer's instructions. This plasmid can be used for sequencing and *in-vitro* transcription; it also incorporates lacZ  $\alpha$ -peptide sequence for blue / white color screening. The completed ligation reaction was transformed into chemically competent TOP10F' *Escherichia coli* and the resulting mixture plated onto LB-agar-ampicillin plates previously coated with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (Promega) and isopropyl- $\beta$ -D-thiogalactoside (Promega), then incubated overnight at 37° C.

*Colony PCR and identification of different clones from a given band:*

Because cloned DNA samples were likely heterogenous<sup>137</sup> it was considered necessary to screen all the different clones obtained rather than choosing one clone to screen for differential expression. Rather than choose 10 random clones to screen (some or even all of which may have been identical), we choose to identify those clones that were different as candidates for further study. Ten white colonies were chosen and pCRII inserts and flanking regions were amplified by colony PCR in a 20  $\mu$ l volume. Reactions were performed in a volume of 20  $\mu$ l containing 2 mM MgCl<sub>2</sub>, 0.2 mM of each deoxynucleoside triphosphate, 0.5 units of ampli-taq gold (Perkin Elmer), 1  $\mu$ M of M13+ (g t a a a a c g a c g g c c a g) and M13- (c a g g a a a c a g c t a t g a c) primers, 15 mM Tris-HCl pH 8.0, and 50 mM KCl. Each white colony was picked with a sterile pipette tip, touched onto marked squares in a fresh LB-agar-ampicillin plate, and then mixed by gentle pipetting in the PCR mixture. Cycle parameters were 95° C for 9 minutes 30 seconds (hot start); 35 cycles of 95° C for 30 seconds, annealing for 45

seconds and 72<sup>0</sup> C for 45 seconds; with a final extension step of 10 minutes at 72<sup>0</sup> C. The first annealing temperature was 59<sup>0</sup> C. At subsequent cycles the annealing temperature was reduced by 0.5<sup>0</sup> C increments until an annealing temperature of 55<sup>0</sup> C was reached. There were 27 cycles with annealing temperature of 55<sup>0</sup> C. High but decreasing stringency in the first 8 cycles was used to increase the specificity of primer binding and DNA amplification in the first few cycles (touch down PCR).

The first step in identification of different clones of near identical length was to rule out those clones that were significantly different from the correct length from the agarose gel analysis. This step was important because the PCR mixture was generally used directly in the cloning reaction so “background” DNA generated during this PCR reaction could potentially be cloned. Inserts of the correct length were kept for further analysis; any remaining ones were discarded. The second step is to identify different clones and flanking regions that are near identical in length. To do this colony PCR products were resolved twice on 1.5% (w/v) agarose gels containing bisbenzimide-PEG (H.A.-Yellow, Hans-Analytik, Germany) or phenyl neutral red-PEG (H.A.-Red, Hans-Analytik). These compounds can induce differential migration of equally sized DNA species based on sequence differences. They act as DNA ligands that bind specific sequence motifs and thus alter frictional coefficients of DNA within the gel according to differences in sequence.<sup>147</sup> Bis-benzimide-polyethylene glycol (PEG) polymer interacts with A plus T sequence motifs. Phenyl neutral red-PEG interacts with G

plus C sequence motifs.<sup>141</sup> Bands that migrated differently in either of the two gels were interpreted to represent different clones.

### **Northern blotting**

*RNA preparation:* Ten µg of total RNA was fractionated under denaturing conditions using a 1.1% (w/v) agarose horizontal gel containing 0.22 M formaldehyde, 20 mM 3-(N-morpholino)-propanesulfonic acid, 5 mM sodium acetate, pH 7.0, and 1 mM di-sodium EDTA, at 5 volts / cm. The RNA was transferred to nylon (Zeta-Probe® GT, Bio-Rad, Hercules, CA) by capillary blotting overnight in 1.5 M sodium chloride and 150 mM sodium citrate pH 7.0, then incubated at 80<sup>0</sup> C for 30 minutes to cross-link RNA to nylon. Membranes were stored dry at 25<sup>0</sup> C until required.

*Probe preparation and labeling:* Probes for analysis of candidate genes from TD were made by M13 primer amplification of the appropriate plasmid clone. All other probes were produced by PCR using gene specific primers. Forward and reverse primers used were: 18S rRNA (ccggggcattcgattgtg, cgggccgggtgaggttc), vimentin (gcgccaggccaaacaagaa, cccccacagtaagccaaacaca), α-tubulin (cgtccccgtccgtcttgc, ggggcgtcgatgtccagggttc), neurofilament-M (ctgcggggcgagaaacg, gtccaccccccggccctacctcag), CaM-Kin II α-subunit (gaccggggatgactgtttgag, ggggtggagatggatgttgta), and *trkB* (agcggactgtggattcgttg, aaccgcgttcatcctctccata). All probes were quantified by gel electrophoresis. Alpha-tubulin product was cloned as described earlier. Twenty-five ng of probe DNA was labeled using 50 µCi [ $\alpha$ -<sup>32</sup>P]-dATP (3000

Ci/mmol) (Amersham) by random priming in a total volume of 20  $\mu$ l using Strip-EZ<sup>TM</sup> DNA (Ambion, Austin, TX). In brief, this variation of the random primer labeling approach uses random decamers, a 3'-5' exonuclease free klenow enzyme, and a modified dCTP that allows for easy membrane stripping and re-probing. Low molecular weight  $^{32}$ P containing compounds were removed using a G50 spin column (ProbeQuant<sup>TM</sup> G-50 Micro Columns, Amersham).

*Hybridization:* Membranes were pre-hybridized by soaking in ULTRAhyb<sup>TM</sup> (Ambion) for 30 minutes at 42<sup>0</sup> C. The labeled probe was denatured, by heating to 95<sup>0</sup>C for 5 minutes, it was then added to the pre-hybridization solution and the mixture incubated overnight at 42<sup>0</sup> C. Membranes were then washed twice for 5 minutes at 42<sup>0</sup> C in 0.1% SDS, 0.03 M sodium citrate, pH 7.0, and 0.3 M sodium chloride. This was followed by two high stringency washes for 15 minutes at 42<sup>0</sup> C in 0.1% SDS, 1.5 mM sodium citrate pH 7.0 and 0.015 M sodium chloride. Wet membranes were wrapped in Saran wrap®. Kodak BioMax MS film (Amersham) was pre-flashed using a Sensitize<sup>TM</sup> pre-flash unit (Amersham), then exposed to membranes with an enhancer screen (BioMax Transcreen HE, Amersham) at -70<sup>0</sup> C for 24-72 hours. Pre-flashing light exposure had been calibrated to ensure a linear response of film. As necessary, membranes were stripped using Strip-EZ<sup>TM</sup> DNA (Ambion), and then re-probed with other candidate genes. Membranes were finally probed with the constitutively expressed chicken 18S rRNA. Autoradiographs were digitized

and bands quantified by densitometry using NIH image software. Data analyzed represented gene band densities as a ratio of 18S rRNA densities.

*Identification of differentially expressed mRNA transcripts:* Sequencing was contracted to the Virginia Tech Bioinformatics Institute. Sequence homology searches were performed through the National Center for Biotechnology Information (NCBI) using the BLAST network service.<sup>148, 149</sup>

## **Results**

### **Clinical signs**

No clinical signs were detected in the period between dosing and euthanasia 24 hours later. Clinical evidence of OPIDN was present in sentinel hens treated with PSP at 7-9 days.

### **Brain esterase activities**

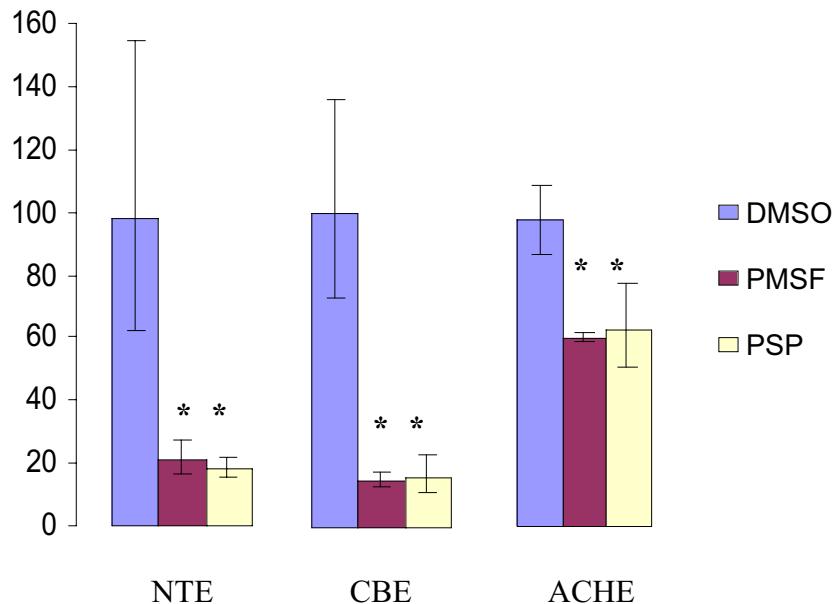
Data was log normally distributed so results are presented as geometric means and confidence intervals (Figure 4). While treatments PSP and PMSF produce in similar effects on NTE activity, only PSP results in development of OPIDN.

### **Targeted display**

One hundred and fifty primer combinations were screened. Most primer combinations produced about 100 bands per gel, in the size range 100-600 bp (Figure 5). Band patterns exhibited good reproducibility when PCR reactions and gels were repeated using the same, or different cDNA from the same hen. Three putative differentially expressed genes were identified. These were designated P1, P2 and P3 (Figure 6). PCR and gels were repeated at least once and banding pattern reproducibility was confirmed before proceeding to Northern analysis. P1 and P3, and P2, were down and upregulated, respectively, in PSP as compared to DMSO treated birds. Occasionally there were ‘aberrant’ bands characterized by strong presence or absence of a band in one hen only. The banding patterns of the gels were always reproducible. Heterogeneity of the three bands that were

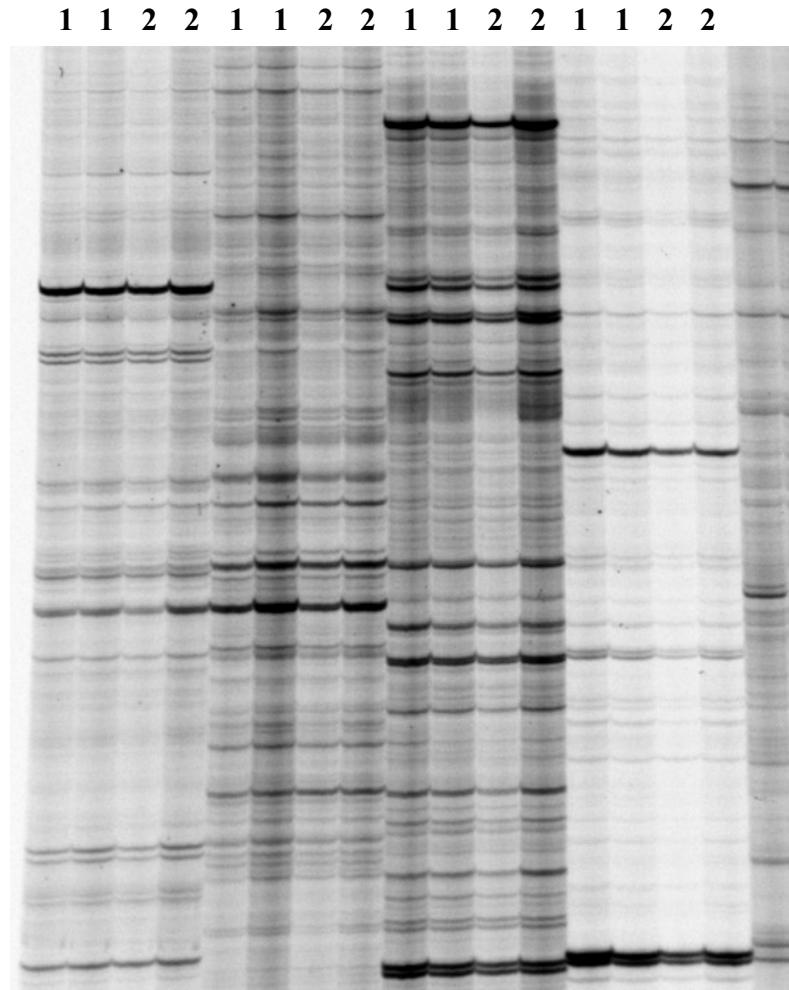
excised and processed for Northern blotting was not detected following screening using DNA binding ligands (Figure 7).

**Figure 4. *Gallus gallus* brain esterase activities 24 hours post dosing with PSP, PMSF and DMSO**



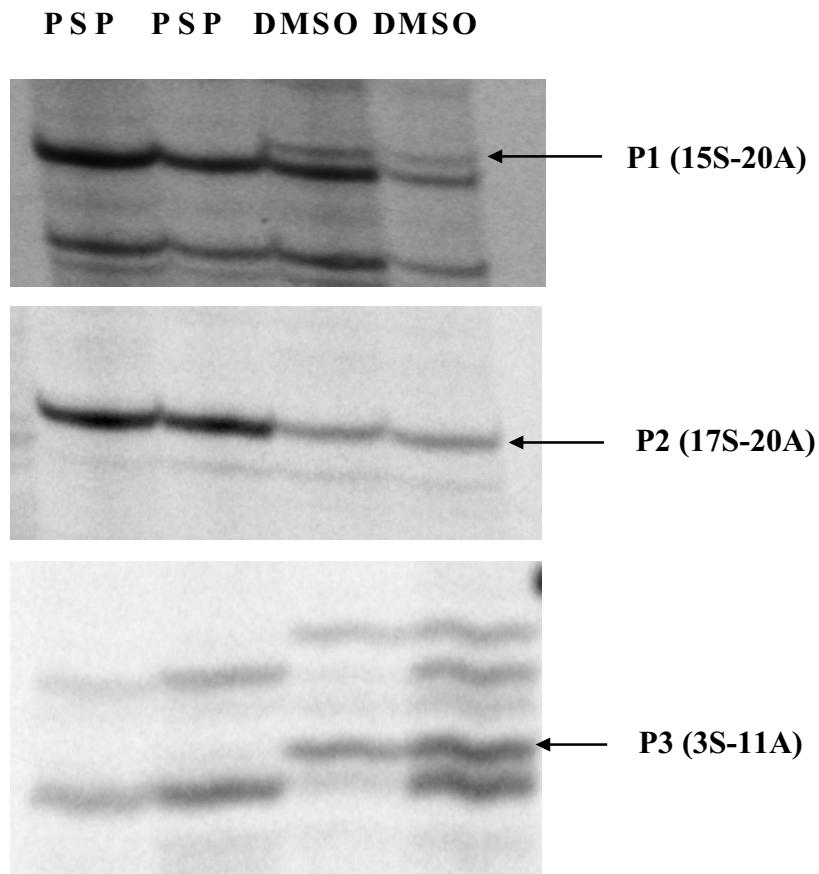
Whole brain esterase activities 24 hours after dosing im with PSP (2 mg / kg), PMSF (90 mg / kg) or DMSO (0.5 mls / kg). Data are expressed as percentage of DMSO controls (n=3). Geometric means and 95% confidence intervals are shown. Log<sub>e</sub> transformed data was analyzed using the GLM procedure of SAS (version 6.12, SAS Institute Inc., Cary, NC). Contrasts were used to compare PSP and PMSF treatment groups with controls for each esterase. An asterisk indicates significant difference from DMSO controls (p<0.01). Although statistically significant inhibition of acetylcholinesterase was observed, hens did not show clinical signs of acute cholinergic neurotoxicity. NTE = neurotoxic esterase, CBE = carboxylesterase, ACHE = acetylcholinesterase.

**Figure 5. Targeted Display Autoradiograph**



Shown is part of a targeted display gel autoradiograph. Each set of four lanes represents different primer combinations. Lanes: 1 = PSP, and, 2 = DMSO treated hens. Within each set of four lanes, the same number represents independently treated hens. Putative differentially expressed genes are not present in this autoradiograph.

**Figure 6. Putative Differentially Expressed Genes**



Portions of three targeted display autoradiographs are shown. Putative differentially expressed genes are designated P1, P2 and P3. The primer combinations used for each rtPCR reaction are shown in parentheses. The above banding patterns were repeatable using the same, or different cDNA, from the same hens.

**Figure 7. Processing Differentially Expressed Genes**

Fig. 7a

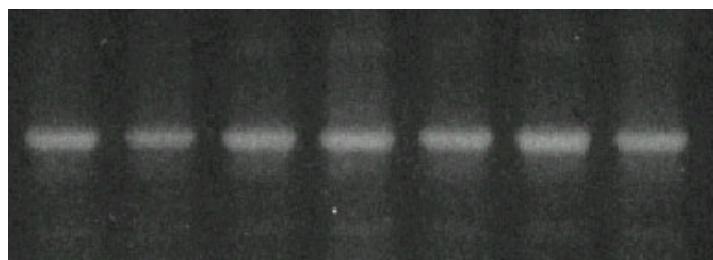
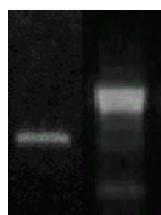


Fig. 7b



Up to 10 colony PCR products were resolved on an agarose-HA red and agarose-HA yellow gel to determine whether inserts of the same length represented different sequences. Figure 7a shows a HA-yellow gel containing seven PCR products that had all migrated the same distance, suggesting they represent the same sequence. Using HA-yellow / red agarose gels, no evidence was found that cloned products representing P1, P2 and P3 were heterogenous. Figure 7b shows a positive control reaction. Both lanes represent an identical PCR reaction run on a normal agarose gel (left lane), or a HA-yellow / agarose gel (right lane). The control sample was produced by excising a longitudinal region of gel comprising of more than one band.

### **Northern blotting**

Spinal cord transcript was not detected for *trk A*, *trk C*, and NGF- $\beta$ . Attempts were made using [ $^{32}$ P]-labeled DNA probes as described, and RNA probes (method not described). All other genes were detected with varying degrees of ease using [ $^{32}$ P]-labeled DNA probes.

The  $\alpha$ -tubulin probe identified two bands approximately 2.5 and 4.5 kb in length. The 2.5 kb  $\alpha$ -tubulin transcript showed significant ( $p<0.001$ ) down regulation in the PSP (38%) and PMSF (28%) treatment groups compared to the DMSO controls (Figure 8). Statistically significant expression changes or possible trends towards significance were not observed for the 4.5 kb transcript, or for vimentin, *trk B*, neurofilament-M, and CaM-Kin II  $\alpha$ -subunit (data not shown).

The putatively differentially expressed gene P1 was estimated to be about 6-7 kb in length based upon its position relative to 18S and 28S rRNA bands. In PSP treated hens it was down regulated 26% ( $p=0.0278$ ) compared to DMSO controls (Figure 9). Significant changes in expression of this transcript in PMSF treated birds were not identified.

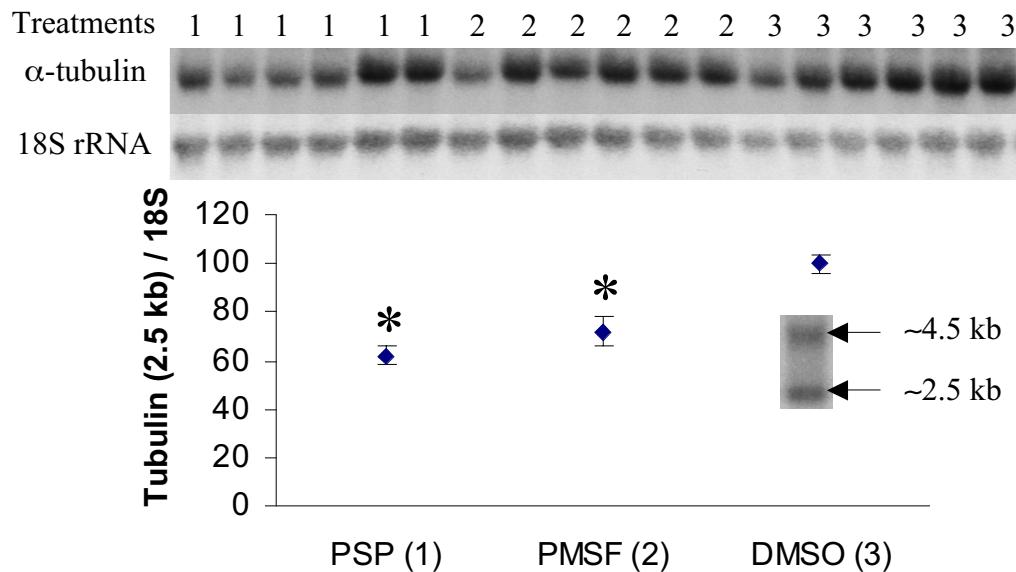
The transcript P2 was long. Its approximate size could not be estimated because it was much longer than the ~5 kb 28S rRNA band, and larger size markers were not used. Mean expression values for PSP and PMSF treated hens compared to DMSO treated controls were 14% and 12% lower. However, these effects were not statistically significant ( $p<0.05$ ). There was considerably more

variability in the response to PSP, than to PMSF and DMSO. Coefficients of variation for PSP, PMSF and DMSO treatment groups were 60%, 20% and 14%, respectively.

Transcript P3 was estimated to be about 5-6 kb in length. Significant mean expression changes for P3 transcript in PSP and PMSF treated hens compared to DMSO treated birds were not identified. Mean expression of P3 transcript in PSP treated birds was decreased 22% compared to DMSO controls. The p value was just greater than 0.05 suggesting a trend towards statistical significance.

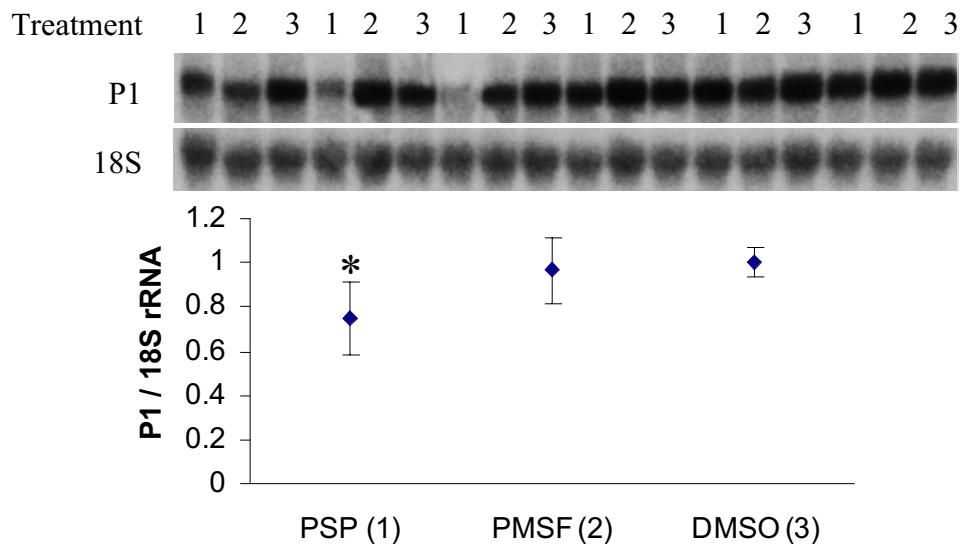
All three of the differentially expressed genes were sequenced. P2 represented an open reading frame with 82% DNA homology to human KIAA1307. This gene was identified from a large size fractionated human brain cDNA library.<sup>150</sup> P1 and P3 did not contain significant open reading frames on either strand. P1 has 100% homology to a region of expressed sequence tag designated pgp1n.pk010.m23 (see Appendix B).

**Figure 8. Expression of Spinal Cord  $\alpha$ -Tubulin mRNA (2.5 kb) 24 hours post exposure to PSP, PMSF, and DMSO**



The  $\alpha$ -tubulin probe used revealed two transcripts  $\sim$ 2.5 kb and  $\sim$ 4.5 kb in length. Only results for the 2.5 kb transcript are shown here. Ratios of  $\alpha$ -tubulin to 18S rRNA were taken to normalize for variability in gel loading. Graph shows mean  $\pm$  SEM of these ratios. Treatments PSP, PMSF or DMSO are represented by the numbers, 1, 2, and 3, respectively. There is significant ( $p < 0.001$ ) down regulation of  $\alpha$ -tubulin transcript in PSP and PMSF treated hens of 38%, and 28%, respectively ( $n=6$ ). Significant differences were not identified for the 4.5 kb transcript.

**Figure 9. Expression of Spinal Cord P1 mRNA 24 hours post exposure to PSP, PMSF, and DMSO**



The P1 probe used revealed one transcript of approximately 6-7 kb in length. Ratios of P1 to 18S rRNA were taken to normalize for variability in gel loading. Graph shows mean  $\pm$  SEM of these ratios. Treatments PSP, PMSF, and DMSO are represented by the numbers, 1, 2, and 3, respectively. There is small (26%), but statistically significant ( $p=0.0278$ ) down regulation of P1 transcript in PSP versus DMSO treated hens ( $n=6$ ). Within the PSP treatment group not all birds responded the same (see discussion).

## **Discussion**

Previous work reported that the neuropathy-inducing OP ester DFP induces changes in the expression of mRNA transcripts for chicken spinal cord neurofilaments L, M and H,<sup>11,12</sup> vimentin,<sup>9</sup> GFAP,<sup>9</sup>  $\alpha$ -tubulin,<sup>10</sup> c-fos,<sup>11</sup> c-jun,<sup>12</sup> and CaM-Kin II  $\alpha$ -subunit.<sup>7</sup> However, whether changes in gene expression are mechanistically relevant events to OPIDN is not known. If similar expression changes occur with other OPIDN inducing compounds, then this could provide supportive evidence that the changes are of some significance. In this study, the expression of transcripts for neurofilament-M,  $\alpha$ -tubulin, vimentin, and CaM-Kin II were determined 24 hours after exposure to an OPIDN inducing dose of PSP. Expression changes were only identified for an ~2.5 kb  $\alpha$ -tubulin transcript, which was down regulated 38%. An  $\alpha$ -tubulin transcript was down regulated 4 and 69%, in the spinal cord of DFP treated chickens at 24 and 48 hours, respectively.<sup>10</sup> While it is not known whether these  $\alpha$ -tubulin transcripts are identical, it does provide associative evidence that these expression changes could be of relevance to OPIDN. However, in this study PSP induced expression changes appeared to be mirrored by a similar change, albeit of less magnitude, in PMSF treated birds. This suggests that this response could be the result of a non-OPIDN specific mechanism, and thus is insufficient or perhaps even unnecessary for axonal degeneration. Clearly, the relevance of expression changes of  $\alpha$ -tubulin needs further clarification. This will depend initially on determining

whether expression changes occur at the protein level. Alpha-tubulin is a major constituent of microtubules, which form a scaffold for the structural organization of the cell interior, and for highways for fast axonal transport. The effect a small expression change of this protein might exert on fast orthograde, or retrograde axonal transport, or other processes, is not known. It is possible that a non-neuropathic NTE inhibitor such as PMSF may induce changes in fast axonal transport, even though manifestation of neurotoxicity does not occur. The issue is discussed further in chapter 5.

Following PSP exposure expression changes were not detected in mRNA encoding for neurofilament-M 12-48 hours later, or for vimentin and CaM-Kin II 24 hours later (data not shown) even though DFP induced responses in these mRNA transcripts,<sup>7-9</sup> indicating that not all OPIDN inducing compounds induce the same expression changes. This is not surprising given the structural diversity of OPIDN inducing compounds,<sup>2, 40</sup> and other differential effects of these compounds, such as on calcium-magnesium activated ATPase<sup>81</sup> and acetylcholinesterase.<sup>151</sup>

The work of Pope *et al*<sup>13</sup> showed that spinal cord extracts from chickens exposed to PSP were able to promote neuritic outgrowth in human SH-SY5Y neuroblastoma cells. This provided the rationale for investigating neurotrophic factor / receptor expression in this study. The failure to identify transcripts for *trk A*, *trk C* and NGF-β indicates that these genes are expressed at a low level in the

adult hen (GG) spinal cord. More sensitive approaches such as quantitative PCR may be needed to evaluate these gene transcripts. More work may be indicated in this area.

The targeted display provided evidence for low frequency and low amplitude gene expression changes occurring in this model 24 hours after PSP exposure. Only for gene P1 were statistically significant mean expression changes identified in confirmatory Northern blotting experiments. The lack of parallel expression changes did not occur in PMSF treated birds, suggesting that this change could be related to NTE inhibition and aging. This change is investigated further in chapters 3 and 4, and discussed in chapter 5. For P2 there was increased variability of response in the PSP treatment group, but the mean change was not increased, as it was in the TD autoradiographs. It is thought unlikely that the scarcity of findings relates to failure of this method. Targeted display autoradiograph banding patterns were found to have good reproducibility, even when using different cDNA prepared at different times from the same RNA. However, the exact number of different genes screened is not known, because the rate of redundancy in the banding pattern can only be estimated. It is possible that there are more changes in gene expression that were not identified using this approach.

Targeted display was chosen to screen cDNA for this study over differential display (DD), because it was thought that it would expedite the process of gene identification, due to amplification of protein coding regions

(open reading frames) of mRNA, rather than 3-prime un-translated regions. Of five bands that were isolated and sequenced, two represented open reading frames (ORF). While this is an improvement over DD, it did not give quite the same level of success in ORF identification that was reportedly achieved when this technique was applied on a rat cell line.<sup>114</sup> This may be a reflection of the fact that birds are phylogenetically more distant than rats to humans, the species for which these primers were designed.

It was expected that the bands isolated would be heterogenous based on results reported with DD.<sup>138</sup> Fortunately, evidence of this was not found when colony PCR reactions were screened using HA-red / yellow agarose gels. It is possible that heterogeneity was missed; this could occur if different DNA species of the same length contained the same frequency of specific motifs. However, it is thought more likely that the lack of band heterogeneity is a reflection of the primers, annealing temperature, and by ‘chance’ short lengths of PCR products of interest (see Appendix B). Shorter products migrate further through the gel resulting in better resolution, reducing the chance of cross contamination of bands during excision.

The occurrence of ‘aberrant’ bands representing large variation between hens in the same treatment group in the TD autoradiographs is interpreted to result from variability in gene expression between individual birds, because these changes were always reproducible. Variability in expression and / or response to PSP is also evident in the Northern blotting (Figures 8 and 9) even though some

changes were statistically significant. The reason(s) for this are not known, but it is thought most likely the result of individual hen variability. It is possible that all the hens show the same response to PSP, but that they would be variable in the time of onset of this change post-exposure. Alternatively, not all hens may exhibit the same pattern of gene responses to PSP. If a gene expression change is necessary for OPIDN induction, then it should occur in all treated birds. While there are strain differences in susceptibility to OPIDN in White Leghorn *G. gallus*,<sup>152</sup> highly variable clinical responses to PSP within a strain have not been reported.

Remaining work in this dissertation focuses on further characterizing the gene expression changes identified in this chapter. In chapter 3 temporal aspects of gene expression change are characterized. Chapter 4 examines cellular expression and differential expression of these genes.

## **CHAPTER 3: Temporal Changes in Phenyl Saligenin Phosphate-Induced Spinal Cord Expression of $\alpha$ -Tubulin, P1, P2 (KIAA1307) and P3 Transcripts**

### **Introduction**

Exposure to any one of a sub-set of organophosphorus (OP) esters results in degeneration of distal regions of long, large diameter axons within the central and peripheral nervous system of the adult domestic fowl (*Gallus gallus*) 7-21 days after a single dose exposure.<sup>2</sup> This toxicosis is called organophosphorus induced delayed neurotoxicity (OPIDN). It is readily induced by phenyl saligenin phosphate (PSP) and di-isopropyl-phosphorofluoridate (DFP). OPIDN is always preceded by essentially irreversible inhibition of neurotoxic esterase (NTE) and aging of the bound OP compound.

Changes in spinal cord mRNA transcript expression have been demonstrated to occur 24 hours after OPIDN inducing doses of PSP (chapter 2). Expression changes were observed for an ~2.5 kb transcript encoding  $\alpha$ -tubulin, and for an unknown gene transcript, named P1. However, expression changes were not detected for several cytoskeletal genes that have previously been reported to be altered at this time following exposure to DFP, for example, neurofilament-M.<sup>8</sup> There are several possible explanations for this lack of concordance between findings with PSP and DFP. These include the possibilities that, 1.) gene expression responses may differ temporally between PSP and DFP, and, 2.) expression changes for these genes may occur with DFP, but not with all

other OPIDN inducing compounds. DFP differs from PSP in that it inhibits acetylcholinesterase as well as NTE, and that neuropathy inducing doses of DFP are sufficient to cause cholinergic poisoning due to acetylcholinesterase inhibition.<sup>151</sup>

In studies reported previously (chapter 2) gene P1 was identified as being down regulated in spinal cord of hens 24 hours after treatment with PSP, but not in hens treated with phenylmethylsulfonyl fluoride. PMSF is not an OP ester, but it will inhibit NTE, although this inhibition is more readily reversed than that caused by OP esters.<sup>37</sup> The gene transcript designated P1 was identified by screening ~15000 gel bands using targeted display (TD). Transcripts P2 and P3 were also identified by TD. However, subsequent Northern blot measurements while indicating some alterations in PSP treated birds, did not reveal statistically significant alterations.

Studies reported in chapter 2 noted that  $\alpha$ -tubulin and P1 transcripts were down regulated 38% and 26%, respectively. Possible explanations for the low magnitude of the observed changes were considered and included, 1.) sub-optimal time point for identifying gene expression changes, 2.) expression changes only occurring in a sub-population of the cells that express the gene resulting in a dilution effect, and, 3.) large magnitude spinal cord transcript expression changes may not occur in PSP induced OPIDN .

In this section temporal issues of transcript expression are addressed using the same experimental system as described in chapter 2. Firstly, the issue of

whether larger expression changes of  $\alpha$ -tubulin and P1 transcripts occur at time points other than 24 hours is addressed. Secondly, the issue of whether there are temporal differences in gene expression for mRNA encoding for neurofilament-M was examined. This transcript is increased in spinal cord 24 hours after exposure to DFP, but not after exposure to PSP.

## **Specific Question and Rationale**

### **1. What is the temporal pattern of changes in expression for $\alpha$ -tubulin and P1 gene transcripts?**

An ~2.5 kb transcript for  $\alpha$ -tubulin and a second transcript designated P1 both exhibited low level decreased expression in the spinal cord of hens 24 hours after PSP exposure (chapter 2). However, nothing is known concerning the expression of these genes at other times following exposure. Information regarding the initiation and persistence of these alteration(s) in gene expression, and the magnitude of expression change may provide additional insights into the mechanisms of OPIDN.

While expression changes previously identified for P2 and P3 by TD were not statistically significant on subsequent Northern blots (see chapter 2), these genes were also studied further. As Northern blots were performed on separate birds from those analyzed by TD, it was hypothesized that the failure to find significant changes may be the result of variability in temporal gene expression response between experiments, due to unknown factors. Examination of P2 and P3 gene expression over several time points may help to resolve this issue.

The experiment designed to address this and the following question examines transcript expression at 12, 24, 36 and 48 hours. These time points were chosen for a variety of reasons. Firstly, as some transcripts can be up or down regulated rapidly, and then return to normal levels, it was considered important that the time points be not too distant from one another. Secondly, the

goal was to concentrate in the period prior to that at which morphologic, electrophysiologic or clinical changes occur, i.e. 4 days after exposure to PSP.<sup>123</sup>

## **2. Do PSP induced hen spinal cord neurofilament-M transcript expression changes occur in the 12-48 hour period?**

Numerous gene transcripts have been reported to exhibit changes in expression in the spinal cord of hens 24 hours after dosing with DFP. These genes encode several cytoskeletal proteins,<sup>8-10</sup> transcription factors,<sup>11, 12</sup> and a protein kinase.<sup>7</sup> If these alterations are necessary for OPIDN induction, then they should be observed following exposure to any OPIDN inducing compounds, at the same or similar time points. If changes in neurofilament-M expression do not occur then it suggests that expression changes are the result of DFP induced, but not OPIDN related effects. Spinal cord expression changes were not observed for neurofilament-M, vimentin or calcium calmodulin protein kinase II (CaM-KinII) 24 hours after dosing with PSP, even though expression changes had been reported after DFP exposure.<sup>7-9</sup>

Neurofilament-M transcript was reported to be upregulated ~225% in the spinal cord of DFP treated hens 24 hours post-exposure.<sup>8</sup> Western blot analysis revealed that this change in transcript level was reflected at the protein level.<sup>112, 153</sup> The transcript encoding for neurofilament-M was chosen to address this question in the present studies because this gene exhibits the highest changes in expression at 24 hours in DFP treated hens compared to other genes reported to be

altered in this system. These issues were addressed using the same experiment used to address question 1 above.

## **Experimental Design and Analysis**

A factorial design with two treatment factors and blocking was used.<sup>133</sup> Treatment factors were compound (PSP, PMSF or DMSO) and time (12, 24, 36, 48 hours). The experiment was performed in three blocks to overcome logistic problems in sacrificing numerous birds simultaneously. Within each block\*compound\*time combination, 1-2 birds were present. There were 3-5 hens per treatment group. Data was analyzed by performing pre-planned comparisions of PSP and PMSF with DMSO controls at each time point, using the general linear model procedure in SAS (version 6.12, SAS Institute, Cary, NC).

## **Materials and Methods**

Adult female White Leghorns (in lay) were acclimatized for 7-10 days at 25<sup>0</sup>C with an 8 hour light 16 hour dark cycle. Unlike experiments reported in chapter 2, hens were in lay. There was therefore known variability between experiments. Dosages were identical to those used previously (PSP 2 mg/kg, PMSF 90 mg/kg, DMSO 0.5 ml/kg all by im injection). Euthanasia was by intravenous pentobarbital injection. Spinal cords were snap frozen in liquid nitrogen, and maintained there until RNA extraction (4-66 hours later). Brains were frozen on dry ice, then stored at -70<sup>0</sup>C until prepared for the measurement of esterase activities. Standard Northern blotting procedures were used to evaluate transcript expression as described in chapter 2. Total RNA was resolved on a formaldehyde-agarose gel prior to transfer and hybridization with the <sup>32</sup>P-labelled probe of interest. To normalize for differences in gel loading blots were then probed to determine 18S rRNA expression. Linearity of film response was ascertained by excising bands, counting radioactivity using a scintillation counter, and plotting log (cpm) transformed scintillation counts against the corresponding densitometry measurements. Ribosomal RNA (18S) bands were also counted for some of the blots. Ratios of cpm for gene of interest / cpm 18S rRNA were determined whenever gene transcripts were not superimposed or contiguous on the Northern blots.

## **Results**

### **1. What is the temporal pattern of transcript expression change for $\alpha$ -tubulin and P1 gene transcripts?**

Alterations were observed in the expression of both  $\alpha$ -tubulin transcripts. The quantity of the ~2.5 kb transcript increased as a function of time in all treatment groups (Figure 10). While PSP treated birds exhibited lower levels of expression of the ~2.5 kb transcript as compared to DMSO controls, this difference was only statistically significant at 48 hours (33% decrease,  $p=0.0479$ ). There was 18% down regulation at 24 hours but this was not statistically significant. Though expression changes were not statistically significant at 24 hours, changes tended toward significance in the same direction as reported in chapter 2. Previously, changes in expression of the ~4.5 kb  $\alpha$ -tubulin transcript were not identified at 24 hours. Results from this experiment support those findings (Figure 11). However, alterations were found before and after 24 hours. In PSP treated hens at 12 hours this transcript was upregulated 38% ( $p=0.0125$ ), while at 48 hours it was down regulated 28% ( $p=0.0576$ ). The expression change noted at 12 hours was not mirrored by a similar change in PMSF treated birds. At 48 hours in PMSF treated hens, the 4.5 kb  $\alpha$ -tubulin transcript was down regulated 42% ( $p=0.0062$ ).

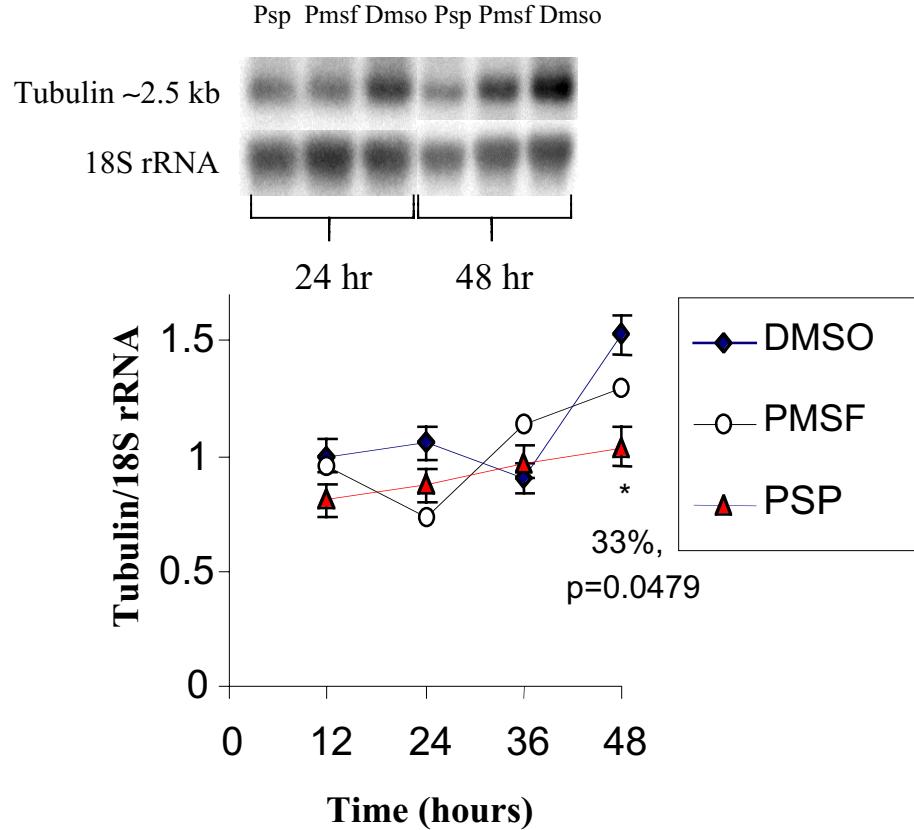
Gene P1 was down regulated 26% ( $p=0.0278$ ) at 24 hours in the previous experiment (chapter 2). In this experiment, down regulation (22%) was evident at 12 hours only ( $p=0.0011$ ) (Figure 12). Gene P2 was upregulated 26% ( $p=0.0055$ )

at 12 hours (Figure 12). However, expression data from this gene appears to contain a large amount of variability between treatments. Gene P3 did not show any significant treatment effects. However, expression at 12 hours was decreased for all treatments compared to combined treatment expression at other time points ( $p<0.01$ ) (Figure 14). Expression ratios generated by scintillation counting (data not shown) were found to be in close agreement to densitometry data.

**2. Do PSP induced hen spinal cord neurofilament-M transcript expression changes occur in the 12-48 hour period?**

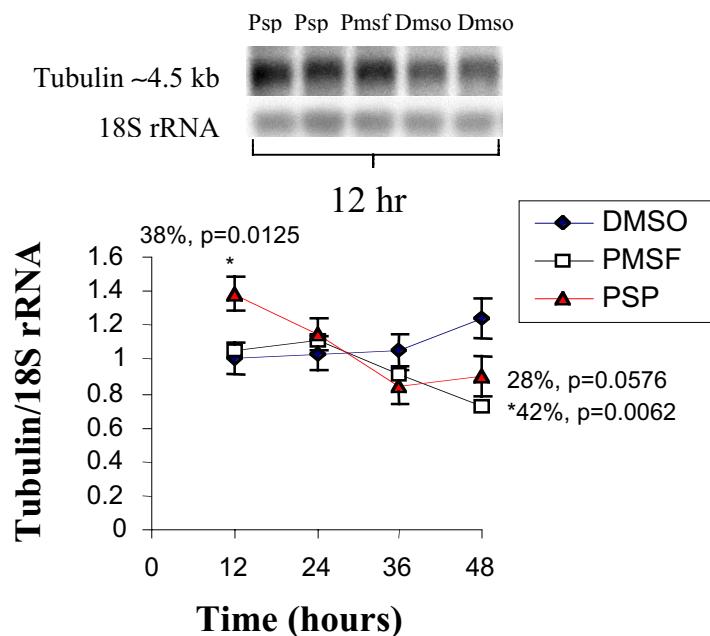
Significant differences, or possible trends towards significance, were not detectable between PSP or PMSF, and DMSO treatment groups (data not shown).

**Figure 10. Response of  $\alpha$ -tubulin (~2.5 kb) transcript levels in spinal cord to treatment with neuropathic esterase inhibitor (PSP), non-neuropathic esterase inhibitor (PMSF), and vehicle (DMSO)**



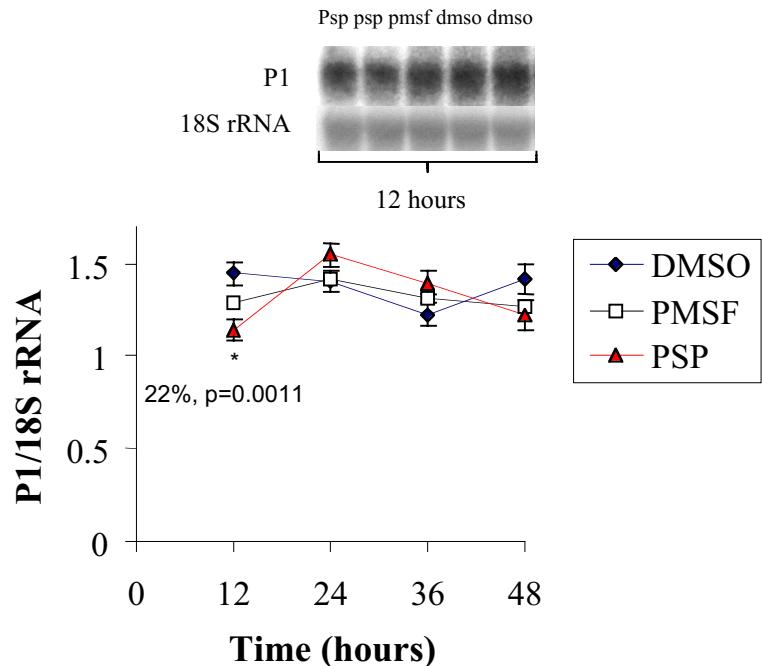
Shown is expression of an ~2.5 kb  $\alpha$ -tubulin transcript in the spinal cords of hens 12-48 hours after treatment with PSP, PMSF or DMSO. Least square means  $\pm$  SE are shown. PSP treated birds at 48 hours have significantly less transcript than DMSO birds (33% decrease,  $p=0.0479$ ). *Post-hoc* contrasts reveal that, 1.) PSP mean (averaged across time) is significantly lower (18%,  $p=0.0204$ ) than DMSO mean (averaged across time), and, 2.) there is significant increase in transcript level across time when averaged across treatments. The 48 hour mean is 36% greater than 12 hour mean ( $p=0.0008$ ).

**Figure 11. Response of  $\alpha$ -tubulin (~4.5 kb) transcript levels in spinal cord to treatment with neuropathic esterase inhibitor (PSP), non-neuropathic esterase inhibitor (PMSF), and vehicle (DMSO)**



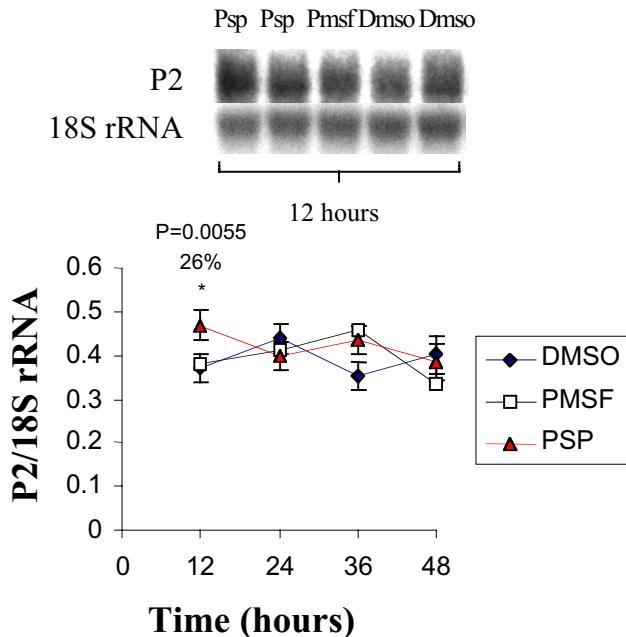
Shown is expression of an ~4.5 kb  $\alpha$ -tubulin transcript in the spinal cords of hens 12-48 hours after treatment with PSP, PMSF or DMSO. Least square means  $\pm$  SE are shown. PSP treated birds at 12 and 48 hours have 38% more and 28% less transcript than DMSO birds ( $p=0.0125$  and  $0.0576$ , respectively). PMSF treated birds have 42% less transcript at 48 hours compared to DMSO birds ( $p=0.0062$ ). Autoradiograph shows representative findings from one of three blocks.

**Figure 12. Response of P1 transcript levels in spinal cord to treatment with neuropathic esterase inhibitor (PSP), non-neuropathic esterase inhibitor (PMSF), and vehicle (DMSO)**



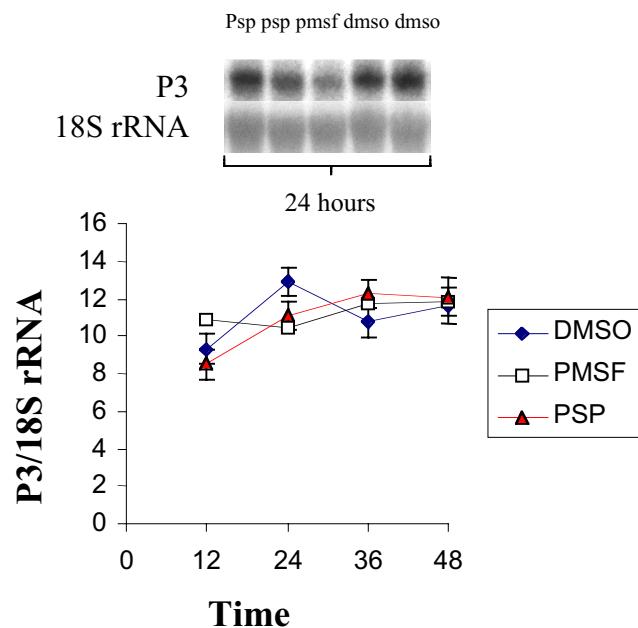
Shown is expression of a gene P1 transcript in the spinal cords of hens 12-48 hours after treatment with PSP, PMSF or DMSO. Least square means  $\pm$  SE are shown. PSP treated birds at 12 hours have 22% less transcript than DMSO birds ( $p=0.0011$ ). Autoradiograph shows representative findings from one of three blocks.

**Figure 13. Response of P2 transcript levels in spinal cord to treatment with neuropathic esterase inhibitor (PSP), non-neuropathic esterase inhibitor (PMSF), and vehicle (DMSO)**



Shown is expression of a gene P2 transcript in the spinal cords of hens 12-48 hours after treatment with PSP, PMSF or DMSO. Least square means  $\pm$  SE are shown. PSP treated birds at 12 hours have 26% more transcript than DMSO birds ( $p=0.0055$ ). Autoradiograph shows representative findings from one of three blocks.

**Figure 14. Response of P3 transcript levels in spinal cord to treatment with neuropathic esterase inhibitor (PSP), non-neuropathic esterase inhibitor (PMSF), and vehicle (DMSO)**



Shown is expression of a gene P3 transcript in the spinal cords of hens 12-48 hours after treatment with PSP, PMSF or DMSO. Least square means  $\pm$  SE are shown. Significant treatment effects are not present at any time points. The 12 hour mean (averaged across treatments) is significantly less than 24, 36, 48 (20% decrease) hour means ( $p=0.0009, 0.0061, 0.0045$ , respectively), as revealed by *post-hoc* contrasts. Autoradiograph shows representative findings from one of three blocks.

**Table 2. Summary of gene expression data**

	<b>α-tubulin ~2.5kb</b>	<b>α-tubulin ~4.5kb</b>	<b>P1 transcript</b>	<b>P2 transcript</b>	<b>P3 transcript</b>
Targeted display at 24 hours	ND	ND	PSP ↓	PSP ↑	PSP ↓
Northern at 24 hours (chapter 2)	PSP ↓ 38% PMSF ↓ 28%	NC	PSP ↓ 26% PMSF NC	PSP NC PMSF NC	PSP NC PMSF NC
Time course Northern (chapter 3)	PSP ↓ 33% at 48 hours. PSP shows NS at other times.	PSP ↑ 38% at 12 hours and ↓ 28%* at 48 hours. PMSF ↓ 42% at 48 hours.	PSP ↓ 22% at 12 hours. PMSF NC	PSP ↑ 26% at 12 hours. PMSF NC	PSP NC PMSF NC

Shown is a table summarizing gene expression data from chapters 2 and 3.

Arrows refer to direction of expression change in spinal cords of PSP or PMSF treated, compared to DMSO treated hens. Expression changes unless otherwise noted are significant at p<0.05. ↑ = upregulated; ↓ = down regulated; ND = not determined; NC = no expression change; NS = possible trend towards significant expression change. \* p=0.0576.

## **Discussion**

These results indicate that PSP treatment altered  $\alpha$ -tubulin transcript responses. Tubulin, in the form of  $\alpha\beta$ -heterodimer units, forms the basic building block of microtubules.<sup>154</sup> Microtubules are involved in many essential processes including intra-cellular transport, cell division, and ciliary and flagellar motility. Numerous isotypes exist for both  $\alpha$  and  $\beta$  tubulin. These are defined by gene-encoded isotypes, in addition to a variety of post-translational modifications. The latter include polyglutamylation, polyglycylation, acetylation, C-terminal tyrosination/detyrosination, phosphorylation and palmitoylation.<sup>154-156</sup> (Not all of these modifications have been reported in mammalian or avian axons). There is therefore considerable opportunity for development of heterogenous structure within specific tubulin families. There is evidence that different isotypes have different cellular functions. Specific  $\alpha$ -tubulin isotypes are differentially regulated in the developing, versus the adult rat brain.<sup>157</sup> The dynamics of microtubule growth and shortening is also affected by the isotype composition of  $\alpha\beta$ -tubulin heterodimers.<sup>158</sup> These observations raise the possibility that the ratio of different  $\alpha$ -tubulin isotypes could be as important as absolute expression level in determining microtubule functional state.

The two  $\alpha$ -tubulin transcript isotypes studied displayed differences in expression. At 12 hours levels of the ~4.5 kb transcript increased ~38%, while the ~2.5 kb transcript decreased ~20%, raising the possibility that alterations in

the ratios of their gene products occur. The levels of both transcripts decreased at 48 hours. A similar response was reported in spinal cord of DFP treated hens at 48 hours post exposure.<sup>10</sup> However, it remains to be determined if these expression changes are carried through to the protein level. The two  $\alpha$ -tubulin transcripts identified in this study differ considerably in size. The ~2.5 kb transcript matches the expected length of most  $\alpha$ -tubulin full length transcripts as determined by DNA database searches. The ~4.5 kb transcript is longer than  $\alpha$ -tubulin transcripts from *G. gallus*, and mammalian species. The larger transcript may represent a previously unidentified  $\alpha$ -tubulin transcript. In the present study both transcripts required equal film exposure times to develop similar band intensities. It is probable that they represent similar sequences expressed at a similar level consistent with both being within the  $\alpha$ -tubulin family. Length differences may be a function of length of un-translated region (UTR) sequence within the transcript, because  $\alpha$ -tubulin proteins do not appear to vary much in length based on protein database searches. Variability in UTRs could lead to differences in mRNA stability, transport, and site of translation. Finally, since the exact  $\alpha$ -tubulin isotypes identified by the probe used are not known, the possibility that one of the transcripts represents a pseudogene cannot be fully ruled out. Alpha-tubulin pseudogenes have been identified in the rat<sup>159</sup> and chicken.<sup>160</sup> However, it is considered unlikely that a pseudogene would be expressed at such a high level suggested by the 45-60 minute exposure times

required for Northern analysis. Clarification of these issues will depend on further characterization of tubulin isotypes in *G. gallus*.

Transcript P1 exhibited a small decrease in abundance at 12 hours, but not at other time points in our experiments. While this result is not in exact agreement with the previous Northern blot experiment for this gene (chapter 2), it does suggest that in general this gene transcript is down-regulated to a small degree during the first 12-24 hours after PSP exposure. The disparity in response over time between the two studies could be because down-regulation is transient and showed some variability between experiments. Inter-experiment variability could relate to a number of factors, including differences in reproductive status (laying versus not laying eggs) between experiments. Unfortunately, gene P1 has not yet been identified, despite attempts to obtain additional sequence information using 5'-RACE. Whether a low magnitude expression change is biologically significant will depend on a number of factors including, 1.) if change in the expression of the transcript is carried through to the protein level, 2.) presence or absence of other proteins performing the same function, and, 3.) if the protein acts at a rate limiting step in a pathway or process. Gene P2 exhibited a small increase in expression at 12 hours only in PSP, but not PMSF treated birds. Given that statistically significant changes in gene expression were not found in the previous Northern experiment measuring responses at 24 hours, the relevance of this finding is equivocal.

For the ~2.5 kb transcript encoding for  $\alpha$ -tubulin, and for the P3 gene transcript there were significant changes in gene expression across time when averaged across the three treatments. In some cases the effect of time was as great in magnitude as the PSP or PMSF treatment effects. While the reason for these changes has not been investigated further, the results obtained thus far suggest a potential effect of the vehicle. Other possibilities, such as age effects, are considered unlikely. An age effect over a 48 hour period would be more likely in the rapidly developing nervous system, not in adult hens.

Dimethyl sulfoxide (DMSO) is used extensively as a solvent for hydrophobic substances in experimental toxicology, as an analgesic in veterinary medicine, and for the treatment for urticaria in humans.<sup>161</sup> In 1965 DMSO was brought under control by the Food and Drug Administration after it was found that it caused changes in the refractive index of lens of experimental animals.<sup>162</sup> There are several publications indicating that DMSO can be directly neurotoxic, as well as modulate the effect of other compounds. For example, the tail nerve conduction velocity is reduced in rats given intra-peritoneal DMSO at 0.36 ml/kg/day for 10 days.<sup>163</sup> DMSO has also been reported to potentiate the suppressive effect of lidocaine on synaptic transmission in bullfrog sympathetic ganglia,<sup>164</sup> and was implicated in a case of peripheral neuropathy in a man also taking suldinac for arthritis.<sup>162</sup> When directly applied to nerve, DMSO induces action potential block.<sup>161</sup> Given these findings the possibility that these results

reflect interactions between DMSO and PSP should be considered. However, hens in this experiment were only given a single 0.5 mls / kg im dose of DMSO. The possibility of solvent effects could be addressed by repeating the experiment using a different vehicle or including untreated hens in the experiment. Ethanol is also an effective solvent for PSP or PMSF, however, it has the disadvantage of being neurotoxic.<sup>165</sup> Corn oil has been used to dilute liquid OP esters such as DFP but is not suitable for dissolving the solids, PSP and PMSF.

The absence of detectable changes in the expression of neurofilament-M transcript in spinal cord between 12-48 hours after PSP exposure was somewhat unexpected based on the notable effect reported for DFP.<sup>8</sup> This raises questions as to the necessity of neurofilament-M expression changes in the progression of OPIDN.

To summarize, this work supports and extends earlier work (see chapter 2) regarding gene expression in pre-clinical OPIDN. Gene transcript changes are still considered low in magnitude, especially for genes P1 and P2. In the next chapter, one issue that is addressed is whether low magnitude expression changes result from dilution effects due to differential expression being restricted to a sub-population of expressing cell types.

## **CHAPTER 4: Cellular and Differential Cellular Spinal Cord Expression of α-Tubulin, P1 and P2 (KIAA1307) in Phenyl Saligenin Phosphate Treated *Gallus Gallus***

### **Introduction**

Data presented so far (chapters 2 and 3) indicate that PSP induces changes in the pattern of spinal cord mRNA expression prior to the occurrence of any morphological, clinical or electrophysiological alterations. Furthermore, certain of the observed changes occur in PSP, but not PMSF treated birds suggesting that they could be related to NTE inhibition and aging. However, these changes are fewer in number and of lower magnitude than originally expected. The methods used thus far have relied upon isolating whole spinal cord RNA from tissue homogenates. Consequently, only gross expression changes will be evident and not those which may be of greater relative magnitude in specific cells or regions. This problem is of special importance in tissues that are highly heterogenous, such as the CNS. Knowledge of cellular expression of the genes being studied could provide valuable mechanistic information. In the work described below differential cellular expression of α-tubulin, P1 and P2 were studied in spinal cord of PSP compared to vehicle treated birds. Additionally, as P1 and P2 are poorly characterized, their transcript expression was determined in different regions of the CNS and PNS as well as extra-neurally.

## **Specific Questions and Rationale**

### **1. What spinal cord neuronal/cell types express the transcripts for $\alpha$ -tubulin,**

**P1 and P2 (KIAA1307)?** Knowledge of the spinal cord cell types that express transcripts that are differentially expressed in OPIDN may provide useful clues to the mechanism of this toxicity. This information is of special importance for the uncharacterized P1 and P2 transcripts. For example, there is indirect evidence that OPIDN results from abnormal glial-neuronal interaction. Mutated *Sws* leads to glial hyperwrapping, vacuolization, and neuronal apoptosis.<sup>44, 45</sup> Neurotoxic esterase and *Sws* also share homology to the regulatory sub-unit (RI $\alpha$ ) of protein kinase A.<sup>44, 45</sup> This sub-unit forms part of a protein complex suggested to be involved in neuron-glia interactions<sup>46</sup> including the induction of neuron-glia adhesion molecules in neurons.<sup>47</sup> In light of the observations listed above it is important to determine whether transcripts found to have altered expression in OPIDN are expressed by neurons and / or glial cells. Additionally, for genes of unknown function, determination of cellular expression patterns may provide clues as to the functions of their protein products. For example, if a transcript is expressed at similar levels in all neurons it will suggest that the gene is not related to the function of a specific neurotransmitter system, but is more likely related to general neuronal function.

### **2. Is the expression of P1 and P2 (KIAA1307) restricted only (or predominantly) to neurons that undergo axonal degeneration in OPIDN? It**

is possible that neurons possessing long axons, which are more susceptible to OPIDN, express proteins that are not present, or are present in much larger or smaller quantities, in neurons that have short axons. Abnormal regulation of such a hypothetical protein could lead to axonal degeneration, assuming that this protein is necessary for axonal maintenance. The notion that a certain protein could be expressed by a discrete neuronal sub-population sensitive to a certain type of injury is precedented. Evidence indicates that neural stannin expression is occurs mainly within trimethyl-tin sensitive neurons.<sup>166</sup>

**3. What spinal cord cell types display differential expression of  $\alpha$ -tubulin, P1 and P2 transcripts following exposure to PSP?** Given the heterogeneity of spinal cord and the low magnitude of the transcript expression changes identified by Northern blotting (~20-40%) the possibility that altered expression may be occurring only in a sub-set of cells expressing a specific gene was considered. It is possible that a large magnitude gene expression change is occurring in a neuronal sub-population, and that this effect is diluted by other cells that do not differentially regulate this gene. Answering this question could provide additional corroborative data to support previous results of Northern blotting. The results of the prior time course experiment were used to select the time point to evaluate transcript expression by *in-situ* hybridization (ISH). Evaluation of P1 and P2 was performed at 12 hours post-dosing. Alpha-tubulin was only evaluated at 48 hours, the time at which both  $\alpha$ -tubulin isotypes were down regulated (chapter 3). The  $\alpha$ -tubulin probe identified two isotypes by Northern blotting.

Presuming that the probe identified these two isoforms by ISH then evaluation at a time at which one was upregulated, and the other down regulated (12 hours) would be futile. The effects would cancel out because the  $\alpha$ -tubulin probe used in this study cannot distinguish between the two  $\alpha$ -tubulin isoforms when used for ISH.

**4. Are P1 and P2 transcripts expressed elsewhere within the central and peripheral nervous system, or extra-neurally?** As P1 and P2 (chicken KIAA1307) transcripts are uncharacterized additional information about expression would be useful. This may provide functional clues, for example, a gene expressed universally may be involved in energy or membrane metabolism. Finding multi-organ expression of a gene would not lessen its potential significance with regard to OPIDN. Neurotoxic esterase is also expressed in blood lymphocytes<sup>167</sup> and non-susceptible neurons<sup>48</sup> even though significant toxicity with neuropathic OP ester compounds is only noted in neurons with long wide axons.

Non-radioactive ISH was considered the most appropriate method to address all the above questions. It has the advantages of high resolution and sensitivity, and importantly, the ability to use ‘unknown’ DNA as templates for probe synthesis. It has the disadvantage of measuring mRNA, not protein. Using an alternative approach to Northern blotting that gives quantitative as well as spatial information could provide further evidence to support earlier findings.

## **Experimental Design**

Four adult White Leghorn hens were randomly assigned to PSP and DMSO treatment groups in two separate experiments in which sacrifices were performed at 12 or 48 hours. The times used correspond to the points of maximum alterations in expression found for P1 and P2, and,  $\alpha$ -tubulin, respectively (see chapter 3).

## **Materials and Methods**

### ***Treatments and perfusion***

Hens were dosed im with 2 mg/kg PSP or 0.5 ml/kg DMSO. At appropriate time points they were catheterized in the brachial vein. Deep anesthesia was induced with pentobarbital. Two thousand units of heparin sulfate were infused iv. This was followed by 0.9% (w/v) sodium chloride for two minutes then by freshly prepared 4% (w/v) paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4, for 15 minutes. Flow rates were 90-100 mls / minute and solutions were at 25<sup>0</sup> C. Carcasses were stored at 4<sup>0</sup> C. Tissues were dissected within 4 hours after perfusion, post-fixed for a further 3 hours then processed routinely overnight into paraffin. Spinal cords from different birds were embedded into the same block so that they could be processed for ISH under 'identical' conditions. Paraffinized tissues were stored at 4<sup>0</sup> C.

### ***In-situ hybridization***

All materials coming into contact with tissue were treated to render them RNase free. 0.05% (v/v) diethylpyrocarbonate (DEPC) was added to all water used to make solutions, glassware was baked at 160<sup>0</sup> C for 4 hours, or treated with 0.5 M sodium hydroxide for 30 minutes then rinsed in DEPC treated water. Plastic tubes were autoclaved or cleaned with RNaseZAP® (Ambion, Austin, TX). Slides were washed for 1 hour in ethanol, dried at 60<sup>0</sup> C, immersed into 2% (v/v) 3-aminopropyl-triethoxysilane (Sigma) in acetone for 1 minute, washed in acetone, then baked at 160<sup>0</sup> C for 4 hours. Ten µm sections of paraffinized tissues

were prepared in a clean water bath, dried at 25<sup>0</sup> C, and stored at 4<sup>0</sup>C in a dust free environment. Sections were used within 2 weeks following preparation.

All reagents used for the preparation of riboprobes were from Roche (Indianapolis, IN) unless otherwise specified. Plasmids containing P1, P2 and  $\alpha$ -tubulin were cut and linearized using appropriate restriction enzymes. They were extracted from the 40  $\mu$ l aqueous layer with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v) then re-extracted in an equal volume of chloroform to remove residual phenol. DNA was precipitated from the aqueous fraction by the addition of one ninth volume of 3 M sodium acetate, pH 7.0, then 2.5 volumes ethanol followed by centrifugation at 12000 x g for 15 minutes. The pellets were washed in 80% ethanol, air dried then dissolved in 10  $\mu$ l DEPC treated water. One  $\mu$ g of linearized plasmid was used in a 20  $\mu$ l *in-vitro* transcription reaction. This reaction included 1 mM ATP, GTP and CTP, 1.3 mM UTP, 0.7 mM digoxigenin-11-uridine-5'-triphosphate, 20 units RNase inhibitor (SUPERase.In<sup>TM</sup>, Ambion), 50 units SP6 (Ambion) or T7 (Life-Technologies) RNA-polymerase and supplied buffers in a total volume of 20  $\mu$ l. Transcription was for 2 hours at 37<sup>0</sup> C. The reaction was stopped by the addition of 2  $\mu$ l of 0.2 M di-sodium EDTA, pH 8.0. RNA was then precipitated by the addition of 22  $\mu$ l of 0.8 M lithium chloride (0.4 M final concentration), followed by four volumes (176  $\mu$ l) of cold 100% ethanol. Samples were incubated at -70<sup>0</sup> C overnight. Pellets were washed briefly in 1 ml of cold 80% (v/v) ethanol, dissolved in 100  $\mu$ l of formamide. Ten  $\mu$ l aliquots were stored at -70<sup>0</sup> C. (Post-labeling DNase 1

digestion did not improve the method). Probe yields were estimated to be ~5 µg / reaction. Sense / anti-sense probe concentrations were compared so that similar concentrations could be used during hybridization reactions; this was by measuring uv light absorption between wavelengths of 190-300 nm on probe dilutions.

Wax was removed from tissue sections by incubation at 60<sup>0</sup> C for 10-12 minutes. The following was then performed at 25<sup>0</sup> C: Two 10 minute incubations in xylene, one 10 minute incubation in ethanol:xylene (50:50 v/v), one 10 minute wash in ethanol, one 10 minute wash in 95% (v/v) ethanol, one 5 minute wash in 70% (v/v) ethanol, and one 10 minute wash in water. The following steps were performed at 25<sup>0</sup> C to increase mRNA target accessibility for probe and improve signal-to-noise ratio: One 20 minute incubation in freshly prepared 0.2 M HCl followed by one 10 minute wash in water. One 20 minute incubation at 37<sup>0</sup> C in 20 mM Tris.HCl, pH 7.4, containing 2 mM CaCl<sub>2</sub> and 10-50 µg / ml proteinase K (concentration had to be optimized). The reaction was stopped by the addition of 0.2 M Tris.HCl, pH 7.4, and 0.1 M glycine for 5 minutes at 25<sup>0</sup> C. Slides were then equilibrated in water for 5 minutes followed by 70% (v/v) then 100% ethanol for 4 minutes each.

Hybridization was performed in buffer containing 0.5 µg / ml riboprobe. The hybridization buffer was comprised of 50% (v/v) formamide, 10% (w/v) dextran sulfate, 0.05% (v/v) Triton-X-100, 500 µg/ml herring sperm DNA, 0.05 % (v/v) polyvinyl pyrrolidone, 5X sodium chloride / sodium citrate pH 7.0

(SSC)<sup>d</sup>. All hybridization reagents were from Sigma except for the herring sperm DNA (Roche). One batch of hybridization buffer was prepared and stored in aliquots at -20<sup>0</sup>C. Probe was mixed thoroughly with hybridization buffer then heated to 85<sup>0</sup> C for 10 minutes. Eighty µl of hybridization solution was used per slide. The solution was covered with a Hybridization Cover Slip (Electron Microscopy Sciences, Fort Washington, PA) sealed with nail hardener then incubated for 15-18 hours at 52<sup>0</sup> C in a humidification chamber.

Cover slips were removed by soaking in 2X SSC for 5-10 minutes. This was followed by washing in 2X SSC for 30 minutes at 50<sup>0</sup> C, followed by 1X SSC for 15 minutes. This was followed by three ten minute washes in 1 X SSC, one for 15 minutes in 0.1X SSC, then one for 5 minutes in 150 mM NaCl, 100 mM Tris.HCl, pH 7.4 (buffer 1), all at 25<sup>0</sup>C. Slides were blocked in buffer 1 containing 2% (v/v) normal goat serum (NGS) for 0.5 hours at 37<sup>0</sup> C. They were then incubated with 1/300-1/400 dilution of anti-digoxigenin-alkaline phosphatase (Fab fragments) in buffer 1 containing 2% NGS for 2 hours at 37<sup>0</sup> C. This was followed by three fifteen-minute washes in buffer 1 at 25<sup>0</sup> C. Antibody was detected by incubating for 0.5-24 hours in the dark in 100 mM TRIS.HCl, pH 9.5, containing 50 mM magnesium sulfate, 0.4 mg / ml nitro blue tetrazolium, 0.19 mg / ml 5-bromo-4-chloro-3-indolyl phosphate and 2 mM levamisole (Sigma). Following development slides were rinsed twice in water then cover slips mounted in Fluoromount G (Electron Microscopy Sciences).

---

<sup>d</sup> 20 x SSC comprises 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0

For quantification, images were captured under identical conditions using a Nikon eclipse E600 microscope and a Nikon Coolpix 990 digital camera. Sets of four images representing birds from one experiment were grouped, and the image flattened before minor image adjustments were made globally. For each gene transcript studied the ISH procedure was repeated at least twice on different sections of the same tissues to determine reproducibility. Evaluation of expression of transcripts in different tissues / regions / cells was by visual examination using a bright field microscope. In addition, for each hen background subtracted neuronal cytoplasmic staining intensity was quantified in five randomly chosen motor neurons from the lateral-ventral gray matter using The Image Processing Tool Kit (version 3.0) and Adobe Photoshop (version 5.0) software. For the PSP and DMSO treatment groups, the mean of ten neurons over two hens was calculated. Statistical comparison of transcript staining data between the two treatment groups was not carried out because there were only two hens per group.

## **Results**

Transcripts encoding  $\alpha$ -tubulin, P1, and P2 (KIAA1307) were detected using the above method. Development times required were ~4 hours for  $\alpha$ -tubulin, ~0.5 hours for P1, and ~24 hours for P2 suggesting that P2 is expressed at a much lower level than the other two genes. The reproducibility of the ISH procedure for  $\alpha$ -tubulin and P1 was good, while that for P2 was poor (perhaps because the quantity of transcript was at the limit of detection by this method).

### **1. What spinal cord neuronal/cell types express the transcripts for $\alpha$ -tubulin, P1 and P2 (KIAA1307)?**

Within lumbo-sacral spinal cord, transcript for  $\alpha$ -tubulin was expressed predominantly within motor neurons having long axons (those most laterally within anterior gray matter) and to a lesser extent in all other neuronal types (Figure 15). No expression could be detected within glial or other cell types. All spinal cord neurons expressed P1 transcript (Figure 16a). However, this transcript was also expressed within other cell types. Weak staining was present within cells that may represent oligodendrocytes and astrocytes, and also ependymal cells and choroid plexus epithelial cells. In spinal cord the pattern of expression of P2 was similar in distribution to that of P1. Some motor neurons exhibited single round puncta of nuclear signal in addition to diffuse cytoplasmic staining (Figure 17).

### **2. Is the expression of P1 and P2 (KIAA1307) restricted to neurons that undergo axonal degeneration in OPIDN?**

Expression of transcripts for P1 and P2 were not restricted to PSP sensitive neurons such as those of the ventral horn or DRG.

**3. What spinal cord cell types differentially regulate  $\alpha$ -tubulin, P1 and P2 transcript expression following exposure to PSP?**

Alpha-tubulin transcript was down regulated at 48 hours in lumbo-sacral spinal cord of PSP versus DMSO treated birds. Based on visual examination down regulation was diffuse across all neuronal types and was not restricted to affected neuronal sub-populations (Figure 18). PSP treated hens had a mean neuronal staining of the transcript for  $\alpha$ -tubulin in ventro-lateral motor neurons that was 35% of controls (individual neuron range was 18-70% of control mean). P1 transcript was down regulated at 12 hours in PSP treated birds. Based on visual examination down regulation was diffuse across all neuronal types (Figure 19). Staining of the gray matter neuropil was also weaker in PSP treated birds. This suggests that P1 may be expressed in dendrites or the astrocytic processes. Density measurements revealed that PSP treated hens a mean neuronal staining of the transcript for gene P1 in ventro-lateral motor neurons that was 29% of controls (individual neuron range was 24-36% of control mean). P2 transcript did not show repeatable changes in expression levels between treatment groups (data not shown).

**4. Are P1 and P2 transcripts expressed elsewhere within the central and peripheral nervous system, and extra-neurally?**

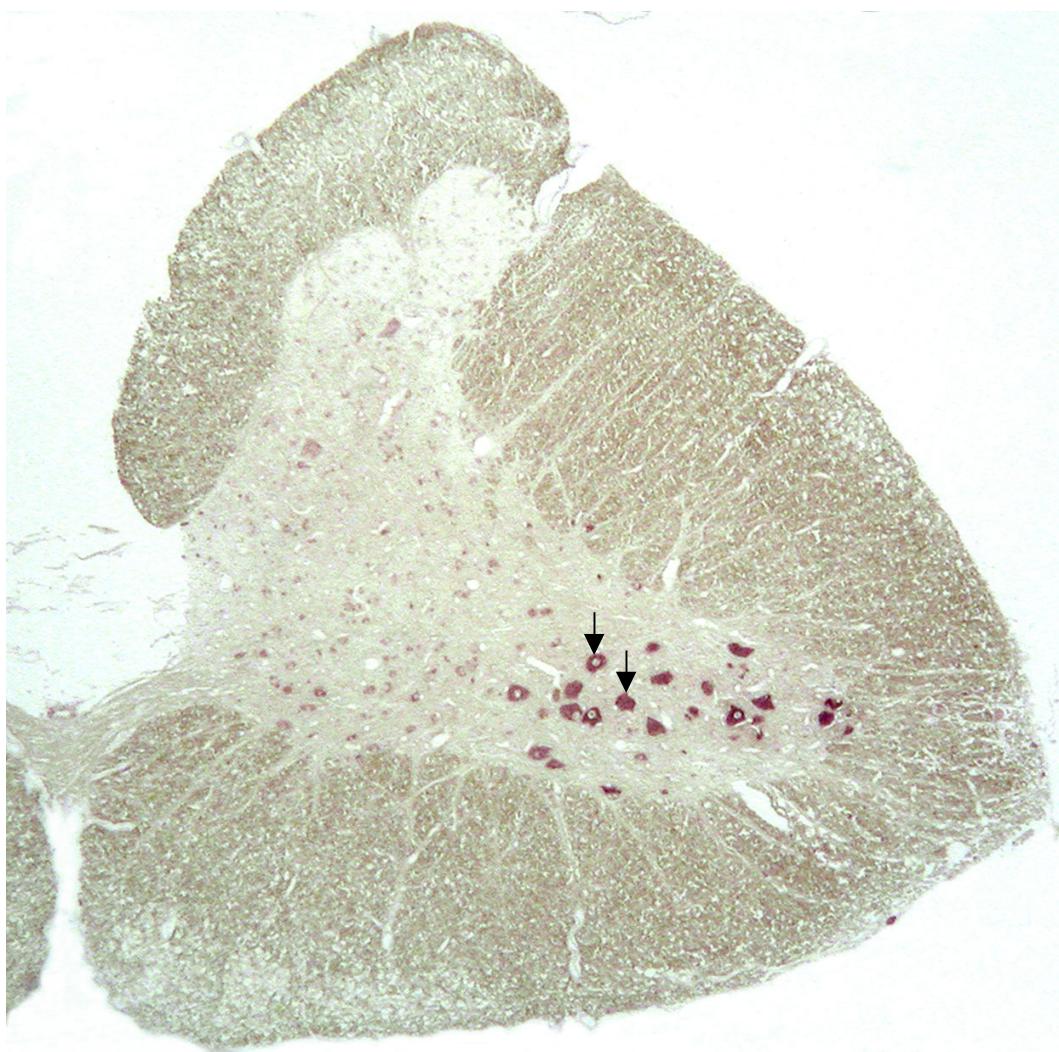
P1 transcript was strongly expressed in a variety of cell types throughout the brain (Figures 16 and 20). Diffuse, variable cytoplasmic expression was detected in all neurons. Expression appeared highest in large neurons such as those of the dorsomedial vestibular and olfactory nuclei of the brain stem, tuberculum olfactorium, lamina medullaris ventralis, hippocampus dorsalis, parahippocampus, optic lobe, striatum and cerebral cortex and cerebellar Purkinje neurons. Weak neuronal expression was present within small neurons such as those of the molecular layer (stellate and basket neurons) and inner granule cell layer of the cerebellar cortex. Moderate expression was detected in ependymal and choroid plexus epithelium, and cells interpreted to be oligodendrocytes. Staining in cells interpreted to be astrocytes was weak or equivocal. Within choroid plexus epithelium, the transcript was localized mainly in the region basal to the nucleus. Elsewhere, it was diffusely cytoplasmic. Within the peripheral nervous system (PNS), strong staining was detected within dorsal root ganglia and sympathetic ganglia (adrenal ganglia) neurons, but not within the adrenal gland itself. Weak staining was present within peripheral nerve in cells thought to be endoneurial fibroblasts. Weak staining was detected in small intestinal epithelial cells and proprial lymphocytes. Table 2 summarizes extra-CNS expression of P1 transcript.

P2 transcript was expressed throughout the CNS in a variety of cell types. All neurons exhibited diffuse cytoplasmic expression of this gene. In addition some neurons exhibited single round puncta characteristic of a nuclear location.

Expression in other cells types was also detected, but to a much smaller degree than for neurons. Staining in white matter cells interpreted to be oligodendrocytes was weak and diffusely cytoplasmic. Staining in gray matter cells interpreted to be astrocyte was very weak. Moderate staining was also present within ependymal and choroid plexus epithelial cells. Within the PNS staining was detected within dorsal root ganglia and sympathetic ganglia (adrenal ganglia) neurons. Weak staining was present within peripheral nerve cells. Extraneurally, this gene was expressed more widely than P1. Transcript was identified within the kidney (distal convoluted tubule epithelial cells, glomerular visceral epithelial cells), splenic lymphoid cells, adrenal gland epithelial cells, myocytes, cardiomyocytes, pulmonary air capillary macrophages, hepatic sinusoidal macrophages, and arterial smooth muscle cells (Figures 21 and 22). Transcript expression within cortical epithelial cells was nuclear, cytoplasmic signal was not identified. Table 3 summarizes extra-CNS expression of P2 transcript.

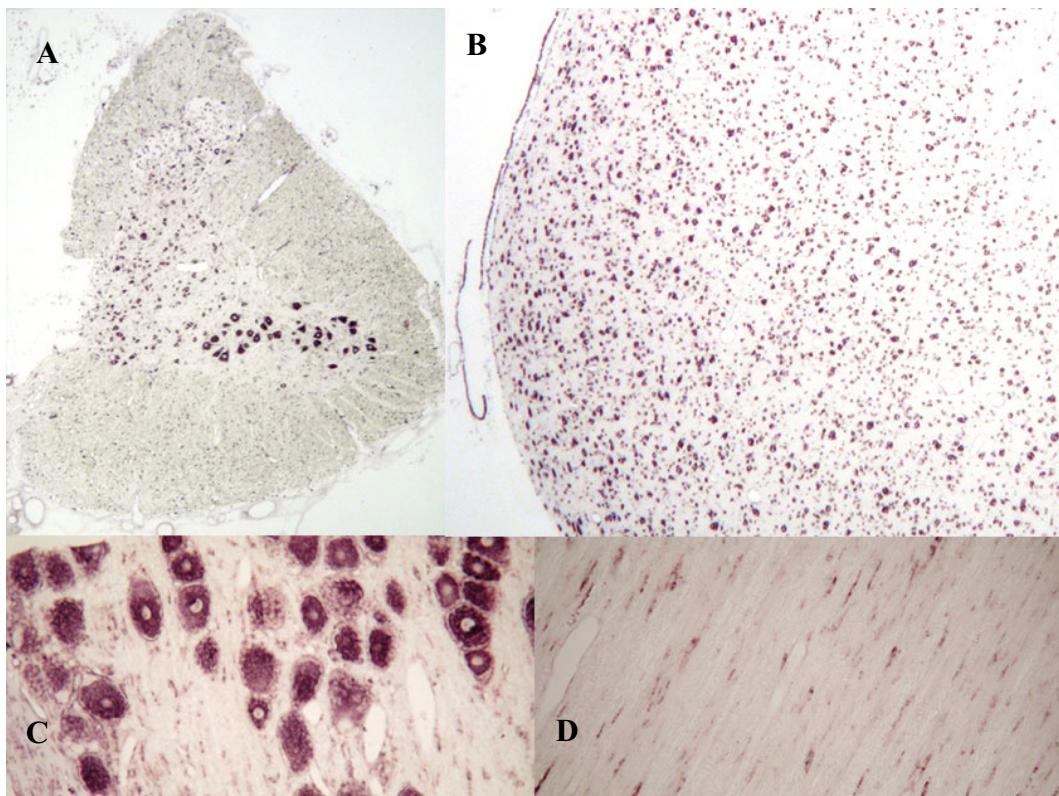
An unexpected finding on H&E spinal cord preparations was the presence of granules within the cytoplasm of motor neurons of lumbo-sacral spinal cord. Granules were present regardless of treatment or time of sacrifice. This is discussed further in appendix C.

**Figure 15. Lumbosacral spinal cord  $\alpha$ -tubulin expression**



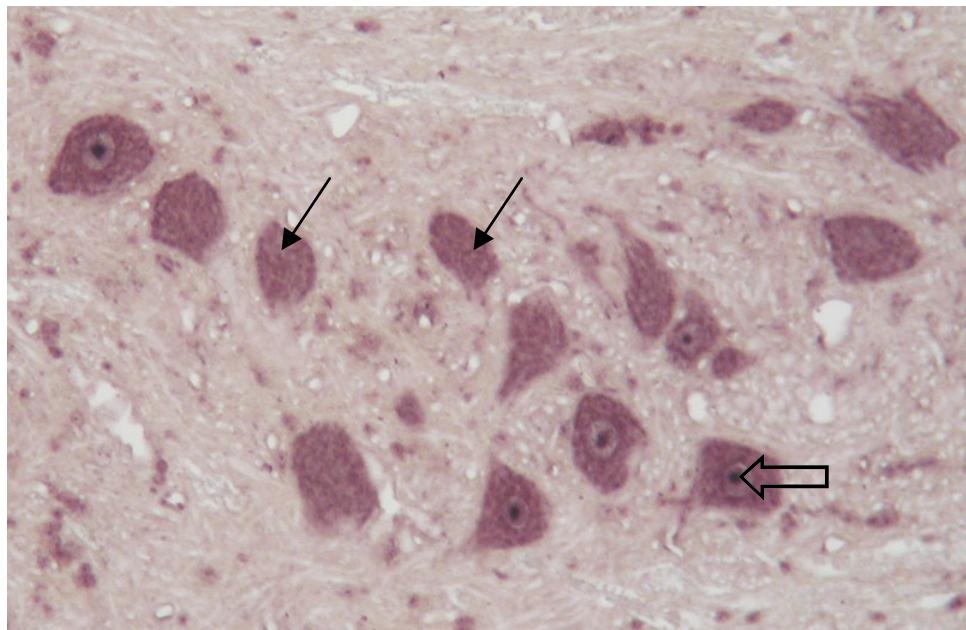
Shown is a hemi-transverse section of spinal cord from a DMSO treated hen. There is  $\alpha$ -tubulin transcript staining within neurons throughout the gray matter. Large neurons within the lateral anterior horn, representing those that project long axons exhibit strong cytoplasmic staining (arrows).

**Figure 16. Neural tissue P1 transcript expression**



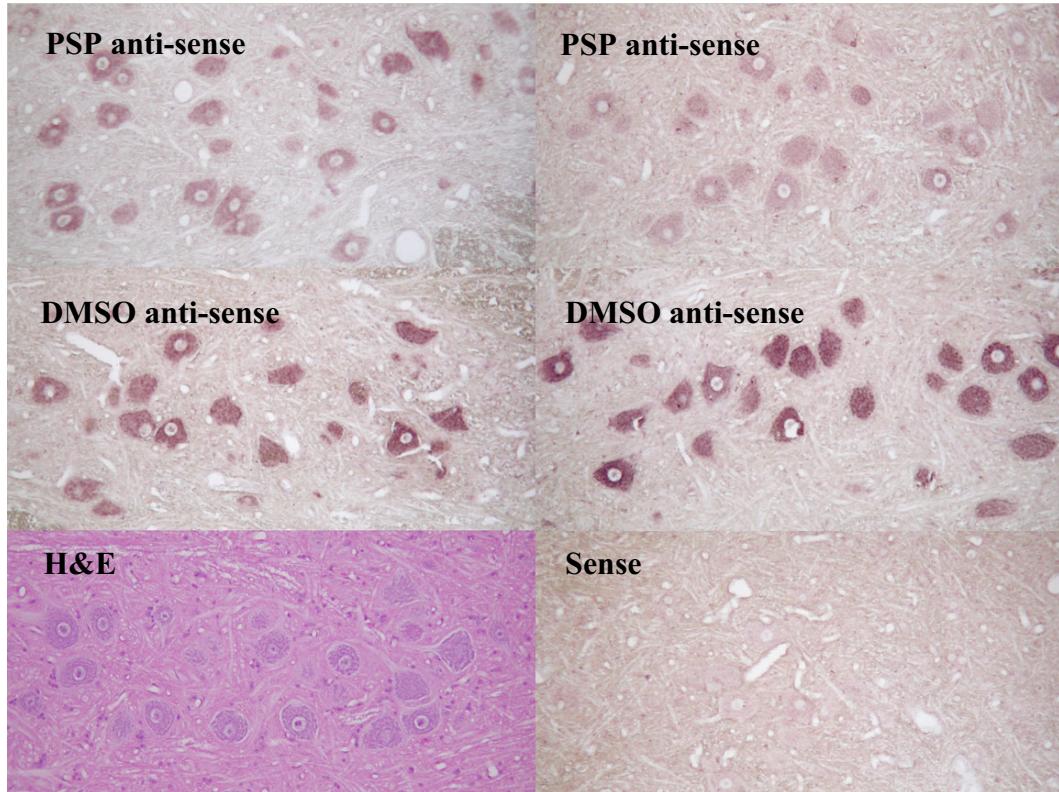
Shown are different nervous system regions from a single hen treated with DMSO. Strong neuronal staining of P1 transcript is present within spinal cord neurons, especially those of the lateral anterior horn (A), throughout the cerebral hemispheres (B) and within dorsal root ganglion neurons (C). Weaker P1 staining was present in peripheral nerve (D) in cells thought to be endoneurial fibroblasts, and in spinal oligodendrocytes (A).

**Figure 17. Expression of P2 transcript in spinal cord anterior horn**



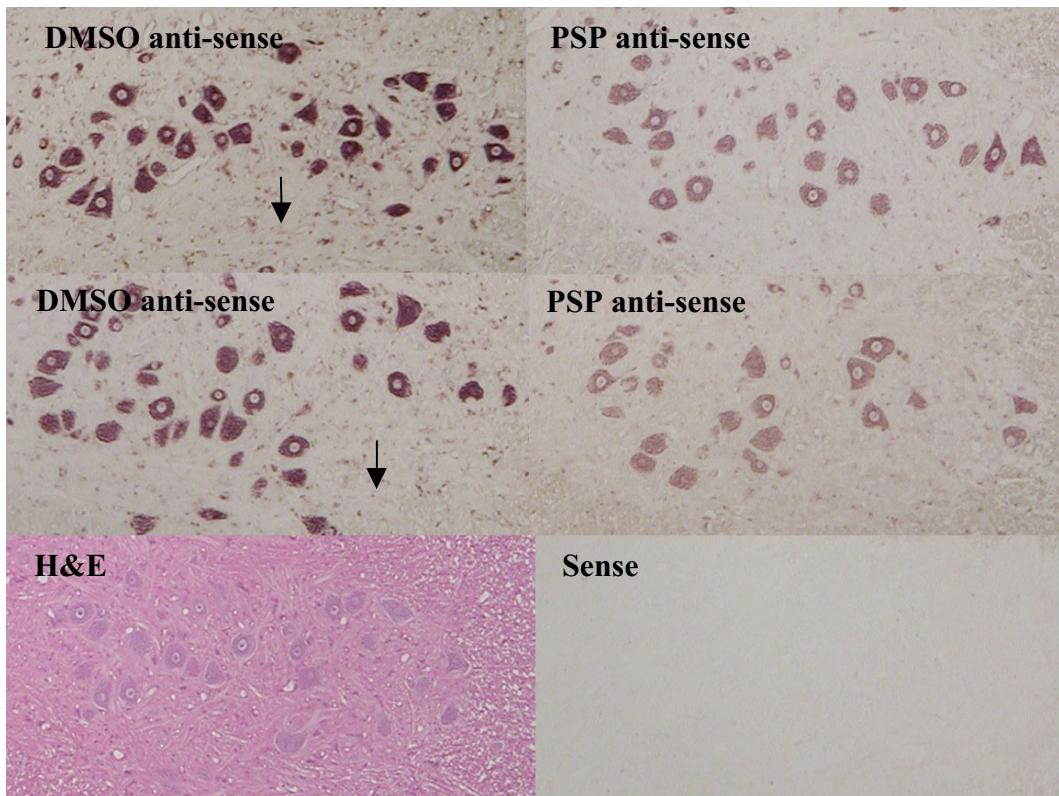
Shown is spinal cord from a hen treated with DMSO. The closed arrows indicate the presence of motor neurons exhibiting diffuse cytoplasmic staining in addition to puncta of nuclear staining (open arrow). Weak neuropil staining may represent astrocyte or dendritic processes.

**Figure 18. Alpha-tubulin transcript expression in lumbosacral lateral anterior horn 48 hours after exposure to PSP or DMSO**



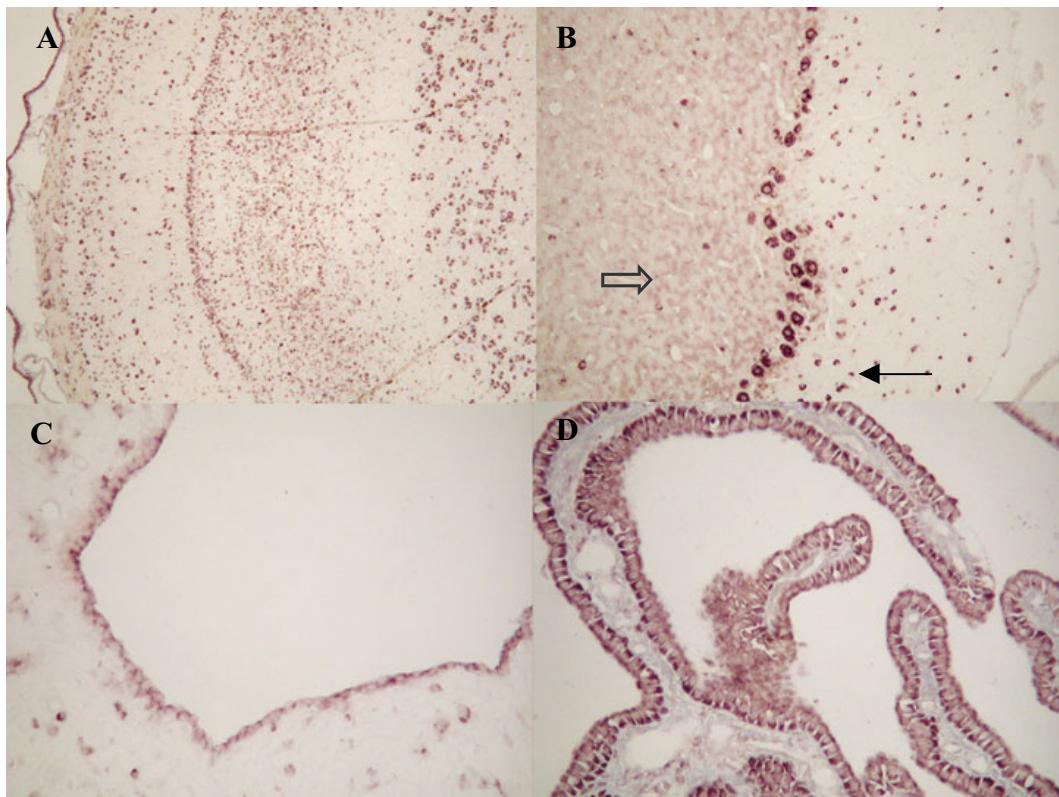
Shown are ventral gray matter horns from hens treated with PSP or DMSO. Less staining is present in large motor neurons of PSP versus DMSO treated birds using  $\alpha$ -tubulin anti-sense probe. Sense probe demonstrates lack of background cell staining. Region of anterior horn is also represented by H&E staining.

**Figure 19. P1 transcript expression in lumbosacral lateral anterior horn 12 hours after exposure to PSP or DMSO**



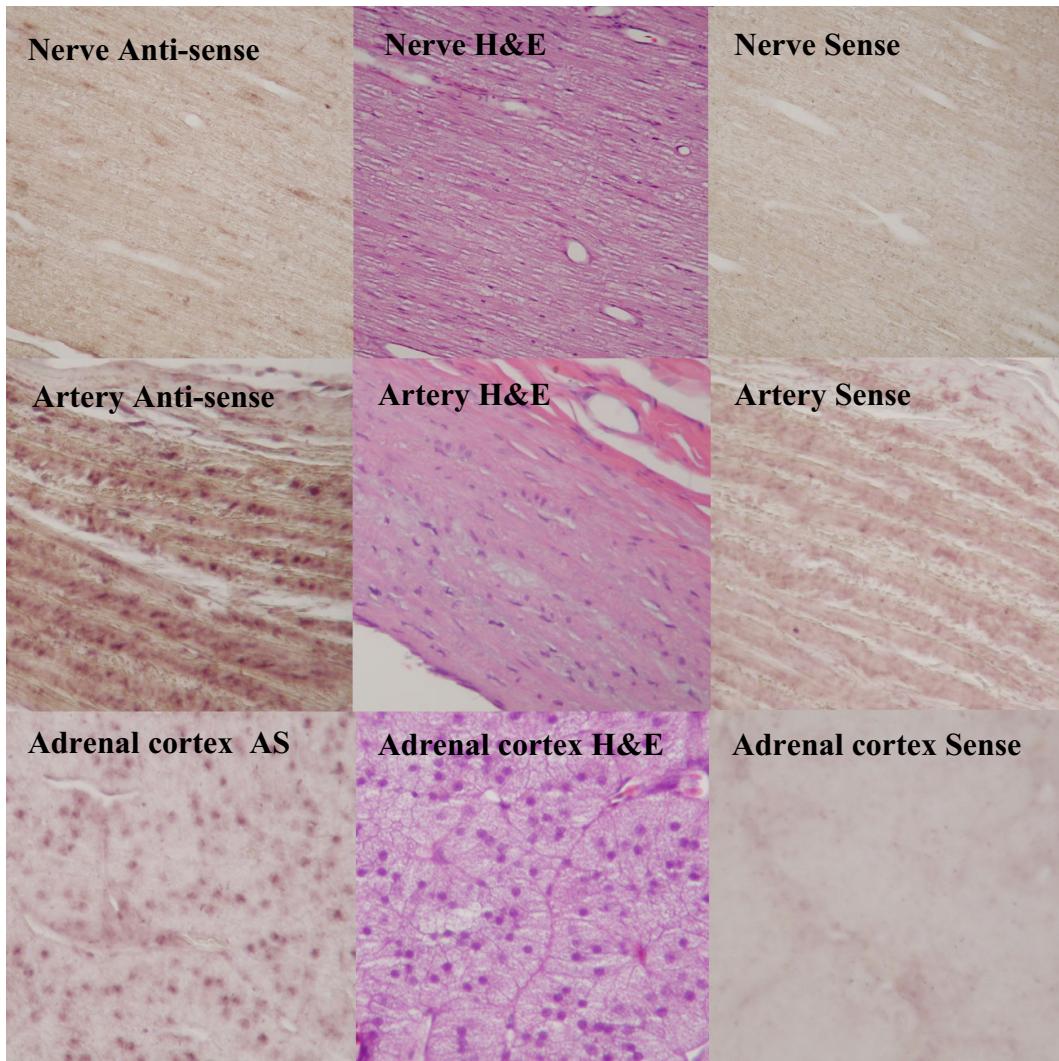
Shown are ventral gray matter horns from separate birds treated with PSP or DMSO. Less staining is present in large motor neurons of PSP versus DMSO treated birds using P1 anti-sense probe. Staining from cells interpreted to be astrocytes also appears less in PSP treated birds (arrows). Sense probe demonstrates lack of background cell staining. Region of anterior horn is also represented by H&E staining.

**Figure 20. Neural tissue P1 transcript expression continued**



Shown are different brain regions from a single hen treated with DMSO. There is neuronal staining of P1 transcript throughout all layers of the optic lobe (A), and within the molecular, granule and Purkinje cell layers of the cerebellar cortex (B). Purkinje cells strongly express P1 (closed arrow) while expression is much weaker in granule neurons (open arrow). Expression is also present within ependymal (C) and choroid plexus (D) epithelial cells.

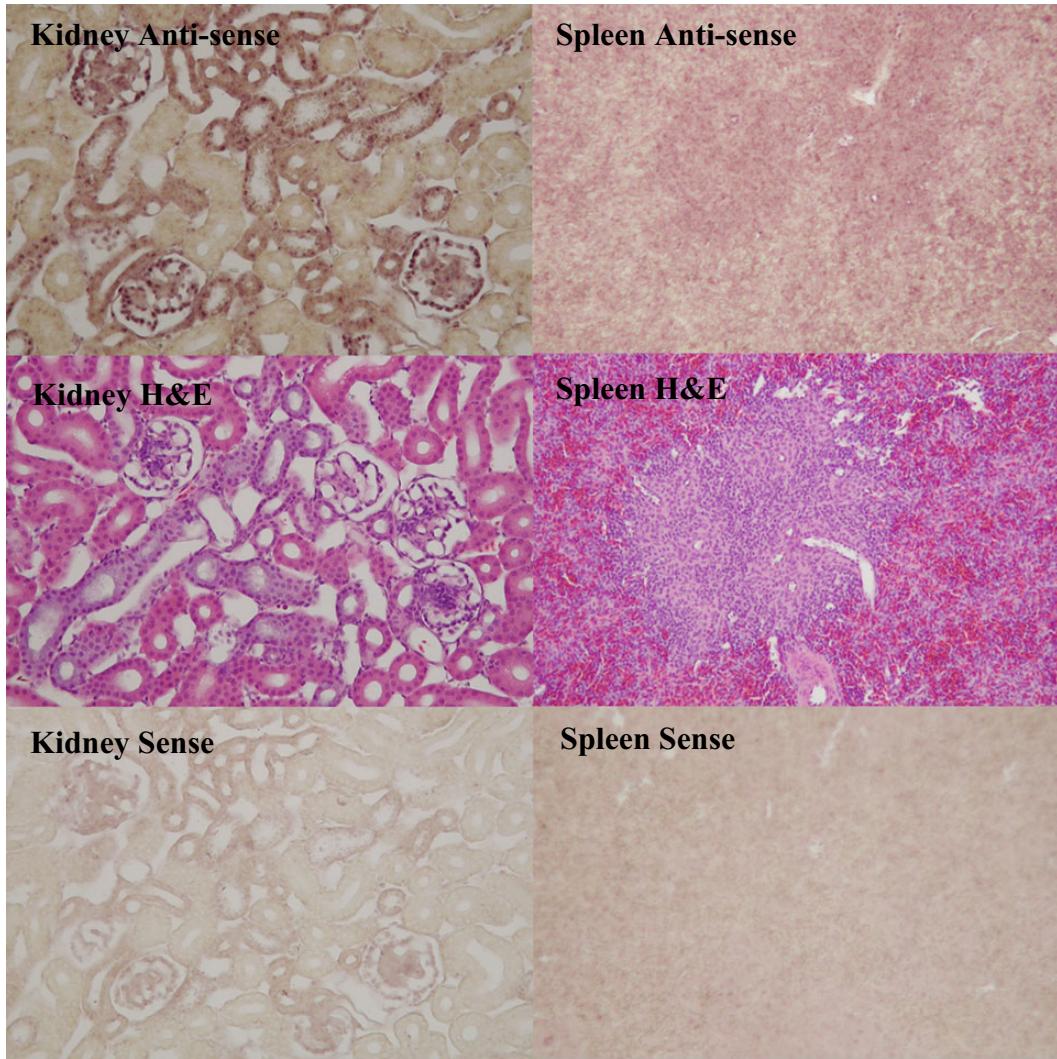
**Figure 21. Extraneural P2 transcript expression**



Shown are different tissues from a single hen treated with DMSO. P2 transcript is present within peripheral nerve, arterial smooth muscle cells, and adrenocortical epithelial cells. Long development times were required during ISH as evidenced by background staining on sense images. The peripheral nerve cell

type expressing P2 is thought to be an endoneurial fibroblast but is not known with certainty. Within adrenocortical epithelial cells staining is nuclear.

**Figure 22. Extraneural P2 transcript expression continued**



Shown are different tissues from a single hen treated with DMSO. Staining is present within kidney and spleen. Renal staining is present within glomerular and distal convoluted tubular epithelial cells. Splenic staining is mainly within lymphoid cells.

**Table 3. Expression of P1 transcript outside central nervous system**

Tissue	Relative Expression	Predominant Cell Type
Spleen	-	
Kidney	-	
Skeletal muscle	-	
Lung	-	
Heart	-	
Liver	-	
Adrenal gland	-	
Sympathetic ganglia	++	Post-ganglionic neurons
Dorsal root ganglia	+++	Neurons
Peripheral nerve	+	Endoneurial fibroblasts?
Small intestine	+	Epithelial cells and lymphocytes
Adipose tissue	-	
Artery	-	

ISH was optimized on brain then used to determine expression in other tissues. – indicates no staining detected; +, ++ and +++ indicate weak, moderate and strong staining compared to other tissues examined using the same probe. Relative levels of expression were assigned following microscopic examination of tissue sections.

**Table 4. Expression of P2 transcript outside central nervous system**

Tissue	Relative expression	Predominant Cell Type
Spleen	++	Lymphocytes
Kidney	+++	Distal convoluted tubules, glomerular epithelial cells
Skeletal muscle	±	Myocytes
Lung	+	Air capillary macrophages?
Heart	+	Cardiomyocytes
Liver	+	Sinusoidal macrophages?
Adrenal gland	++	'Cortical' epithelial cells
Sympathetic ganglia	++	Post ganglionic neurons
Dorsal root ganglia	+++	Neurons
Peripheral nerve	+	Endoneurial fibroblasts / Schwann cells?
Small intestine	-	
Adipose tissue	-	
Artery	++	Smooth muscle cells

ISH was optimized on brain then used to determine expression in other tissues. – indicates no staining detected; ± indicates equivocal signal; +, ++ and +++ indicate weak, moderate and strong staining compared to other tissues examined using the same probe. Relative levels of expression were assigned following microscopic examination of tissue sections.

## **Discussion**

The observations of this chapter regarding expression of transcripts for  $\alpha$ -tubulin and P1 support findings of the time course study described in chapter 3. (ISH for gene P2 was not considered quantitative). They also provide additional information regarding expression in normal hens and in hens exposed to PSP. Within the spinal cord,  $\alpha$ -tubulin, and genes P1 and P2, were all detected predominatly within neurons.

Changes in the expression of both  $\alpha$ -tubulin and P1 were observed across all spinal cord neurons and were not restricted to motor neurons. This suggests that while only some neurons undergo axonal degeneration, many neurons may be affected by PSP. If this is correct then certain feature(s) of neurons with long large diameter axons may make them more sensitive to PSP induced injury. In OPIDN, degenerative changes have been identified in the medulla oblongata, in addition to long axon extremities.<sup>32, 168</sup> While these changes are not the most striking morphological feature of OPIDN, their occurrence supports the suggestion that a spectrum of neuronal sensitivity exists to neuropathic OP esters that may be related to axonal length and width.

Expression of the P1 transcript also appeared to be down regulated in astrocytes of PSP treated birds. There is little evidence for a significant role of astrocytes in OPIDN. However, astocytes differentially express glial fibrillary acidic protein and vimentin transcripts in DFP induced OPIDN.<sup>9, 169</sup> No changes were observed in the expression of the vimentin transcript in spinal cord

by Northern blotting 24 hours after exposure to PSP (data not shown). This finding further suggests that the changes in gene expression that take place in the spinal cord in response to PSP and DFP are different, raising into question the significance of these changes.

The presence of apparent P2 transcript staining in the nuclei of some neurons and adrenal cortical epithelial cells is unusual. Localization in other cell types appeared entirely cytoplasmic even though nuclei were easily seen using a microscope. The biological significance of this is unknown, but likely has no relevance to OPIDN as distribution of P2 altered from that which appeared in control hens was not noted following exposure to PSP. The probe used to identify P2 corresponds to a region of an open reading frame, so the nuclear signal observed was not pre-mRNA. The underlying causes of difficulties with performing repeatable ISH analysis of P2, as well as the lack of observable effect in these experiments, likely relate to the long development times that were required to obtain a signal, indicating that the transcript was at the limit of detection. The ISH approach must therefore be considered qualitative for P2 transcript.

Expression of P1 and P2 in essentially all CNS neurons, as well as other CNS cells, PNS and extra-neural tissues does not detract from their potential mechanistic significance. Rather, it suggests that if these genes are mechanistically necessary, they are likely insufficient in and of themselves to mediate the down stream effects of OPIDN. Other factors presumably must be

present for axonopathy to develop. Expression of the P1 transcript is interesting in that it is present in many neural cell types (neurons, astrocytes, oligodendrocytes, ependymal epithelial cells), but has quite limited expression outside the nervous system. The relevance of this is unknown as the gene has not yet been characterized. It should be noted that the ISH procedure was optimized on CNS tissue only. It is possible that optimization was not appropriate for P1 detection in non-neural tissues. The extra-neuronal expression of P2 (KIAA1307) was in keeping with previous studies using human tissues.<sup>150</sup>

Understanding the relevance of the above findings will depend on understanding the functions of these genes. Unfortunately, P1 has not yet been identified (despite attempts using 5'-RACE). Gene P2 has a human homologue that was called KIAA1307. This was identified from a human brain cDNA library. However, sequence analysis of this gene failed to identify a possible function.<sup>150</sup> Down regulation of  $\alpha$ -tubulin transcript suggests the possibility of down regulation at the protein level. If this occurs, then there could be effects on fast orthograde and retrograde axonal transport, and other cellular functions. This could relate to evidence indicating altered fast orthograde<sup>57</sup> and retrograde<sup>59</sup> axonal transport in OPIDN.

## **CHAPTER 5: General Discussion, Future Directions and Conclusion**

### **General Discussion**

Organophosphorus ester induced delayed neurotoxicity (OPIDN) is a manifestation of exposure of susceptible mammals and birds to specific organophosphorus (OP) esters, such as phenyl saligenin phosphate (PSP) and di-isopropyl phosphorofluoridate (DFP).<sup>2</sup> The most striking nervous system lesions that develop following OP ester exposure are degeneration of the distal regions of long large diameter axons within the central and peripheral nervous system. The foundations for the hypotheses developed and tested in this dissertation are represented by two bodies of work relating to OPIDN: 1.) evidence implicating neurotoxic esterase (NTE) as the prime molecular target in OPIDN, especially, the mechanistic relevance of NTE inhibition and aging in OPIDN induction,<sup>105, 170</sup> and, 2.) data demonstrating effects of DFP on gene expression within different regions of the nervous system in domestic fowl (*G.gallus*).<sup>7-12, 112</sup> The hypotheses tested in this dissertation were that, 1.) changes in gene expression occur in adult hen lumbo-sacral spinal cord 24 hours after exposure to an OPIDN-inducing dose of PSP, and that, 2.) some or all of these gene expression changes do not occur following exposure to an equivalent esterase inhibiting dose of the non-neuropathic NTE inhibitor, PMSF.

The hypotheses for this dissertation represented an attempt to determine if a direct association exists between NTE inhibition and aging, and, changes in the

expression of specific genes. This hypothesis was considered important because it has the potential to tie together two disparate areas of OPIDN research, namely, effects of neuropathic esters on NTE, and later gene expression changes. The results obtained indicated that changes in gene expression occur following administration of PSP, an NTE inhibiting neuropathy inducing OP ester, that do not occur following NTE inhibition by non-neuropathy inducing PMSF, and that these changes are evident within 48 hours of PSP exposure. Transcript expression changes were not restricted to susceptible neurons. In addition, it is emphasized that because PSP and PMSF very likely have other differential effects on cells, it cannot be assumed that a change occurring in PSP but not PMSF treated hens is causally related to NTE inhibition and aging.

Data provided in chapters 2, 3 and 4 provide some evidence for an association between NTE inhibition and aging, and specific changes in gene expression. However, these changes were low in frequency and magnitude. It remains to be determined whether there is a direct mechanistic link between these two processes, or whether these represent chance associations. One gene was found by targeted display (TD) cDNA screening to be differentially expressed by Northern and *in-situ* hybridization analysis in subsequent experiments. This gene (P1) remains to be fully identified, even though it exhibits 100% homology with an expressed sequence tag (pgp1n.pk010.m23). The two alpha-tubulin transcripts also demonstrated expression changes following PSP exposure. An  $\alpha$ -tubulin

transcript has previously been shown to be differentially expressed following DFP exposure in hens.<sup>10</sup>

The absence of detectable changes in spinal cord transcript expression changes for vimentin, CaM-Kin II  $\alpha$ -subunit, neurofilament-M with PSP, but not DFP<sup>7-9</sup> is interesting. Disparity could be for several reasons including differential toxicant-induced effects, different bird strain responses, unidentified vehicle effects on gene expression,<sup>e</sup> vehicle-toxicant interactions,<sup>f</sup> or experimental conditions. If differences are due to differential effects of DFP and PSP then it raises the question of the significance of these effects. As both DFP and PSP readily induce OPIDN it is expected that any changes in gene expression that are mechanistically necessary occur with both compounds. However, DFP has notable capability to induce inhibition of acetylcholinesterase and cholinergic poisoning at doses that cause OPIDN, whereas PSP does not.<sup>151</sup> It is important that results from this type of study are not over-interpreted. For example, in acrylamide and hexane induced axonopathy, neurofilament cross-linking<sup>171</sup> and modification<sup>172</sup> have been implicated as being important. However, a later study

---

<sup>e</sup> Results of DFP gene expression studies cited in this dissertation appear to report vehicle controls only at one time point. The vehicle had not previously been shown to have no effect on gene expression for the gene(s) being studied, using exactly the same model system.

<sup>f</sup> DFP, a liquid, was diluted in propylene glycol in the studies cited; PSP, a solid, was dissolved in DMSO.

using mice lacking axonal neurofilaments showed that these proteins are not necessary for toxicosis to develop.<sup>173</sup>

The transcript coding for  $\alpha$ -tubulin is the only transcript that was demonstrated to be altered at the transcript level following DFP or PSP exposure in *G.gallus*. Following DFP exposure spinal cord  $\alpha$ -tubulin transcript was down regulated about 31% on day 2, and upregulated to 200% on day 10.<sup>10</sup> The increase on day 10 is consistent with the axon reaction, changes occurring in the neuronal cell body following axotomy. For example, rat facial nucleus tubulin transcript expression increases considerably following facial axotomy.<sup>86</sup> The decrease noted on day 2 is consistent with the findings of this study where both the ~2.5 and ~4.5 kb  $\alpha$ -tubulin were down regulated, 33% and 28%, respectively, following exposure to PSP. The significance of this down regulation is not known. The down regulation noted for the 4.5 kb transcript was clearly associated with a similar expression change in the PMSF treated birds, while that for the 2.5 kb transcript was not. Using the rationale developed in this dissertation, this suggests that down regulation of the 2.5 kb transcript may be of greater mechanistic importance in OPIDN than down regulation of the 4.5 kb transcript. Alpha-tubulin transcript isotypes are not well characterized in *G.gallus*. However, there are at least five expressed genes in this species.<sup>160</sup> It is not known whether the transcript described in the DFP study represents one of those studied here. Given that tubulin is a major component of microtubules, it

would be interesting to know the relationship between alterations in tubulin expression, and effects on fast axonal transport. As  $\alpha$ -tubulin transcript expression changes were identified following PMSF treatment, it would also be interesting to know whether PMSF can induce changes in fast orthograde axonal transport. It has already been demonstrated that PMSF does not affect retrograde axonal transport in di-*n*-butyl-2,2-dichlorovinyl phosphate induced OPIDN.<sup>59</sup>

Gene expression change as a mechanism of xenobiotic induced toxicity is preceded. Examples include dioxin<sup>174</sup> and peroxisome proliferators.<sup>98</sup> These act through the Ah receptor and peroxisome proliferator-activated receptors, respectively. Both of these proteins interact with DNA following ligand binding and affect gene expression. However, there is no evidence that NTE acts in this way. Neurotoxic esterase is membrane associated<sup>39</sup> which precludes direct interaction with DNA. If there is a biochemical pathway linking NTE inhibition and aging with gene expression changes it might involve multiple steps. Alternatively, this study does not rule out the possibility that gene expression changes identified are caused by effects of PSP not associated with OP compound aging on NTE. If gene expression changes are important mechanistic events leading to axonal degeneration in OPIDN then it does not appear to be a result of large numbers of expression changes. Perhaps, early alteration of expression of one or a few critical gene products is sufficient to initiate axonal degeneration. Alternatively, gene expression changes may not be necessary for OPIDN induction.

Lumbo-sacral spinal cord, giving rise to the ischiatic nerve (corresponding to spinal nerves 25-30), was chosen for this study. This region contains both the cell bodies of motor neurons whose axons undergo degeneration, and the distal end of the medial pontine spinal tract (MPST) that also degenerates in OPIDN. Therefore, both ‘ends’ of affected neurons (and associated glial cells) were examined utilizing a single tissue. The targeted display approach used here had the potential to detect expression changes in motor neuron cell bodies, support cells associated with MPST axons, and other sites. However, tissue heterogeneity has the potential to dilute out effects occurring in specific cell sub-populations, making detection of expression changes in individual cell types more difficult. In this study (chapter 4) expression changes for  $\alpha$ -tubulin and P1 occurred across all neurons, and for P1 perhaps astrocytes also. Another group of investigators examined gene expression in multiple regions of the nervous system, and tried to identify a link between gene expression change and susceptibility of tissue to degeneration in OPIDN.<sup>8, 10</sup> Examination of nervous system regions not affected by axonal degeneration may provide indirect evidence for specific gene involvement in OPIDN. However, NTE itself is expressed in regions where axonal degeneration does not occur.<sup>48</sup> It is therefore possible that mechanistically significant gene expression changes occur in susceptible and non-susceptible nervous system regions. Additionally, a gene expression change occurring only in a susceptible nervous system region does not imply that the alteration is mechanistically significant because it may not be related to OPIDN.

initiation. For these reasons differential expression of  $\alpha$ -tubulin and P1 was only examined in the spinal cord, a region susceptible to OPIDN.

Other regions of the nervous system, such as OPIDN-susceptible peripheral nerves or dorsal root ganglion (DRG) could have been examined for gene expression changes. However, obtaining sufficient RNA from peripheral nerve and DRG for TD would have been problematic. Having said this, an advantage of using peripheral nerve is that possible gene expression changes in nerve cells can be sought within a simpler tissue where axonal degeneration occurs. This tissue would perhaps be the choice if the specific hypothesis tested related to the role of gene expression changes in axon-support cell interactions in OPIDN. This hypothesis is viable, especially given the evidence that *Drosophila* NTE may be involved in neuronal-glial interactions.<sup>44</sup> Theoretically, dorsal root ganglia would have been excellent for this type of study because they represent a simpler tissue than whole spinal cord. They contain primary sensory neurons, glial cells and other support cells. They have been used previously for similar investigations in the rat.<sup>88</sup> However, in adult domestic fowl this presents practical problems because DRG are small, probably because peripheral sensibility is less well developed in birds.<sup>175</sup> Obtaining sufficient RNA for TD and Northern blotting would have been problematic.

There has been discussion in the literature as to whether neuropathic OP esters act on neuronal cell bodies or on their axons. Experiments utilizing unilateral femoral artery injections of DFP in cats show that ‘axonal’ exposure

can induce lesions similar to OPIDN.<sup>55</sup> NTE is transported into axons.<sup>48</sup> However, it is not known whether lesions are induced by only cell body exposure. While it has been reported that neuropathic OP esters act primarily on axons,<sup>176</sup> this does not rule out the possibility that the mechanism would still involve gene expression changes in neurons. Results presented in this dissertation that demonstrates down regulation of expression of  $\alpha$ -tubulin, for example, suggest this possibility. However, even if OP esters act on axons, interference with retrograde neurotrophin signaling back to the neuronal nucleus could affect gene transcription secondarily, leading to axonal degeneration.

## **Future Directions**

Findings from this research raise several new questions and possibilities for research. These are outlined below.

Further characterization of gene P1 is a logical next step. While the sequence identified has 100% homology with a chicken EST (pgp1n.pk010.m23) this did not enable identification of the gene. Five prime RACE was used. Future attempts could use different permutations of 5'-RACE, or screening of a chicken brain cDNA library. Given the rate of *G.gallus* genome sequencing, regular DNA data base searches using this sequence are indicated. Identification of this gene could provide new questions or avenues of work.

Several questions generated by this research could be readily addressed. These are outlined below-

**1. Is differential expression of α-tubulin, P1 and P2 transcript carried through to the protein level?** Expression of α-tubulin, P1 and P2 protein in PSP treated hen spinal cord could be measured. Antibodies to α-tubulin are already available; some of these may cross react with tubulin in this species. The importance of this is that, as stressed in this dissertation, altered regulation of mRNA does not imply altered protein expression. Finding expression changes carried through to the protein level could increase the significance of findings.

**2. What is the mechanistic significance of the detected gene expression changes?** Experiments could be designed to further evaluate the mechanistic significance of transcript (or protein) expression changes. For example,

comparisons could be made between PSP, PMSF, and PMSF then PSP treated hens. As pre-treatment with PMSF is protective, if an expression change occurred in PSP treated hens, but not in protected or PMSF treated hens, it would provide further evidence that the gene is of mechanistic relevance to OPIDN. If expression change occurred in the protected group, but not the PSP or PMSF groups, it would suggest that the change is the result of an interaction between PSP and PMSF, and probably does not relate to OPIDN. The above experiment could be combined with an additional treatment group consisting of birds treated with PSP then PMSF, as this promotes OPIDN.<sup>51</sup> These promoted birds would be expected to show more marked or earlier gene expression changes than PSP treated or protected birds if the gene studied is of mechanistic significance.

**3. Do changes in transcript expression of spinal cord  $\alpha$ -tubulin, P1 and P2 also occur following treatment with other neuropathic OP compounds?**

Genes P1 and P2 could be evaluated following DFP treatment. All three could be evaluated following treatment with other neuropathy-inducing OP esters such as mipafox or TOTP.

**4. Do reported DFP induced changes in fast orthograde axonal transport occur following exposure to PMSF and PSP?** Previous work examining effects of neuropathic OP esters on fast orthograde axonal transport have focused on DFP.<sup>57, 58</sup> Tubulin is a major component of microtubules, the scaffold on which fast axonal transport occurs. PMSF has been shown in this study to affect tubulin gene expression. Therefore, it would be interesting to know whether PMSF

affects fast orthograde axonal transport. If PMSF affects fast axonal transport in a similar manner to DFP then it suggests that transport changes may not be necessary for OPIDN induction. The relevance of DFP induced changes in fast axonal transport would be strengthened if similar responses occurred after PSP exposure. The type of study suggested here has also already been performed for retrograde axonal transport in OPIDN. Di-*n*-butyl-2,2-dichlorovinyl phosphate (DBDCVP) induced a 70% decrease in retrograde axonal transport 7 days after exposure. There was no effect of PMSF, but this compound was protective when given before DBDCVP.<sup>59</sup>

**5. Did the observed changes in transcript expression in this study result from interactions between PSP and DMSO?** Given the effect of DMSO on expression of transcripts for  $\alpha$ -tubulin and P3 (50% of genes studied temporally), the question arises as to the relative importance of an interaction between PSP and DMSO (see above). This issue could be addressed experimentally by using a different vehicle for PSP and PMSF, such as ethanol. Ethanol is unfortunately also neurotoxic,<sup>165</sup> however, as it has different properties to DMSO, it may be useful for addressing the issue of compound interactions.

## **Conclusion**

Organophosphorus induced delayed neurotoxicity is a condition characterized by degeneration of long wide axons within nerves and the spinal cord. NTE is the primary target for OPIDN, and is expressed in susceptible and non-susceptible neurons. This study shows that PSP induced gene expression changes occur in the clinically silent period before clinical toxicosis develops, and that some of these changes do not occur following exposure to PMSF, a non-neuropathic compound that also targets NTE. Expression changes occurred in all neurons within a spinal cord level indicating that while expression changes may be necessary for OPIDN induction they are not sufficient. The factors that result in degeneration restricted to long wide diameter axons in OPIDN need further clarification.

## LITERATURE CITED

1. Jortner BS, Shell L, el-Fawal H, Ehrich M. Myelinated nerve fiber regeneration following organophosphorus ester-induced delayed neuropathy. *Neurotoxicology* 1989; 10:717-26.
2. Ehrich M, Jortner BS. Organophosphorus-Induced Delayed Neuropathy. In: Kreiger R, ed. *Handbook of Pesticide Toxicology*. Vol. 2. San Diego: Academic Press, 2001:988-1012.
3. Clothier B, Johnson MK. Reactivation and aging of neurotoxic esterase inhibited by a variety of organophosphorus esters. *Biochem J* 1980; 185:739-47.
4. Johnson MK. The primary biochemical lesion leading to the delayed neurotoxic effects of some organophosphorus esters. *J Neurochem* 1974; 23:785-9.
5. Johnson MK. Organophosphorus and other inhibitors of brain 'neurotoxic esterase' and the development of delayed neurotoxicity in hens. *Biochem J* 1970; 120:523-31.
6. Johnson MK, Lauwerys R. Protection by some carbamates against the delayed neurotoxic effects of di-isopropyl phosphorofluoridate. *Nature* 1969; 222:1066-7.
7. Gupta RP, Bing G, Hong JS, Abou-Donia MB. cDNA cloning and sequencing of Ca<sup>2+</sup>/calmodulin-dependent protein kinase IIalpha subunit

- and its mRNA expression in diisopropyl phosphorofluoridate (DFP)-treated hen central nervous system. Mol Cell Biochem 1998; 181:29-39.
8. Gupta RP, Lin WW, Abou-Donia MB. Enhanced mRNA expression of neurofilament subunits in the brain and spinal cord of diisopropyl phosphorofluoridate-treated hens. Biochem Pharmacol 1999; 57:1245-51.
  9. Damodaran TV, Abou-Donia MB. Alterations in levels of mRNAs coding for glial fibrillary acidic protein (GFAP) and vimentin genes in the central nervous system of hens treated with diisopropyl phosphorofluoridate (DFP). Neurochem Res 2000; 25:809-16.
  10. Damodaran TV, Abdel-Rahman A, Abou-Donia MB. Altered time course of mRNA expression of alpha tubulin in the central nervous system of hens treated with diisopropyl phosphorofluoridate (DFP). Neurochem Res 2001; 26:43-50.
  11. Gupta RP, Damodaran TV, Abou-Donia MB. C-fos mRNA induction in the central and peripheral nervous systems of diisopropyl phosphorofluoridate (DFP)-treated hens. Neurochem Res 2000; 25:327-34.
  12. Damodaran TV, Rahman AA, Abou-Donia MB. Early differential induction of C-jun in the central nervous system of hens treated with diisopropylphosphorofluoridate (DFP). Neurochem Res 2000; 25:1579-86.

13. Pope C, diLorenzo K, Ehrich M. Possible involvement of a neurotrophic factor during the early stages of organophosphate-induced delayed neurotoxicity. *Toxicol Lett* 1995; 75:111-7.
14. De Nicola AF, Moses DF, Gonzalez S, Orti E. Adrenocorticoid action in the spinal cord: some unique molecular properties of glucocorticoid receptors. *Cell Mol Neurobiol* 1989; 9:179-92.
15. Lidsky TI, Manetto C, Ehrich M. Nerve conduction studies in chickens given phenyl saligenin phosphate and corticosterone. *J Toxicol Environ Health* 1990; 29:65-75.
16. Ehrich M, Jortner BS, Gross WB. Dose-related beneficial and adverse effects of dietary corticosterone on organophosphorus-induced delayed neuropathy in chickens. *Toxicol Appl Pharmacol* 1986; 83:250-60.
17. Cavanagh JB. Toxic substances and the nervous system. *Br Med Bull* 1969; 25:268-73.
18. Couri D, Milks M. Toxicity and metabolism of the neurotoxic hexacarbons n-hexane, 2- hexanone, and 2,5-hexanedione. *Annu Rev Pharmacol Toxicol* 1982; 22:145-66.
19. Pappolla M, Penton R, Weiss HS, Miller CH, Jr., Sahenk Z, Autilio-Gambetti L, Gambetti P. Carbon disulfide axonopathy. Another experimental model characterized by acceleration of neurofilament transport and distinct changes of axonal size. *Brain Res* 1987; 424:272-80.

20. Troncoso JC, Griffin JW, Price DL, Hess-Kozlow KM. Pathology of the peripheral neuropathy induced by p-bromophenylacetylurea. *Lab Invest* 1982; 46:215-23.
21. Tilson HA. The neurotoxicity of acrylamide: an overview. *Neurobehav Toxicol Teratol* 1981; 3:445-61.
22. Abou-Donia MB. Organophosphorus ester-induced delayed neurotoxicity. *Annu Rev Pharmacol Toxicol* 1981; 21:511-48.
23. Roger H, Recordier M. Les polynevrites phosphocreasotiques. *Ann medicine* 1934; XXXV:44-63.
24. Morgan JP. The Jamaica ginger paralysis. *JAMA* 1982; 248:1864-7.
25. Dennis DT. Jake walk in Viet Nam. *Ann Intern Med* 1977; 86:665-666.
26. Smith HV, Spalding JMK. Outbreak of paralysis in Morocco due to ortho-cresyl phosphate poisoning. *The lancet* 1959; II:1019-1021.
27. Ecobichon DJ. Organophosphorus Ester Insecticides. In: Ecobichon DJ, Joy RM, eds. *Pesticides and Neurological Diseases*. Boca Raton: CRC Press, 1994:171-249.
28. Shaw IC, Parker RM, Porter S, Quick MP, Lamont MH, Patel RK, Norman IM, Johnson MK. Delayed neuropathy in pigs induced by isofenphos. *Vet Rec* 1995; 136:95-7.
29. Morgan JP, Penovich P. Jamaica ginger paralysis: Forty seven year follow up. *Arch Neurol* 1978; 35:530-532.

30. Bouldin TW, Cavanagh JB. Organophosphorous neuropathy. II. A fine-structural study of the early stages of axonal degeneration. Am J Pathol 1979; 94:253-70.
31. Bouldin TW, Cavanagh JB. Organophosphorous neuropathy. I. A teased-fiber study of the spatio-temporal spread of axonal degeneration. Am J Pathol 1979; 94:241-52.
32. Tanaka D, Jr., Bursian SJ. Degeneration patterns in the chicken central nervous system induced by ingestion of the organophosphorus delayed neurotoxin tri-ortho-tolyl phosphate. A silver impregnation study. Brain Res 1989; 484:240-56.
33. Johnson MK. A phosphorylation site in brain and the delayed neurotoxic effect of some organophosphorus compounds. Biochem J 1969; 111:487-95.
34. Johnson MK. The delayed neurotoxic effect of some organophosphorus compounds. Identification of the phosphorylation site as an esterase. Biochem J 1969; 114:711-7.
35. Johnson MK. The target for initiation of delayed neurotoxicity by organophosphorus esters: biochemical studies and toxicological applications. Reviews in Biochemical Toxicology 1982; 4:141-211.
36. Ehrich M, Jortner BS, Padilla S. Relationship of neuropathy target esterase inhibition to neuropathology and ataxia in hens given organophosphorus esters. Chem Biol Interact 1993; 87:431-7.

37. Johnson MK. Mechanism of protection against the delayed neurotoxic effects of organophosphorus esters. *Fed Proc* 1976; 35:73-4.
38. Clothier B, Johnson MK. Rapid aging of neurotoxic esterase after inhibition by di-isopropyl phosphorofluoridate. *Biochem J* 1979; 177:549-58.
39. Atkins J, Glynn P. Membrane association of and critical residues in the catalytic domain of human neuropathy target esterase. *J Biol Chem* 2000; 275:24477-83.
40. Johnson MK. Organophosphorus esters causing delayed neurotoxic effects: mechanism of action and structure activity studies. *Arch Toxicol* 1975; 34:259-88.
41. Glynn P, Read DJ, Guo R, Wylie S, Johnson MK. Synthesis and characterization of a biotinylated organophosphorus ester for detection and affinity purification of a brain serine esterase: neuropathy target esterase. *Biochem J* 1994; 301:551-6.
42. Glynn P, Read DJ, Lush MJ, Li Y, Atkins J. Molecular cloning of neuropathy target esterase (NTE). *Chem Biol Interact* 1999; 119-120:513-7.
43. Acierno JS, Jr., Kennedy JC, Falardeau JL, Leyne M, Bromley MC, Colman MW, Sun M, Bove C, Ashworth LK, Chadwick LH, Schiripo T, Ma S, Goldin E, Schiffmann R, Slaugenhaupt SA. A physical and

- transcript map of the MCOLN1 gene region on human chromosome 19p13.3-p13.2. *Genomics* 2001; 73:203-10.
44. Kretzschmar D, Hasan G, Sharma S, Heisenberg M, Benzer S. The swiss cheese mutant causes glial hyperwrapping and brain degeneration in *Drosophila*. *J Neurosci* 1997; 17:7425-32.
  45. Lush MJ, Li Y, Read DJ, Willis AC, Glynn P. Neuropathy target esterase and a homologous *Drosophila* neurodegeneration-associated mutant protein contain a novel domain conserved from bacteria to man. *Biochem J* 1998; 332:1-4.
  46. Mews M, Meyer M. Modulation of Schwann cell phenotype by TGF-beta 1: inhibition of P0 mRNA expression and downregulation of the low affinity NGF receptor. *Glia* 1993; 8:208-17.
  47. Kallunki P, Jenkinson S, Edelman GM, Jones FS. Silencer elements modulate the expression of the gene for the neuron- glia cell adhesion molecule, Ng-CAM. *J Biol Chem* 1995; 270:21291-8.
  48. Glynn P, Holton JL, Nolan CC, Read DJ, Brown L, Hubbard A, Cavanagh JB. Neuropathy target esterase: immunolocalization to neuronal cell bodies and axons. *Neuroscience* 1998; 83:295-302.
  49. Moser M, Stempfl T, Li Y, Glynn P, Buttner R, Kretzschmar D. Cloning and expression of the murine *sws/NTE* gene. *Mech Dev* 2000; 90:279-282.

50. Forshaw PJ, Atkins J, Ray DE, Glynn P. The catalytic domain of human neuropathy target esterase mediates an organophosphate-sensitive ionic conductance across liposome membranes. *J Neurochem* 2001; 79:400-6.
51. Massicotte C, Inzana KD, Ehrich M, Jortner BS. Neuropathologic effects of phenylmethylsulfonyl fluoride (PMSF)-induced promotion and protection in organophosphorus ester-induced delayed neuropathy (OPIDN) in hens. *Neurotoxicology* 1999; 20:749-59.
52. el-Fawal HA, Jortner BS, Ehrich M. Effect of verapamil on organophosphorus-induced delayed neuropathy in hens. *Toxicol Appl Pharmacol* 1989; 97:500-11.
53. Carrington CD, Abou-Donia MB. Axoplasmic transport and turnaround of neurotoxic esterase in hen sciatic nerve. *J Neurochem* 1985; 44:616-21.
54. Lowndes HE, Baker T, Riker WF, Jr. Motor nerve dysfunction in delayed DFP neuropathy. *Eur J Pharmacol* 1974; 29:66-73.
55. Howland RD, Lowndes HE, Baker T, Richardson RJ. DFP mononeuropathy: evidence for a peripheral site of initiation. *Brain Res* 1980; 184:248-51.
56. Schwartz JH, De Camilli P. Chapter 5. Synthesis and trafficking of neuronal proteins. In: Kandel ER, Schwartz JH, Jessell TM, eds. *Principles of Neural Science*. New York: McDraw Hill, 2000:99-103.

57. Reichert BL, Abou-Donia MB. Inhibition of fast axoplasmic transport by delayed neurotoxic organophosphorus esters: a possible mode of action. Mol Pharmacol 1980; 17:56-60.
58. Carrington CD, Lapadula DM, Abou-Donia MB. Acceleration of anterograde axonal transport in cat sciatic nerve by diisopropyl phosphorofluoridate. Brain Res 1989; 476:179-82.
59. Moretto A, Lotti M, Sabri MI, Spencer PS. Progressive deficit of retrograde axonal transport is associated with the pathogenesis of di-n-butyl dichlorvos axonopathy. J Neurochem 1987; 49:1515-22.
60. Gupta RP, Abou-Donia MB. In vivo and in vitro effects of diisopropyl phosphorofluoridate (DFP) on the rate of hen brain tubulin polymerization. Neurochem Res 1994; 19:435-44.
61. James KA, Austin L. The effect of DFP on axonal transport of protein in chicken sciatic nerve. Brain Res 1970; 18:192-4.
62. Bradley WG, Williams MH. Axoplasmic flow in axonal neuropathies. I. Axoplasmic flow in cats with toxic neuropathies. Brain 1973; 96:235-46.
63. Pleasure DE, Mishler KC, Engel WK. Axonal transport of proteins in experimental neuropathies. Science 1969; 166:524-5.
64. Liu CH, Higgins RJ, Buster D, Sanborn JR, Wilson BW. The effect of organophosphates on a chicken brain or sea urchin egg kinesin-driven microtubule motility assay. Toxicol Lett 1993; 69:239-47.

65. Abou-Donia MB. Involvement of cytoskeletal proteins in the mechanisms of organophosphorus ester-induced delayed neurotoxicity. *Clin Exp Pharmacol Physiol* 1995; 22:358-9.
66. Lapadula ES, Lapadula DM, Abou-Donia MB. Biochemical changes in sciatic nerve of hens treated with tri-o-cresyl phosphate: increased phosphorylation of cytoskeletal proteins. *Neurochem Int* 1992; 20:247-55.
67. Abou-Donia MB, Lapadula DM, Suwita E. Cytoskeletal proteins as targets for organophosphorus compound and aliphatic hexacarbon-induced neurotoxicity. *Toxicology* 1988; 49:469-77.
68. Jensen KF, Lapadula DM, Anderson JK, Haykal-Coates N, Abou-Donia MB. Anomalous phosphorylated neurofilament aggregations in central and peripheral axons of hens treated with tri-ortho-cresyl phosphate (TOCP). *J Neurosci Res* 1992; 33:455-60.
69. Jortner BS, Perkins SK, Ehrich M. Immunohistochemical study of phosphorylated neurofilaments during the evolution of organophosphorus ester-induced delayed neuropathy (OPIDN). *Neurotoxicology* 1999; 20:971-5.
70. Mayford M. Ca<sup>2+</sup>/calmodulin-dependent protein kinase and neuronal function. In: Marks F, ed. *Protein Phosphorylation*. New York: VCH Publishers Inc., 1995:149-177.
71. Yamamoto H, Fukunaga K, Tanaka E, Miyamoto E. Ca<sup>2+</sup>- and calmodulin-dependent phosphorylation of microtubule-associated protein

- 2 and tau factor, and inhibition of microtubule assembly. *J Neurochem* 1983; 41:1119-25.
72. Czernik AJ, Pang DT, Greengard P. Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent phosphorylation sites in rat and bovine synapsin I. *Proc Natl Acad Sci U S A* 1987; 84:7518-22.
73. Hashimoto Y, Soderling TR. Regulation of calcineurin by phosphorylation. Identification of the regulatory site phosphorylated by Ca<sup>2+</sup>/calmodulin-dependent protein kinase II and protein kinase C. *J Biol Chem* 1989; 264:16524-9.
74. Ferris CD, Huganir RL, Bredt DS, Cameron AM, Snyder SH. Inositol trisphosphate receptor: phosphorylation by protein kinase C and calcium/calmodulin-dependent protein kinases in reconstituted lipid vesicles. *Proc Natl Acad Sci U S A* 1991; 88:2232-5.
75. Cohn JA, Kinder B, Jamieson JD, Delahunt NG, Gorelick FS. Purification and properties of a multifunctional calcium/calmodulin-dependent protein kinase from rat pancreas. *Biochim Biophys Acta* 1987; 928:320-31.
76. Dash PK, Karl KA, Colicos MA, Prywes R, Kandel ER. cAmp response element-binding protein is activated by Ca<sup>2+</sup>/calmodulin- as well as cAmp-dependent protein kinase. *Proc Natl Acad Sci U S A* 1991; 88:5061-5.

77. Sheng M, Thompson MA, Greenberg ME. CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* 1991; 252:1427-30.
78. Wegner M, Cao Z, Rosenfeld MG. Calcium-regulated phosphorylation within the leucine zipper of C/EBP beta. *Science* 1992; 256:370-3.
79. Hanson PI, Schulman H. Neuronal Ca<sup>2+</sup>/calmodulin-dependent protein kinases. *Annu Rev Biochem* 1992; 61:559-601.
80. el-Fawal HA, Correll L, Gay L, Ehrich M. Protease activity in brain, nerve, and muscle of hens given neuropathy- inducing organophosphates and a calcium channel blocker. *Toxicol Appl Pharmacol* 1990; 103:133-42.
81. Barber D, Hunt J, Ehrich M. Inhibition of calcium-stimulated ATPase in the hen brain P2 synaptosomal fraction by organophosphorus esters: relevance to delayed neuropathy. *J Toxicol Environ Health A* 2001; 63:101-13.
82. Livesey FJ, Hunt SP. Identifying changes in gene expression in the nervous system: mRNA differential display. *Trends Neurosci* 1996; 19:84-8.
83. Lodish H, Baltimore D, Berk A, Lawrence Zipursky S, Matsudaira P, Darnell J. *Molecular Cell Biology*. New York: Scientific American Books, 1995:1344.

84. Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, Bumgarner R, Goodlett DR, Aebersold R, Hood L. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science* 2001; 292:929-34.
85. Su QN, Namikawa K, Toki H, Kiyama H. Differential display reveals transcriptional upregulation of the motor molecules for both anterograde and retrograde axonal transport during nerve regeneration. *Eur J Neurosci* 1997; 9:1542-7.
86. Tetzlaff W, Alexander SW, Miller FD, Bisby MA. Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. *J Neurosci* 1991; 11:2528-44.
87. Kiryu S, Yao GL, Morita N, Kato H, Kiyama H. Nerve injury enhances rat neuronal glutamate transporter expression: identification by differential display PCR. *J Neurosci* 1995; 15:7872-8.
88. Livesey FJ, Hunt SP. Differential display cloning of genes induced in regenerating neurons. *Methods* 1998; 16:386-95.
89. Livesey FJ, JA OB, Li M, Smith AG, Murphy LJ, Hunt SP. A Schwann cell mitogen accompanying regeneration of motor neurons. *Nature* 1997; 390:614-8.
90. Wang XB, Uhl GR. Subtracted differential display: genes with amphetamine-altered expression patterns include calcineurin. *Brain Res Mol Brain Res* 1998; 53:344-7.

91. Wang JF, Chen B, Young LT. Identification of a novel lithium regulated gene in rat brain. *Brain Res Mol Brain Res* 1999; 70:66-73.
92. Vreugdenhil E, de Jong J, Schaaf MJ, Meijer OC, Busscher J, Vuijst C, de Kloet ER. Molecular dissection of corticosteroid action in the rat hippocampus. Application of the differential display techniques. *J Mol Neurosci* 1996; 7:135-46.
93. Fan L, van der Brug M, Chen W, Dodd PR, Matsumoto I, Niwa S, Wilce PA. Increased expression of mitochondrial genes in human alcoholic brain revealed by differential display. *Alcohol Clin Exp Res* 1999; 23:408-13.
94. Lee IJ, Soh Y, Song BJ. Molecular characterization of fetal alcohol syndrome using mRNA differential display. *Biochem Biophys Res Commun* 1997; 240:309-13.
95. Balduini W, Cimino M, Reno F, Marini P, Princivalle A, Cattabeni F. Effects of postnatal or adult chronic acetylcholinesterase inhibition on muscarinic receptors, phosphoinositide turnover and m1 mRNA expression. *Eur J Pharmacol* 1993; 248:281-8.
96. Vendrell M, Zavia NH, Serratosa J, Bondy SC. c-fos and ornithine decarboxylase gene expression in brain as early markers of neurotoxicity. *Brain Res* 1991; 544:291-6.
97. Wilson CL, Safe S. Mechanisms of ligand-induced aryl hydrocarbon receptor-mediated biochemical and toxic responses. *Toxicol Pathol* 1998; 26:657-71.

98. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. *Science* 2001; 294:1866-70.
99. Bartus RT, Elliott PJ, Hayward NJ, Dean RL, Harbeson S, Straub JA, Li Z, Powers JC. Calpain as a novel target for treating acute neurodegenerative disorders. *Neurol Res* 1995; 17:249-58.
100. Gupta RP, Abou-Donia MB. Diisopropyl phosphorofluoridate (DFP) treatment alters calcium- activated proteinase activity and cytoskeletal proteins of the hen sciatic nerve. *Brain Res* 1995; 677:162-6.
101. Carlson K, Ehrich M. Organophosphorus compound-induced modification of SH-SY5Y human neuroblastoma mitochondrial transmembrane potential. *Toxicol Appl Pharmacol* 1999; 160:33-42.
102. Carlson K, Jortner BS, Ehrich M. Organophosphorus compound-induced apoptosis in SH-SY5Y human neuroblastoma cells. *Toxicol Appl Pharmacol* 2000; 168:102-13.
103. Massicotte C. The Effects of Neuropathy-Inducing Organophosphate Esters on Chick Dorsal Root Ganglion Cell Cultures. Ph.D. dissertation, Department of Biomedical Sciences and Pathobiology. Blacksburg: Virginia Tech, 2001.
104. Moyle G. Clinical manifestations and management of antiretroviral nucleoside analog-related mitochondrial toxicity. *Clin Ther* 2000; 22:911-36.

105. Johnson MK. Organophosphates and delayed neuropathy--is NTE alive and well? *Toxicol Appl Pharmacol* 1990; 102:385-99.
106. Glynn P. Neuropathy target esterase. *Biochem J* 1999; 344 Pt 3:625-31.
107. Abou-Donia MB, Viana ME, Gupta RP, Anderson JK. Enhanced calmodulin binding concurrent with increased kinase-dependent phosphorylation of cytoskeletal proteins following a single subcutaneous injection of diisopropyl phosphorofluoridate in hens. *Neurochem Int* 1993; 22:165-73.
108. Gupta RP, Abou-Donia MB. Neurofilament phosphorylation and [<sup>125</sup>I]calmodulin binding by Ca<sup>2+</sup>/calmodulin-dependent protein kinase in the brain subcellular fractions of diisopropyl phosphorofluoridate (DFP)-treated hen. *Neurochem Res* 1995; 20:1095-105.
109. Gupta RP, Abou-Donia MB. Tau phosphorylation by diisopropyl phosphorofluoridate (DFP)-treated hen brain supernatant inhibits its binding with microtubules: role of Ca<sup>2+</sup>/Calmodulin-dependent protein kinase II in tau phosphorylation. *Arch Biochem Biophys* 1999; 365:268-78.
110. el-Fawal HA, Ehrlich MF. Calpain activity in organophosphorus-induced delayed neuropathy (OPIDN): effects of a phenylalkylamine calcium channel blocker. *Ann N Y Acad Sci* 1993; 679:325-9.
111. Gupta RP, Abdel-Rahman A, Wilmarth KW, Abou-Donia MB. Alteration in neurofilament axonal transport in the sciatic nerve of the diisopropyl

- phosphorofluoridate (DFP)-treated hen. *Biochem Pharmacol* 1997; 53:1799-806.
112. Gupta RP, Abdel-Rahman A, Jensen KF, Abou-Donia MB. Altered expression of neurofilament subunits in diisopropyl phosphorofluoridate-treated hen spinal cord and their presence in axonal aggregations. *Brain Res* 2000; 878:32-47.
113. US EPA. Environmental hazard evaluation: Human and domestic animals. [Addendum 10: Neurotoxicity, series 81, 82, and 83. Office of Prevention, Pesticides and toxic substances, Washington, DC. EPA 540/09-1-123. Available from NTIS. Springfield, VA. PB91-154617], 1991.
114. Brown AJ, Hutchings C, Burke JF, Mayne LV. Application of a rapid method (targeted display) for the identification of differentially expressed mRNAs following NGF-induced neuronal differentiation in PC12 cells. *Mol Cell Neurosci* 1999; 13:119-30.
115. Liang P, Pardee AB. Differential display. A general protocol. *Mol Biotechnol* 1998; 10:261-7.
116. Lopez-Nieto CE, Nigam SK. Selective amplification of protein-coding regions of large sets of genes using statistically designed primer sets [see comments]. *Nat Biotechnol* 1996; 14:857-61.
117. Jo H, Zhang H, Zhang R, Liang P. Cloning oncogenic ras-regulated genes by differential display. *Methods* 1998; 16:365-72.

118. Douglass J, McKinzie AA, Couceyro P. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J Neurosci* 1995; 15:2471-81.
119. Salehi M, Hodgkins MA, Merry BJ, Goyns MH. Age-related changes in gene expression in the rat brain revealed by differential display. *Experientia* 1996; 52:888-91.
120. Flanigan TP, Leslie RA. Changes in brain gene expression in psychiatric illness: mRNA differential display provides some clues. *Mol Psychiatry* 1997; 2:451-6.
121. Sarasa M, Terrado J, Mengod G, Climent S, Muniesa P, Palacios JM. Differential expression of alpha-CGRP and beta-CGRP genes within hypoglossal motoneurons in response to axotomy. *Brain Res Mol Brain Res* 1996; 35:269-77.
122. Kiefer R, Lindholm D, Kreutzberg GW. Interleukin-6 and transforming growth factor-beta 1 mRNAs are induced in rat facial nucleus following motoneuron axotomy. *Eur J Neurosci* 1993; 5:775-81.
123. el-Fawal HA, Jortner BS, Ehrich M. Use of the biventer cervicis nerve-muscle preparation to detect early changes following exposure to organophosphates inducing delayed neuropathy. *Fundam Appl Toxicol* 1990; 15:108-20.

124. Cao H, Agarwal SK, Burnside J. Cloning and expression of a novel chicken sulfotransferase cDNA regulated by GH. *J Endocrinol* 1999; 160:491-500.
125. Thomson JM, Waldrip HW, Compton MM. Identification of a differential display product associated with apoptosis in chicken thymocytes. *Dev Comp Immunol* 1997; 21:413-24.
126. Worpenberg S, Burk O, Klempnauer KH. The chicken adenosine receptor 2B gene is regulated by v-myb. *Oncogene* 1997; 15:213-21.
127. Wang DZ, Hu X, Lin JLC, Kitten GT, Solursh M, Lin JJC. Differential displaying of mRNAs from the atrioventricular region of developing chicken hearts at stages 15 and 21. *Front Biosci* 1996; 1:a1-a15.
128. el-Fawal HA, Jortner BS, Ehrich M. Modification of phenyl saligenin phosphate-induced delayed effects by calcium channel blockers: in vivo and in vitro electrophysiological assessment. *Neurotoxicology* 1990; 11:573-92.
129. Massicotte C, Barber DS, Jortner BS, Ehrich M. Nerve conduction and ATP concentrations in sciatic-tibial and medial plantar nerves of hens given phenyl saligenin phosphate. *Neurotoxicology* 2001; 22:91-8.
130. Zimmermann JW, Schultz RM. Analysis of gene expression in the preimplantation mouse embryo: use of mRNA differential display [published erratum appears in Proc Natl Acad Sci U S A 1994 Sep 27;91(20):9664]. *Proc Natl Acad Sci U S A* 1994; 91:5456-60.

131. Wilkinson JQ, Lanahan MB, Conner TW, Klee HJ. Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display. *Plant Mol Biol* 1995; 27:1097-108.
132. King AS, McLelland J. Nervous System. Birds. Their structure and function. Philadelphia: Bailliere Tindall, 1984:237-283.
133. Lentner M. Experimental Design and Analysis. Blacksburg, Virginia, USA: Valley Book Company, 1993:585.
134. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 1992; 257:967-71.
135. Welsh J, Chada K, Dalal SS, Cheng R, Ralph D, McClelland M. Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Res* 1992; 20:4965-70.
136. Drewes G, Ebneth A, Preuss U, Mandelkow EM, Mandelkow E. MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell* 1997; 89:297-308.
137. Callard D, Lescure B, Mazzolini L. A method for the elimination of false positives generated by the mRNA differential display technique. *Biotechniques* 1994; 16:1096-7, 1100-3.

138. Miele G, MacRae L, McBride D, Manson J, Clinton M. Elimination of false positives generated through PCR re-amplification of differential display cDNA. *Biotechniques* 1998; 25:138-44.
139. Zhang H, Zhang R, Liang P. Differential screening of differential display cDNA products by reverse northern. *Methods Mol Biol* 1997; 85:87-93.
140. Muller M, Kruse L, Tabrett AM, Barbara DJ. Detection of a single base exchange in PCR-amplified DNA fragments using agarose gel electrophoresis containing bisbenzimide-PEG. *Nucleic Acids Res* 1997; 25:5125-6.
141. Yoshikawa Y, Mukai H, Asada K, Hino F, Kato I. Differential display with carboxy-X-rhodamine-labeled primers and the selection of differentially amplified cDNA fragments without cloning. *Anal Biochem* 1998; 256:82-91.
142. Wadhwa R, Duncan E, Kaul SC, Reddel RR. An effective elimination of false positives isolated from differential display of mRNAs. *Mol Biotechnol* 1996; 6:213-7.
143. Mohr S, Cullen P, Schmidt R, Cignarella A, Assmann G. Avoidance of false positives in PCR-based mRNA differential display during investigation of nonstandardized tissues or cells. *Clin Chem* 1997; 43:182-4.
144. Correll L, Ehrich M. A microassay method for neurotoxic esterase determinations. *Fundam Appl Toxicol* 1991; 16:110-6.

145. Maniatis Tea. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Larboratory, 1989.
146. Tuma RS, Beaudet MP, Jin X, Jones LJ, Cheung CY, Yue S, Singer VL. Characterization of SYBR Gold nucleic acid gel stain: a dye optimized for use with 300-nm ultraviolet transilluminators. *Anal Biochem* 1999; 268:278-88.
147. Muller W, Hattesohl I, Schuetz HJ, Meyer G. Polyethylene glycol derivatives of base and sequence specific DNA ligands: DNA interaction and application for base specific separation of DNA fragments by gel electrophoresis. *Nucleic Acids Res* 1981; 9:95-119.
148. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; 215:403-10.
149. Gish W, States DJ. Identification of protein coding regions by database similarity search. *Nat Genet* 1993; 3:266-72.
150. Nagase T, Kikuno R, Ishikawa KI, Hirosawa M, Ohara O. Prediction of the coding sequences of unidentified human genes. XVI. The complete sequences of 150 new cDNA clones from brain which code for large proteins in vitro. *Dna Res* 2000; 7:65-73.
151. Ehrich M, Jortner BS, Padilla S. Comparison of the relative inhibition of acetylcholinesterase and neuropathy target esterase in rats and hens given cholinesterase inhibitors. *Fundam Appl Toxicol* 1995; 24:94-101.

152. Ehrich M, Jortner BS, Taylor D, Dunnington EA, Siegel PB. Differences between genetic stocks of chickens in response to acute and delayed effects of an organophosphorus compound. *J Toxicol Environ Health* 1993; 39:539-53.
153. Xie K, Gupta RP, Abou-Donia MB. Protein levels of neurofilament subunits in the hen central nervous system following prevention and potentiation of diisopropyl phosphorofluoridate (DFP)-induced delayed neurotoxicity(1). *Biochem Pharmacol* 2002; 63:11-9.
154. McKean PG, Vaughan S, Gull K. The extended tubulin superfamily. *J Cell Sci* 2001; 114:2723-33.
155. Redeker V, Melki R, Prome D, Le Caer JP, Rossier J. Structure of tubulin C-terminal domain obtained by subtilisin treatment. The major alpha and beta tubulin isotypes from pig brain are glutamylated. *FEBS Lett* 1992; 313:185-92.
156. Regnard C, Desbruyeres E, Denoulet P, Edde B. Tubulin polyglutamylase: isozymic variants and regulation during the cell cycle in HeLa cells. *J Cell Sci* 1999; 112:4281-9.
157. Miller FD, Naus CC, Durand M, Bloom FE, Milner RJ. Isotypes of alpha-tubulin are differentially regulated during neuronal maturation. *J Cell Biol* 1987; 105:3065-73.

158. Panda D, Miller HP, Banerjee A, Luduena RF, Wilson L. Microtubule dynamics in vitro are regulated by the tubulin isotype composition. *Proc Natl Acad Sci U S A* 1994; 91:11358-62.
159. Lemischka I, Sharp PA. The sequences of an expressed rat alpha-tubulin gene and a pseudogene with an inserted repetitive element. *Nature* 1982; 300:330-5.
160. Pratt LF, Cleveland DW. A survey of the alpha-tubulin gene family in chicken: unexpected sequence heterogeneity in the polypeptides encoded by five expressed genes. *EMBO J* 1988; 7:931-40.
161. Larsen J, Gasser K, Hahin R. An analysis of dimethylsulfoxide-induced action potential block: a comparative study of DMSO and other aliphatic water soluble solutes. *Toxicol Appl Pharmacol* 1996; 140:296-314.
162. Reinstein L, Mahon R, Jr., Russo GL. Peripheral neuropathy after concomitant dimethyl sulfoxide use and sulindac therapy. *Arch Phys Med Rehabil* 1982; 63:581-4.
163. Cavaletti G, Oggioni N, Sala F, Pezzoni G, Cavalletti E, Marmiroli P, Petruccioli MG, Frattola L, Tredici G. Effect on the peripheral nervous system of systemically administered dimethylsulfoxide in the rat: a neurophysiological and pathological study. *Toxicol Lett* 2000; 118:103-7.
164. Somei K, Mizuma K, Nara K, Matsuya H, Yoda J, Sasaki K, Kashimoto T. Dimethylsulfoxide potentiates the suppressive effects of lidocaine on

- synaptic transmission in bullfrog sympathetic ganglion. Methods Find Exp Clin Pharmacol 1995; 17:571-6.
165. Torres G, Horowitz JM. Drugs of abuse and brain gene expression. Psychosom Med 1999; 61:630-50.
166. Krady JK, Oyler GA, Balaban CD, Billingsley ML. Use of avidin-biotin subtractive hybridization to characterize mRNA common to neurons destroyed by the selective neurotoxicant trimethyltin. Brain Res Mol Brain Res 1990; 7:287-97.
167. Sigolaeva LV, Makower A, Eremenko AV, Makhaeva GF, Malygin VV, Kurochkin IN, Scheller FW. Bioelectrochemical analysis of neuropathy target esterase activity in blood. Anal Biochem 2001; 290:1-9.
168. Tanaka D, Jr., Bursian SJ, Lehning E. Selective axonal and terminal degeneration in the chicken brainstem and cerebellum following exposure to bis(1-methylethyl)phosphorofluoridate (DFP). Brain Res 1990; 519:200-8.
169. Damodaran TR, Abou-Donia MB. Vimentin gene expression is altered in the central nervous system of hens treated with diisopropylphosphorofluoridate (abstract). Toxicological Sciences 2000; 54:211.
170. Glynn P. Neural development and neurodegeneration: two faces of neuropathy target esterase. Prog Neurobiol 2000; 61:61-74.

171. Lapadula DM, Irwin RD, Suwita E, Abou-Donia MB. Cross-linking of neurofilament proteins of rat spinal cord in vivo after administration of 2,5-hexanedione. *J Neurochem* 1986; 46:1843-50.
172. Lapadula DM, Suwita E, Abou-Donia MB. Evidence for multiple mechanisms responsible for 2,5-hexanedione- induced neuropathy. *Brain Res* 1988; 458:123-31.
173. Stone JD, Peterson AP, Eyer J, Oblak TG, Sickles DW. Neurofilaments are nonessential to the pathogenesis of toxicant-induced axonal degeneration. *J Neurosci* 2001; 21:2278-87.
174. Schmidt JV, Bradfield CA. Ah receptor signaling pathways. *Annu Rev Cell Dev Biol* 1996; 12:55-89.
175. King AS. Comparative Neuroanatomy. Physiological and Clinical Anatomy of the Domestic Mammals. Vol. 1. Oxford: Oxford University Press, 1987:210-219.
176. Lotti M, Caroldi S, Moretto A, Johnson MK, Fish CJ, Gopinath C, Roberts NL. Central-peripheral delayed neuropathy caused by diisopropyl phosphorofluoridate (DFP): segregation of peripheral nerve and spinal cord effects using biochemical, clinical, and morphological criteria. *Toxicol Appl Pharmacol* 1987; 88:87-96.
177. Kopito RR, Sitia R. Aggresomes and Russell bodies. Symptoms of cellular indigestion? *EMBO Rep* 2000; 1:225-31.

178. Kim PS, Arvan P. Endocrinopathies in the family of endoplasmic reticulum (ER) storage diseases: disorders of protein trafficking and the role of ER molecular chaperones. *Endocr Rev* 1998; 19:173-202.

## **APPENDIX A: TARGETED DISPLAY PRIMER DESIGN**

### **Primer Design**

The TD approach used in this dissertation is a variation of classical isotopic differential display (DD) methodology. The only significant difference from DD is primer design. These primers were based on the octamer primers designed by Lopez-Nieto<sup>116</sup> with subsequent modification by Brown.<sup>114</sup> Octamers were designed by statistical analysis of 1000 human protein coding regions of genes to amplify protein coding regions of large numbers of genes.<sup>116</sup> Subsequent extension of 5-prime ends by six base pairs to produce 14-mers all having a 50% GC ratio enabled use in PCR.<sup>114</sup> A total of 30 sense primers and their reverse complement anti-sense primers have been designed. The full set of primers available using this methodology is shown below (Table 4). Each sense primer can be used with all of the anti-sense primers except its own reverse complement. There are therefore a total of 870 (30 x 29) possible primer combinations. As targeted display primers are designed to target and amplify protein coding regions of genes they give a theoretical advantage over DD, an approach in which 3-prime untranslated regions of genes are amplified.

**Table 5. Full set of targeted display primers**

Sense primers		Anti-sense primers	
1s	t g g t g a a a g c a g g a	1a	t g g t g a t c c t g c t t
2s	t g a t g a c a t c g t g g	2a	t g a t g a c c a c g a t g
3s	t g a t g a c c t g a g g a	3a	t g a t g a t c c t c a g g
4s	t g g t g a g a a g c t g a	4a	t g g t g a t c a g c t t c
5s	t g g t g a g t g g a g a a	5a	t g g t g a t t c t c c a c
6s	t g a t g a a g a a g g c c	6a	t g a t g a g g c c t t c t
7s	t g g t g a c a t g a a g g	7a	t g g t g a c c t t c a t g
8s	t g g t g a c c t g g a a a	8a	t g g t g a t t c c a g g
9s	t g g t g a g a a g t g g a	9a	t g g t g a t c c a c t t c
10s	t g a t g a t c c t g g a c	10a	t g a t g a g t c c a g g a
11s	t g a t g a a g a c c c t g	11a	t g a t g a c a g g g t c t
12s	t g g t g a c c a a g a a g	12a	t g g t g a c t t c t t g g
13s	t g a t g a c c t g g a g a	13a	t g a t g a t c t c c a g g
14s	t g a t g a g a c c t g g a	14a	t g a t g a t c c a g g t c
15s	t g a t g a t g c t g a g c	15a	t g a t g a g c t c a g c a
16s	t g a t g a a g a g g a g c	16a	t g a t g a g c t c c t c t
17s	t g g t g a c c a g a t g a	17a	t g g t g a t c a t c t g g
18s	t g a t g a c t a c c t g g	18a	t g a t g a c c a g g t a g
19s	t g g t g a g a g c t g a a	19a	t g g t g a t t c a g c t c

---

20s	t g g t g a t g g a c a a g	20a	t g g t g a c t t g t c c a
21s	t g g t g a a t g c t g g a	21a	t g g t g a t c c a g c a t
22s	t g a t g a c c t c a c c a	22a	t g a t g a t g g t g a g g
23s	t g g t g a c t g c a g a a	23s	t g g t g a t t c t g c a g
24s	t g g t g a g a g g a g a t	24a	t g g t g a a t c t c c t c
25s	t g a t g a t g g a c c t g	25a	t g a t g a c a g g t c c a
26s	t g g t g a c a a g g a g a	26a	t g g t g a t c t c c t t g
27s	t g a t g a c c t g a a g g	27a	t g a t g a c c t t c a g g
28s	t g a t g a c t g c c a a g	28a	t g a t g a c t t g g c a g
29s	t g g t g a g g a g a a a g	29a	t g g t g a c t t t c t c c
30s	t g g t g a t g g a g a g a	30a	t g g t g a t c t c t c c a

---

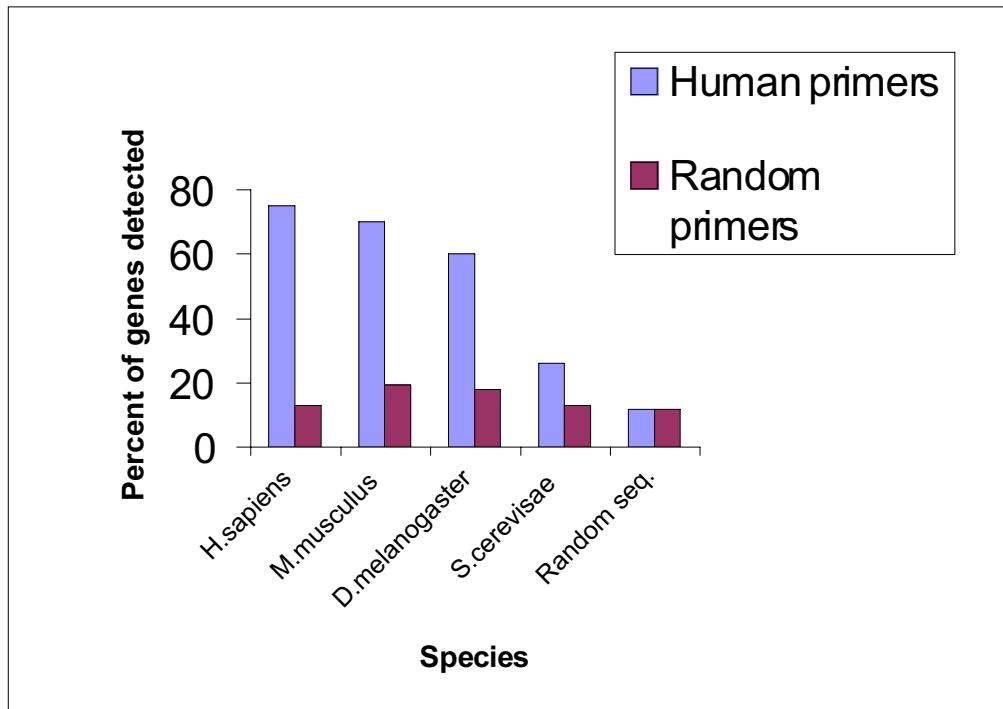
## **Gene Detection Rates using Targeted Display Primers**

Individual eukaryotic cells express about 12000–15000 different mRNA species.<sup>134</sup> The author estimates that chicken spinal cord gray matter expresses ~30000 genes. One hundred and fifty primer combinations were used in this study. About 100 gel bands were identified per primer combination; thus, a total of 15000 bands were screened in this TD study. However, the number of gel bands does not equate with the number of gene transcripts studied. This is because some transcripts are represented more than once. Redundancy in this context is the presence of different amplification products of the same transcript derived from the same or different primer combinations. The rate of band redundancy is unknown, but is estimated by the author to be 10-20%. If redundancy in this system is assumed to be 15% then 12750 different genes were screened, this is 42.5% of the expressed gene pool for this tissue based on the above assumptions.

The work of Lopez-Nieto<sup>116</sup> showed that designed primers had a gene detection rate of ~76% in humans. Gene detection rates are much higher with statistically designed targeted primers versus randomly designed primers (Figure 23). Computer modeling showed that statistically designed primers are theoretically able to target protein coding sequences of non-human species with reducing detection rates moving down the phylogenetic tree (Figure 23).<sup>116</sup> Based on this information it is expected that the gene detection rate in the chicken

is between 60-70%. That is, if all the primer combinations were used then 60-70% of the expressed gene pool would be amplified.

**Figure 23. Comparison of expected gene detection rates by 30 human statistically designed primer pairs and 30 randomly selected octa-nucleotide primer pairs in different species**



From Lopez-Nieto *et al*, Nature Biotechnology, 1996.

Percent of genes detected using eight-mer primers designed in man (*H.sapiens*) and used against other species. Gene detection rates using random sequences are also shown. Gene detection rates decrease moving down the phylogenetic tree. *M.musculus*, mouse; *D.melanogaster*, drosophila; *S.cerevisiae*, a yeast.

## **APPENDIX B: DNA SEQUENCE OF P1, P2 AND P3**

### **Gene P1**

(20A primer)

**TGGTGACTTGTCCAGGAGGTGCAGAGACGGGTTCCAGTTATTCCCTGT**  
ACAGAGCCTCTGCCAACCAACCAGAGCATGGGCTGTAGGGGCAGGAGAGA  
GATCCTGACACTGTGGAAGGACAAAGCAGGAGTGGAAAGCCCAGTGGC  
AGAAACATGCAAGCTGAGCAAGAGCAGTGTCCAAGGCAAGTCTCTTC  
TAAGCAGGCTTCTACTGCCTGGGGGCAGGAAGTGGGATAAG**CTCA**  
**GCATCATCA** (15S primer)

Primers are shown in bold italics. There was 100% homology with expressed sequence tag (EST) pgp1n.pk010.m23 (GenBank Accession: BI393303). This EST comprised 1500 bp of 3-prime untranslated region (UTR) (complete sequence not shown). Patching together sequences of ESTs revealed a UTR of  $\geq 2700$  bp. There was 100% homology between primers and primer binding sites. The entire transcript is  $\sim 6\text{-}7$  kb in length based on Northern analysis.

### **Gene P2**

(20A primer)

**TGGTGACTTGTCCAACGTATGCAGCTCATTGAGAGCCTGCTGTGCACG**  
ACTGCTGCTCCCCATAGCTGAGGCTTGCTGGAAGTTGATCTGAATTGC  
ATCCATTAAGAAATTCAACATCTCCAGCACCAAAGGAGCAAAGGAGA  
AGTTGGCCTGGGACTGTAATTCCCTCTCGACAGCCCTAACACATTCTACA  
CAGGCTGCTCTTCTTGGGTTTC**CATCTGGTCACCA** (17S primer)

Primers are shown in bold italics. There was  $\sim 82\%$  DNA homology with human gene KIAA1307.<sup>150</sup> The sequence shown represents an open reading

frame. It is not known whether the primer sequences are 100% homologous to binding sites.

### Gene P3

(11A primer)

***TGATGACAGGGTCTATAGACAAC***TTACAGCCAATAAGTTCCATATT

ACCAACCAC~~TTCTGG~~AAAAAAAAACCCACAAAACACCACAATCACAC

ACACCA~~CACACTCGAACG~~TTGCT***CCTCAGGT***CATCA (3S primer)

Primers are shown in bold italics. Homologies were not found following DNA data base searches. It is not known whether the primer sequences are 100% homologous to binding sites.

## **APPENDIX C: MORPHOLOGIC ANALYSIS OF ADULT WHITE LEGHORN SPINAL MOTOR NEURONS**

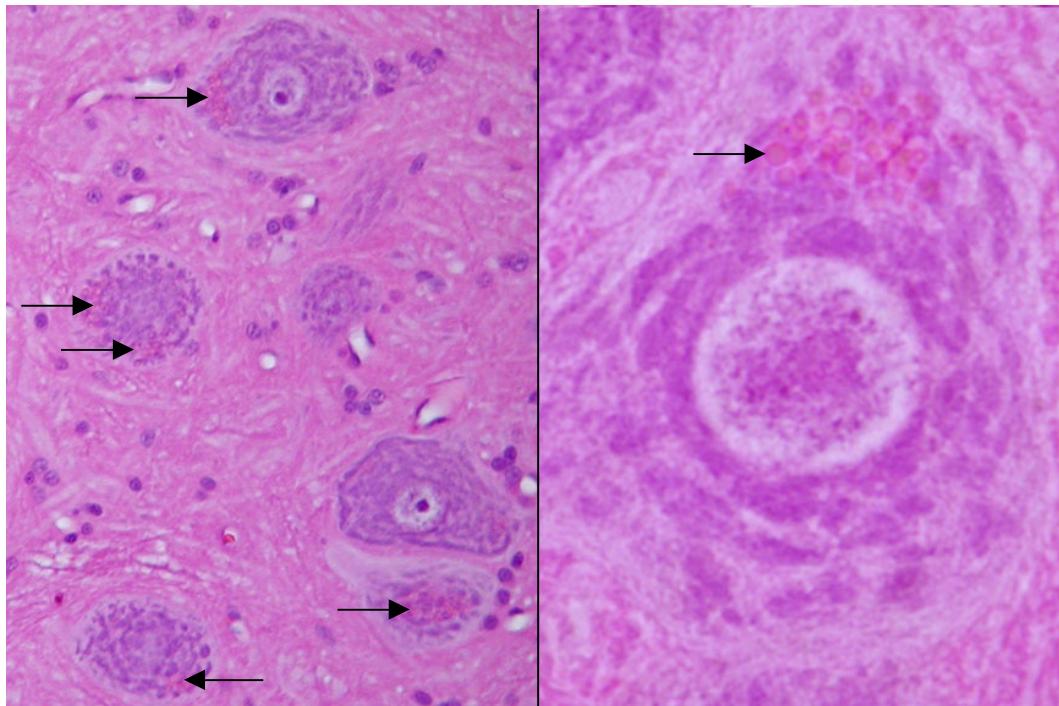
An unexpected finding noted during the *in-situ* hybridization study (chapter 4) was the presence of motor neuron cytoplasmic inclusions that were dark pink with H&E staining (Figure 24). These inclusions were present equally in PSP and DMSO treated birds at 12 hour, and 48 hour sacrifice points, and were restricted almost entirely to large motor neurons. Given the previously noted effects of DMSO on spinal cord gene expression (chapter 3), the possibility of DMSO neurotoxicity was considered. A literature search provided some evidence for DMSO neurotoxicity.<sup>161-163</sup> The author was not aware of this literature before embarking on this study. No further evidence was found that inclusions represent a normal avian morphological feature. The question arose: Are motor neuron inclusions transiently induced by DMSO? To answer this question an experiment was performed in which adult hens were treated with 0.5 mls / kg im DMSO (100%) or saline (0.9%) and sacrificed at 24 hours. Tissues were fixed by immersion or perfusion. The treatment structure is shown below-

<b>Treatment</b>	<b># Birds</b>	<b>Fixation</b>
DMSO	2	Perfusion
DMSO	2	Immersion
Saline	2	Perfusion

All birds had the inclusions on H&E staining. Method of fixation or treatment had no effect on presence of granules. It was concluded that inclusions are most likely a feature of this strain of White Leghorn bird. Ultra-structural examination of perfused tissues revealed inclusions comprised double membrane bound structures with occasional continuity with tubular membranes consistent with rough endoplasmic reticulum, and homogenous osmiophilic centers (Figure 25 and 26). There appeared to be occasional surface ribosomes. Structurally, granules appear to be analogous with Russell bodies, foci of protein dilated rough endoplasmic reticulum found in plasma cells. These proteinaceous inclusions are thought to accumulate when a protein synthesis exceeds its rate of folding and degradation, leading to an accumulation somewhere along the secretory pathway.<sup>177</sup> These features are suggestive of an endoplasmic reticulum storage disease.<sup>178</sup>

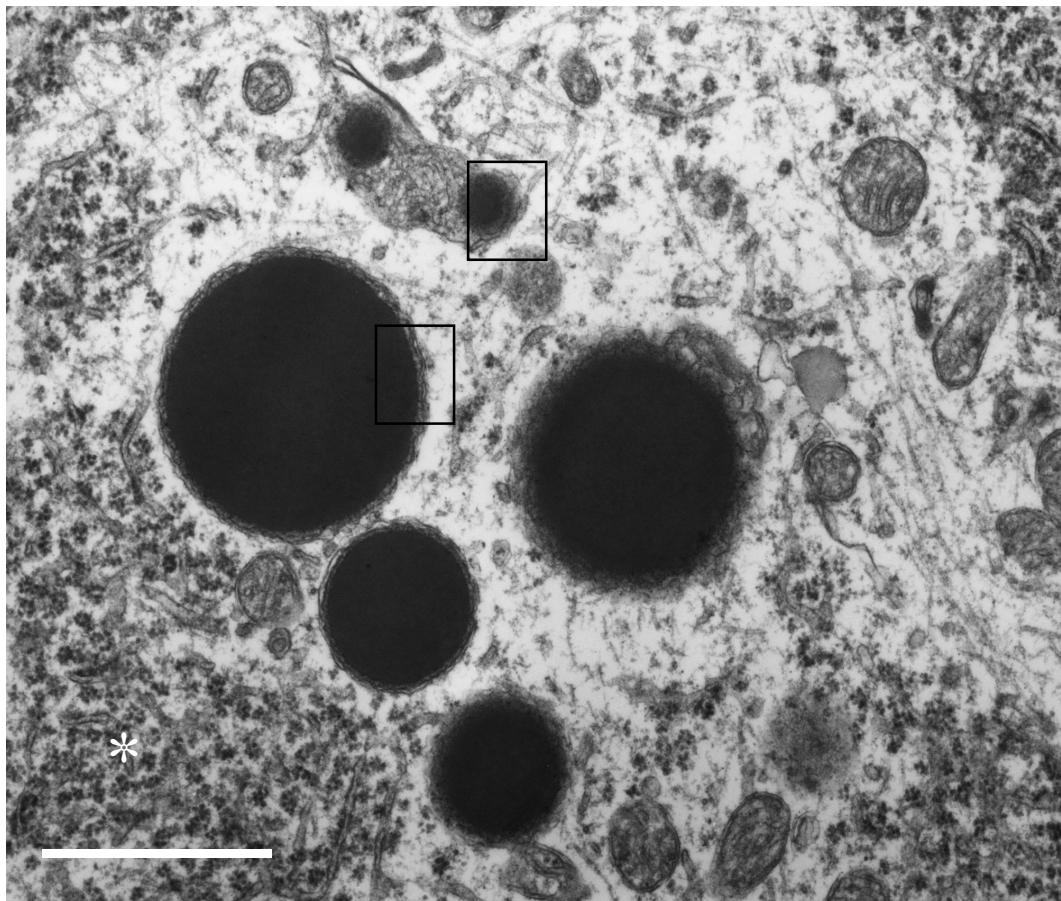
While interesting in themselves, these findings are not thought to have any impact on the findings of this study. These birds do not demonstrate any neurodegenerative changes or clinical deficits up to ~3 years, so the abnormality appears to be sub-clinical. Furthermore, this low antibody strain of White Leghorn chicken was found to be less sensitive to DFP induced OPIDN, compared to other closely related strains.<sup>152</sup>

**Figure 24. Spinal Cord Motor Neuron Cytoplasmic Granules**



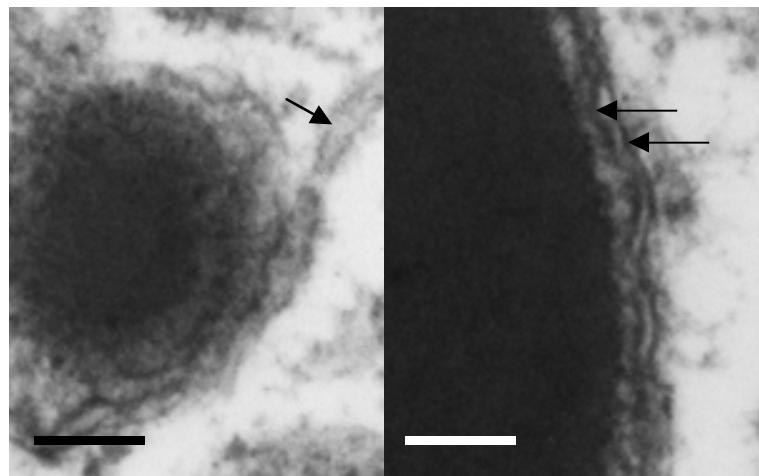
Shown are motor neurons from the lateral-ventral gray matter of lumbo-sacral spinal cord. Within the Nissl substance there are numerous pink rounded granules (arrows). These granules were much less numerous within cervical cord motor neurons. Neurodegenerative changes were not evident.

**Figure 25. Ultra-structural Features of Cytoplasm of Motor Neuron**



Cytoplasmic granules have homogenous osmiophilic centers and are typically present within or at the edge of rough endoplasmic reticulum (\*). Rectangles represent regions shown in figure 26. Bar = 1  $\mu\text{m}$ .

**Figure 26. Ultra-structural Features of Cytoplasmic Granules**



Electron photograph micrographs represent magnifications from figure 25. Left: a structure interpreted to represent an early granule showing continuity with a double membranous structure (arrow) interpreted to be part of the endoplasmic reticulum. Right: a larger granule showing its surrounding double membrane (arrows). Bars = 100 nm.

## **VITA**

Jonathan Fox was born in Inkberrow, England in March 1964. He spent most of his childhood in Warwickshire, England. In 1987 he entered the veterinary degree program at the University of Liverpool. He obtained a Bachelor of Science Degree in Medical Cell Biology in 1990, and a Bachelor of Veterinary Science Degree with distinction in 1993. After becoming a Member of the Royal College of Veterinary Surgeons he decided to pursue a career in pathology and research. He embarked on a two year residency training program in veterinary anatomic pathology at the University of Florida which was successfully completed in 1995. The next 20 months were spent working as a veterinary pathologist at the Texas Veterinary Medical Diagnostic Laboratory in College Station, Texas. In September 1996 he passed qualifying examinations necessary to become a Diplomate of the American College of Veterinary Pathologists. During his veterinary and pathology training Jonathan developed an interest in toxicant-induced nervous system injury. In July 1997 he entered the Ph.D. program at the College of Veterinary Medicine, Virginia Tech with the goal of working on organophosphorus-induced axonopathy.

## Papers

1. Lehning EJ, Jortner BS, **Fox JH**, Arezzo JC, Kitano T, LoPachin RM. Gamma-diketone peripheral neuropathy. I. Quantitative morphometric analyses of axonal atrophy and swelling. *Toxicol Appl Pharmacol* 2000; 165:127-40.
2. Duncan J, R. B., **Fox JH**, Lindsay DS, Dubey JP, Zuccaro ME. Acute sarcocystosis in a captive White-tailed deer in Virginia. *J Wildl Dis* 2000; 36:357-361.
3. **Fox J**, Duncan R, Friday P, Klein B, Scarratt W. Cerebello-olivary and lateral (accessory) cuneate degeneration in a juvenile American Miniature horse. *Vet Pathol* 2000; 37:271-274.
4. **Fox JH**, Homer BL, Kuchinski K. A retrospective necropsy study of the ostrich (*Struthio camelus*) bursa of Fabricius. *Avian Pathology* 1999; 28:491-494.
5. **Fox J**, Li YT, Dawson G, Alleman A, Johnsrude J, Schumacher J, Homer B. Naturally occurring GM2 ganglosidosis in two Muntjak deer with pathological and biochemical features of human classical Tay-Sachs disease (type B GM2 gangliosidosis). *Acta Neuropathol (Berl)* 1999; 97:57-62.
6. Schumacher J, Bennett RA, Fox LE, Deem SL, Neuwirth L, **Fox JH**. Mast cell tumor in an eastern kingsnake (*Lampropeltis getulus getulus*). *J Vet Diagn Invest* 1998; 10:101-4.

7. Deem SL, Heard DJ, **Fox JH**. Avian pox in eastern screech owls and barred owls from Florida. *J Wildl Dis* 1997; 33:323-7.
8. Johnsrude JD, Alleman AR, Homer BL, **Fox JH**, Schumacher J, Harvey JW. Cytological findings in cerebrospinal fluid from two animals with GM2 gangliosidosis. *Vet Clin Path* 1996; 25:80-3.
9. **Fox JH**, Greiner EC, Bain P, Jones R. Malaria in a captive emu (*Dromaius novaehollandiae*) from Florida. *Avian Dis* 1996; 40:477-9.

### Select abstracts

1. **Fox JH**, Ehrich M, Hancock SP, Jortner BS. Phenyl saligenin phosphate induced spinal cord gene expression changes in adult *Gallus gallus*. *Toxicological Sciences* 2002; 66: 315
2. **Fox JH**, Ehrich MF, Barber DS, Jortner BS. Use of targeted display to identify differentially expressed spinal cord mRNA transcripts in chicken organophosphorus induced delayed neurotoxicity. *Toxicological Sciences* 2001; 60:239.
3. Lehning EJ, Jortner BS, **Fox JH**, Chiu FC, LoPachin RM. Gamma-diketone neuropathy: II. Morphometric analyses of axonal atrophy and swelling. *Toxicological Sciences* 1999; 48:285.
4. **Fox JH**, Jortner BS, Lehning EJ, LoPachin RM. Gamma-diketone neuropathy: I. Qualitative neuropathic assessment. *Toxicological Sciences* 1999; 48:285.

5. Lehning EJ, Jortner BS, **Fox JH**, Perkins S, Hinckley J, LoPachin RM. Gamma-diketone neuropathy: Ultrastructural characterization of peripheral nerve fibers. *Toxicological Sciences* 2000; 54:176.