Effects of Diazinon and Diazoxon on Astrocyte-Neuronal Interactions: Inhibition of Neurite Outgrowth by Mechanisms of Oxidative Stress

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Abstract

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Organophosphorus insecticides (OPs) are among the most widely-used class of insecticides in the world. Concern for the developmental neurotoxic potential of OPs has increased in recent years due to mounting *in vitro*, *in vivo*, as well as epidemiological evidence showing adverse developmental effects of these compounds. The purpose of this project was to elucidate the neurotoxic mechanisms of a specific OP, diazinon (DZ), and its oxygen metabolite, diazoxon (DZO). Specifically, this work focuses on the ability for these compounds to adversely affect astrocyte function and impair their ability to foster neurite outgrowth in primary hippocampal neurons. The results demonstrate that both DZ and DZO induce oxidative stress in astrocytes, which subsequently modulates their ability to promote neuronal growth in hippocampal neurons, as well as production and expression of the neuritogenic extracellular matrix protein, fibronectin. Furthermore, the mechanistic *in vitro* studies revealed that these OPs directly inhibit neurite outgrowth in hippocampal neurons. Interestingly, astrocytes protect against such effects of DZ and DZO on neurite outgrowth by increasing neuronal levels of a vital endogenous antioxidant factor, glutathione (GSH). Depleted of GSH, astrocytes are not able to confer protection to neurons against DZ- and DZO-induced inhibition of neurite outgrowth.

Ultimately, this work provides strong evidence for an astrocyte-mediated mechanism of toxicity to these OPs in a developmental neurotoxicity model. These results highlight the importance of astrocytes in supporting neuronal growth and development, and their role in a properly functioning antioxidant defense system in the brain. Thus, the impairment of astrocyte function by DZ and DZO shown here supports the concern for these ubiquitous compounds to adversely affect neurodevelopment, which starts *in utero* and continues on through adolescence.

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GLOSSARY

AChE: Acetylcholine esterase

ACM: Astrocyte-conditioned media

BSO: L-buthionine sulfoximine

CPF: Chlorpyrifos

DMSO: Dimethyl sulfoxide

DZ: Diazinon

DZO: Diazoxon

ECM: Extracellular matrix

FN: Fibronectin

GSH: Glutathione

GSHee: Glutathione ethyl-ester

Mel: Melatonin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide

PBN: N-t-butyl-alpha-phenylnitrone

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DEDICATION

To Harlow,
whose progress will always be more interesting and
more rewarding than any research.

Chapter 1

INTRODUCTION

"To know the brain...is equivalent to ascertaining the material course of thought and will, to discovering the intimate history of life in its perpetual duel with external forces."

-Santiago Ramón y Cajal

1.1 Environmental Toxicants and Neurodevelopment

On both a macro and micro scale, the developing brain is quite different than the adult brain. During development, a multitude of dynamic processes take place, actively forming the neural networks and connections that are relatively unchanged in adult life. This, as well as age-related differences in metabolism and pharmacokinetics, render the developing central nervous system (CNS) particularly vulnerable to injury by exogenous sources (i.e. environmental toxicants). As discussed by Rice and Barone (2000), development of the CNS in humans refers to a process that begins early *in utero* and continues through puberty. Process- and cell-specific behaviors in the brain occur primarily during discrete times in development. For example, in the human brain, the majority of glial cell proliferation occurs in the second half of prenatal development and during the first years of the postnatal period. Synapse formation, on the other hand, starts early in the embryonic period, drastically increases in the early postnatal years, and continues on a lesser scale through puberty (Rice and Barone, 2000).

Seeing as a significant amount of development occurs postnatally in humans, age-specific behaviors support the increased concern for the susceptibility of the developing brain to toxicant exposure. For instance, babies and toddlers have a higher frequency of hand-to-mouth contact and spend more time on or close to the floor than do adults (Xue et al., 2007). Such behaviors increase the frequency and amount of exposure one receives to whatever contaminants may be present in that environment. According to the U.S. Environmental Protection Agency (EPA), children also ingest more soil and dust, and tend to eat more in the amount and types of foods that are contaminated with toxicants such as pesticides (EPA, 2008).

Mounting evidence suggests a link between environmental exposures and various behavioral alterations in children exposed *in utero* or in early phases of development. Prenatal and early postnatal exposure to certain pesticides, especially organophosphorus insecticides such as chlorpyrifos (CPF), have been linked to lower IQ and decreased memory function (Rauh et al., 2011), as well as to increased incidence of attention-deficit hyperactivity disorder (ADHD) and other behavioral impairments (Bouchard et al., 2010; Rohlman et al., 2011). Extensive epidemiological evidence also exists supporting developmental effects of other environmental exposures, lead (Olympio et al., 2009) and second-hand cigarette smoke (Lee et al., 2011) being two well-studied examples.

1.2 The Role of Glial Cells: Glial-Neuronal Interactions

Traditionally, neurotoxic compounds are evaluated on their ability to cause neuronal injury as the primary mechanism of toxicity. In recent years, glial cells have gained recognition as major players in modulating neurotoxicity. Work is now being done by our lab and others demonstrating the adverse effects of various environmental contaminants on glial cells, and the role this damage plays in the mechanisms of neurotoxicity of such compounds.

Astrocytes, a specific class of glial cells, are the most abundant cell type in the brain and are now recognized as playing a vital, active role in brain function and development (Nedergaard et al., 2003). Astrocytes form and maintain the blood-brain barrier, control levels of the neurotransmitters glutamate and dopamine in the synapses, as well as mediate neuronal differentiation, neurite outgrowth, synapse formation, and synaptic transmission (Benarroch, 2005; He and Sun, 2007). The majority of these functions are mediated through the secretion of various proteins, such as growth factors, extracellular matrix (ECM) proteins, and members of the protease system (Bachoo et al., 2004; Moore et al., 2009).

Considering the important role of glial cells in these interactions, more recent studies aim to investigate the ability for environmental contaminants to adversely affect glial cells. Certain OPs, for example, affect glial cell proliferation of primary rat astrocytes and a human astrocytoma cell line (Guizzetti et al., 2005). The metal manganese (Giordano et al., 2009b) and methyl mercury (Yin et al., 2007) have also been shown to increase oxidative stress production in primary rat astrocytes.

Due to the fact that glial cells are targeted by various neurotoxicants, it is easy to understand that damage to these cells may disrupt astrocyte-neuronal interactions and indirectly affect neuronal development. As one example, increased oxidative stress in astrocytes exposed to manganese resulted in decreased neurite outgrowth in primary hippocampal neurons cultured together with these previously-treated astrocytes (Giordano et al., 2009b). Ethanol also appears to exert its neurotoxicity in this way, primarily through the astrocyte-specific muscarinic M3 receptor. In this case, astrocytes' ability to foster neurite outgrowth was impaired by ethanol exposure, resulting in an inhibition of neurite outgrowth of hippocampal neurons (Guizzetti et al., 2010).

1.3 Inside the Matrix: The Importance of Extracellular Matrix Proteins

1.3.1 The Extracellular Matrix and its Role in Neurite Outgrowth

The extracellular matrix (ECM) is best known for its role in providing structural support for organs and tissues by forming the basement membranes of cell layers and substrates for individual cells. In recent years, the ECM is recognized as having a more active role in cell adhesion and cell signaling, ultimately affecting a variety of processes such as cell migration, differentiation, proliferation, and survival (Hynes, 2009). In the case of astrocyte-neuronal interactions, the ECM affects cell behavior by providing a variety of signals, largely in the form of proteins, growth factors, and cytokines (Kiryushko et al., 2004; Zagris, 2001).

Neurite outgrowth is a particularly fundamental process that occurs during brain development and is primarily accomplished through signals from the extracellular space, including ECM proteins, cell adhesion molecules, and other soluble factors that can positively or adversely affect neuritogenesis. Astrocytes can promote neurite outgrowth through the expression and release of "permissive" factors such as adhesive glycoproteins (fibronectin, laminin, and tenascin), glycosaminoglycans (i.e. heparin sulfates), and neurotrophic growth factors (such as nerve growth factor (NGF) and brain-derived neurotrophic growth factor (BDNF)) (Kiryushko et al., 2004). Similarly, they can inhibit neuritogenesis through the release of other factors, including the chondroitin sulfate proteoglycans, neurocan or brevican (Friedlander et al., 1994; Yamada et al., 1997). Work done by Moore and colleagues (2009) adds to this with a proteomic approach that identified various astrocyte-secreted mediators of neuronal

differentiation, including various ECM proteins, growth factors, proteases, and protease modulators. Fibronectin, thrombospondin-1, and plasminogen activator inhibitor-1 (PAI-1) are examples of such factors identified as being involved in neuronal differentiation and were modulated in response to the cholinergic agonist carbachol.

1.3.2 Fibronectin

Fibronectins are a family of glycoproteins that are found throughout the body in basement membranes, extracellular matrices, and in various bodily fluids, including plasma and cerebrospinal fluid. These proteins are not detectable in many parts of the CNS, but have been shown to be synthesized and secreted by astrocytes in the brain. Fibronectin is often found on the surface of or released from astrocytes (Price and Hynes, 1985). Astrocyte-derived fibronectin is a 230 kDa protein containing an extracellular domain (EDA sequence) that is absent in plasma fibronectin (Mahler et al., 1997; Yoshida and Takeuchi, 1991). Fibronectin is encoded by a single gene but is alternatively spliced at three regions to generate 20 different proteins in humans and 12 in rodents (Hynes, 2009). The gene for fibronectin has binding sites for several other ECM proteins, including collagen, heparin, fibrin, and fibrillin.

Fibronectin generally forms a matrix by dimerizing and associating these dimers into fibrils. The fibrils assemble into a cell-associated network that extends between neighboring cells, providing a dynamic structural support for cell-cell interactions and growth. In addition to the binding sites for other ECM proteins, integrin-binding sites are also present and are responsible for most cell-ECM interactions concerning fibronectin. This occurs via integrin receptors, which are heterodimer cell surface receptors that bind specific ECM proteins (Singh et al., 2010). In particular, fibronectin predominantly interacts with the α5β1 integrin receptor, which is involved in synapse formation in neurons (Webb et al., 2007), as well as mediating neurite outgrowth after injury (Gardiner et al., 2007). Both fibronectin and the α5β1 integrin receptor are highly expressed during development, and especially in the developing nervous system and neuronal cells (Lefcort et al., 1992; Liesi et al., 1986).

At the cellular level, fibronectin is indicated in playing a role in a variety of integral processes, including cell adhesion, cell migration, and neurite outgrowth (Kiryushko et al., 2004; Matthiessen et al., 1989; Tom et al., 2004). The spatial and temporal localization of fibronectin during neuronal differentiation suggests that fibronectin is involved in forming the migratory

pathway for the growth cones of axons (Stewart and Pearlman, 1987). Highlighting its role in neuronal development, it has been shown that inhibition of fibronectin by function-blocking antibodies strongly inhibits neurite outgrowth, as shown in primary neurons (Guizzetti et al., 2008) as well as in hippocampal slices (Giordano et al., 2011).

Fibronectin appears to be modulated by a variety of factors, both endogenous and exogenous. Ethanol in rat C6 glioma cells (Ren et al., 2000), kainic acid in hippocampal astrocytes (Mahler et al., 1997), interferon-gamma in primary cortical astrocytes (DiProspero et al., 1997), as well as ascorbate (vitamin C) in human skin fibroblasts (Peterszegi et al., 2002) were found to decrease levels of fibronectin. In astrocytes specifically, various growth factors and cytokines have been shown to up-regulate fibronectin: epidermal growth factor, transforming growth factor-β (TGF-β) 1 and 2, platelet derived growth factor, basic fibroblast growth factor, and interleukin-6 (Gris et al., 2007; Mahler et al., 1997; Martinez and Gomes, 2002; Pasinetti et al., 1993).

1.4 Organophosphorus Insecticides: Diazinon and Diazoxon

1.4.1 Organophosphorus Insecticides (OPs)

Organophosphorus insecticides (OPs) are among the most widely used class of insecticides in the world. While usage has decreased over the last ten years, about 35% of insecticides used in the U.S. are OPs (EPA, 2011). OPs are deliberately released into the environment with the purpose of controlling pest species. Humans are consequently exposed to these compounds in a variety of settings, potentially adversely affecting their health. The primary mechanism of OP toxicity is acetylcholinesterase (AChE) inhibition, which results in the accumulation of the neurotransmitter acetylcholine at muscarinic and nicotinic receptors in the central and peripheral nervous system (Costa, 2006). However, many other potential mechanisms of toxicity have been identified, including alterations to signal transduction, DNA synthesis, glucose homeostasis, and increases in oxidative stress (Adigun et al., 2010; Guizzetti et al., 2005; Lukaszewicz-Hussain, 2010; Mostafalou et al., 2012; Slotkin et al., 2006).

The widely-used OP diazinon (DZ) and its oxygen analog, diazoxon (DZO), are considered neurotoxic, but the mechanism of toxicity is not well understood. While diazinon was banned for residential use in the U.S. in 2004, it is still commonly used in agriculture in the U.S.

and abroad (EPA, 2011). According to the Agency for Toxic Substances & Disease Registry (ATSDR), the general public is primarily exposed to DZ through ingestion of food from crops sprayed with DZ, although these exposures are likely small. Those living in close proximity to crops sprayed with such pesticides, however, are at risk for increased exposure from spray drift and subsequent adverse health effects (ATSDR, 2008). Occupational exposure is a major concern since pesticide applicators and manufacturers are exposed to significant amounts of DZ and other OP compounds. DZ also has a history of use to control pests on livestock and is a large source of occupational exposure for those who conduct the "sheep-dipping" (Virtue and Clayton, 1997).

1.4.2 Developmental Neurotoxicity of OPs

More specifically, many OPs have been identified as developmental neurotoxicants. A plethora of *in vitro* and *in vivo* studies exist to support this claim. For instance, the developing brain is thought to be differentially affected by certain OPs, as young rats are more sensitive to the acute effects of AChE inhibition caused by OP exposure than adults (Pope and Liu, 1997; Won et al., 2001). Long-lasting effects of late gestational and neonatal exposures to OPs have also been shown: early exposure to chlorpyrifos and diazinon affected learning and memory (Icenogle et al., 2004; Levin et al., 2008; Roegge et al., 2006), as well as neural cell development and synaptic function (Slotkin et al., 2008) in adolescent rodents.

Additionally, epidemiological evidence supports correlations between OP exposures and various neurobehavioral deficits. Rohlman and colleagues (2011) reviewed a collection of studies evaluating the link between chronic OP exposure and neurobehavioral changes. In 19 of the 24 studies evaluated, occupational OP exposure consistently correlated with increased neurobehavioral deficits in eight main function areas, including motor speed/coordination, information processing speed, and various measures of attention and memory function (Rohlman et al., 2011). Studies attempting to correlate biomarkers of OP exposure (i.e. urinary metabolites) to neurobehavioral changes have been less successful. However, a few notable exceptions exist, including a recent study by Bouchard and colleagues (2010). This study examined the association between urinary concentrations of dialkyl phosphate metabolites of OPs and attention deficit/hyperactivity disorder (ADHD) in children 8 to 15 years of age. The authors found that children with higher dialkyl phosphate concentrations, dimethyl alkylphosphate (DMAP) in

particular, were more likely to be diagnosed with ADHD. A 10-fold increase in DMAP concentration, the metabolite that results from exposure to dimethyl-containing OPs, correlates to 55% to 72% increased odds of being diagnosed with ADHD (Bouchard et al., 2010).

As with most OP compounds, acetylcholinesterase (AChE) inhibition is the primary mechanism of DZ-induced neurotoxicity. However, more recent evidence indicates that DZ and DZO are developmental neurotoxicants, causing effects in the absence of AChE inhibition. For instance, both the parent compound (DZ) and metabolite (DZO) have been shown to inhibit neurite outgrowth of axonal processes in the N2a mouse neuroblastoma cell line (Axelrad et al., 2003; Flaskos et al., 2007; Sidiropoulou et al., 2009a). DZ and DZO have also been shown to interfere with glial cell differentiation in human and rat cell lines, as well as in primary rat cortical astrocytes (Guizzetti et al., 2005; Sidiropoulou et al., 2009b).

Various aspects of cell signaling dysregulation have also been studied. Investigations in N2a neuroblastoma cells found that sub-lethal concentrations of DZ and DZO (1-10 μM) that inhibit neurite outgrowth also altered the levels of various proteins involved in axonal growth, stress responses, and microtubule structures (Harris et al., 2009b; Sidiropoulou et al., 2009a). In neuronotypic PC12 cells, DZ disrupted DNA synthesis (Slotkin et al., 2007) and adenylyl cyclase cascade signaling (Adigun et al., 2010), often demonstrating more severe effects when exposure coincided with cell differentiation.

1.4.3 OPs and Oxidative Stress

For the purposes of this project, oxidative stress is best described as an imbalance between the production of oxidants and the antioxidant defense system in the body. Reactive oxygen species (ROS) are produced by a variety of mechanisms in the body, some of which are part of normal physiologic functioning, metabolism, and general signal transduction. These agents include hydroxyl radicals (·OH), superoxide anion (O_2·-), and hydrogen peroxide (H_2O_2). Under normal conditions, the body is adept at employing and regulating the ROS produced in utilizing molecular oxygen. In response to various external insults, ROS overproduction and/or mismanagement of the regulatory systems result in a state of oxidative stress. The ROS produced are often reactive, which contributes to their ability to cause lipid peroxidation, enzyme oxidation, and damage to proteins and nucleic acids (Dickinson and Chang, 2011; Matés, 2000).

As reviewed by Lukaszewicz-Hussain (2010), myriad studies exist supporting the ability of various OPs to induce oxidative stress in humans, as well as in animal studies and *in vitro* models. These effects manifest themselves in the form of altered levels and activity of various antioxidant factors, as well as increases in various markers of oxidative stress (i.e. malonyldialdehyde (MDA) production) and ROS. With respect to OPs being neurotoxicants, it is important to note that the brain is particularly susceptible to oxidative damage. This is true due to its high oxygen consumption, high lipid content, and a relatively low amount of endogenous antioxidants (Lukaszewicz-Hussain, 2010; Matés, 2000).

Chlorpyrifos (CPF) is a ubiquitous, neurotoxic OP that has been shown to increase oxidative stress in the brain. Interestingly, its effects appear to be dependent upon duration and time of exposure. For instance, CPF increased ROS production in PC12 cells under conditions of acute toxicity, but not when chronically administered (Crumpton et al., 2000). CPF increased lipid peroxidation in neonatal rats exposed postnatally but not when exposed in the late gestational phase (Slotkin et al., 2005). The authors of the latter study attribute this difference to the treatment period (PD11-14) being the peak time for neuronal cell differentiation and synaptogenesis, providing a critical window for damage. Other studies have also found that acute exposures to CPF in rats increase levels of thiobarbituric acid-reactive substances (TBARS) in the liver (Bagchi et al., 1995; Tuzmen et al., 2008), kidney (Oncu et al., 2002), and brain (Verma and Srivastava, 2001).

Repeated administration of malathion, endosulfan, or CPF to rats also resulted in increased lipid peroxidation and altered antioxidant enzymes in the blood, liver, and lung (Akhgari et al., 2003; Bebe and Panemangalore, 2003). Various antioxidants, including melatonin, vitamin C and E, were shown in several cases to alleviate oxidative stress caused by OPs (Akturk et al., 2006; Giordano et al., 2007; Gultekin et al., 2001). *In vitro* models manipulating the powerful endogenous antioxidant, glutathione (GSH), have also been used to demonstrate the role of oxidative stress in OP-induced toxicity. Cerebellar granule neurons isolated from mice lacking the modifier subunit of glutamate cysteine ligase (GCL), the first and limiting enzyme in the synthesis of GSH, were used to systematically investigate the ability for various OPs (CPF, DZ) and their oxygenated metabolites, chlorpyrifos-oxon (CPFO) and diazoxon (DZO), to induce oxidative stress (Giordano et al., 2007). All of the OPs in this study increased ROS production and lipid peroxidation in cerebellar granule neurons from *Gclm* (+/+)

and *Gclm* (-/-) mice; the entirety of these effects was significantly greater in neurons taken from *Gclm* (-/-) mice.

Oxidative stress due to OP exposure has also been reported in humans. Increased lipid peroxidation and decreased amounts of antioxidant capacity was observed in individuals acutely poisoned with malathion (Ranjbar et al., 2005). In other patients acutely exposed to a variety of OPs, increased levels of malondialdehyde (MDA) and superoxide dismutase (SOD) were observed. Levels of these oxidative markers were not attenuated by traditional OP-poisoning therapy, indicating that the initial increases in lipid peroxidation significantly disturbed cell integrity and contributed to eventual cell death (Vidyasagar et al., 2004). In considering these compounds as threats to neurodevelopment, it is notable that increased lipid peroxidation and DNA fragmentation were observed in fetal cord blood samples obtained from pregnant women exposed to OPs during crop-spraying season. These increases correlated with significantly inhibited fetal butyrylcholinesterase (BChE) activity (Samarawickrema et al., 2008).

1.5 Hypothesis

The overarching hypothesis states that the organophosphorus insecticide (OP) diazinon (DZ) and its oxygen analog, diazoxon (DZO), will modulate glial-neuronal interactions, impair the ability of astrocytes to foster neuronal differentiation, and inhibit neurite outgrowth in primary hippocampal neurons. By increasing oxidative stress in astrocytes, DZ and DZO will adversely affect normal functioning of astrocytes. This includes their ability to support neuronal growth through the production and secretion of specific neuritogenic proteins and other factors. Disruption of such functions by DZ/DZO-induced oxidative stress will ultimately impair neurite outgrowth and differentiation.

1.6 Specific Aims

Specific Aim 1: Determine whether DZ and DZO impair astrocytes' ability to foster neuronal development, as measured by neurite outgrowth of primary hippocampal neurons.

Sub-Aim 1.1: Determine if DZ/DZO-exposed astrocytes cause an inhibition of neurite outgrowth in primary hippocampal neurons.

Sub-Aim 1.2: Determine if DZ and DZO increase oxidative stress in primary cortical astrocytes, as measured by reactive oxidative species (ROS) production as a result of exposure to these compounds.

Sub-Aim 1.3: Evaluate the effects of antioxidant pre-treatment on astrocytes' ability to foster neurite outgrowth in primary hippocampal neurons.

Specific Aim 2: Determine if the neuritogenic protein fibronectin is involved in the effects of DZ and DZO on astrocytes' ability to foster neuronal growth of hippocampal neurons.
Sub-Aim 2.1: Determine if DZ and DZO decrease protein levels of fibronectin produced in and secreted by astrocytes.

Sub-Aim 2.2: Determine if antioxidants prevent decreases in fibronectin levels induced by DZ or DZO in astrocytes.

Specific Aim 3: Evaluate changes in neurite outgrowth of primary hippocampal neurons directly exposed to DZ and DZO.
Sub-Aim 3.1: Determine if DZ and DZO inhibit neurite outgrowth in hippocampal neurons directly exposed to these compounds.

Sub-Aim 3.2: Evaluate the effect of antioxidant pre-treatment on the effects of DZ and DZO on neurite outgrowth of hippocampal neurons.

Sub-Aim 3.3: Evaluate the ability for astrocytes to protect against DZ- and DZO-induced inhibition of neurite outgrowth of hippocampal neurons. Specifically, observe the role of astrocyte-produced glutathione in this protective effect.

Chapter 2

DIAZINON AND DIAZOXON IMPAIR ASTROCYTES' ABILITY TO FOSTER NEURITE OUTGROWTH IN PRIMARY HIPPOCAMPAL NEURONS

2.1 Introduction

There is increasing concern in the public and regulatory spheres over exposure to organophosphorus pesticides (OPs) and their ability to adversely affect neurodevelopment in children (Costa, 2006; Eaton et al., 2008; Eskenazi et al., 2008). The cause for concern resides in the fact that there is widespread exposure of children to OPs, both in rural and urban environments (Barr et al., 2004; Beamer et al., 2008; Eskenazi et al., 2007; Fenske et al., 2002; Lu et al., 2000; Valcke et al., 2006). Additionally, evidence abounds from animal studies indicating that the developing nervous system is more susceptible to the neurotoxicity of OPs than the mature nervous system (Moser et al., 1998; Moser and Padilla, 1998; Pope and Liu, 1997; Won et al., 2001). This is compounded by recent epidemiological studies that link early exposure to OPs and various neurobehavioral deficits in children, such as increased incidence of ADHD and lowered I.Q. (Bouchard et al., 2010; Eskenazi et al., 2007; Rauh et al., 2011; Rohlman et al., 2011).

While acute toxicity to OPs primarily occurs as a result of acetylcholinesterase (AChE) inhibition, the mechanisms of lower-level, chronic exposures on neurodevelopment remain unclear. As reviewed by Lukaszewicz-Hussain (2010), several other studies support the ability of various OPs to induce oxidative stress in humans (Ranjbar et al., 2005; Vidyasagar et al., 2004), in animal models (Jafari et al., 2012; Slotkin et al., 2005; Yilmaz et al., 2012), and in various *in vitro* models (Crumpton et al., 2000; Giordano et al., 2007; Lee et al., 2012; Slotkin and Seidler, 2009). These effects manifest in the form of altered levels and activity of different antioxidant factors, as well as increases in various markers of oxidative stress, including increased lipid peroxidation and levels of reactive oxygen species (ROS).

ROS, including O_2^-, $\cdot OH$, and hydrogen peroxide (H_2O_2), are produced by a variety of enzymatic and chemical processes, many of which are an integral part of normal physiological

functioning and cell signaling (Dickinson and Chang, 2011). In contrast, the overabundance and mismanagement of ROS leads to oxidative stress, which is more recently implicated in the progression of various neurodegenerative diseases, including Alzheimer's (AD) and Parkinson's Disease (PD), as well as Friedriech's ataxia and Amyotrophic lateral sclerosis (Barnham et al., 2004; Potashkin and Meredith, 2006). Additionally, oxidative damage and related mechanisms have been more recently implicated in other instances of neurodevelopmental dysfunction, such as autism spectrum disorders and schizophrenia (Do et al., 2009; Frustaci et al., 2012; Tang et al., 2013). The considerable body of literature presents a range of oxidative stress indicators: oxidative DNA damage in brains of PD and AD patients, for example, as well as increased lipid peroxidation; alterations to various antioxidant enzymes; mitochondrial dysfunction; and metal-ion dysregulation, to list a few.

Underscoring the link between OP-induced oxidative stress and the susceptibility of the developing brain to these exposures, Samarawickrema and colleagues (2008) provide evidence of increased lipid peroxidation in fetal cord blood samples obtained from pregnant women living in a rural farming community that were exposed to OPs during crop-spraying season. These increases correlated with significantly inhibited fetal butyrylcholinesterase (BChE) activity (Samarawickrema et al., 2008). The brain, and specifically the developing brain, is particularly susceptible to oxidative damage. This is true due to its high oxygen consumption, high lipid content, and a relatively low amount of endogenous antioxidants (Lukaszewicz-Hussain, 2010; Matés, 2000). While these facts support the idea that oxidative stress plays a major role in the developmental neurotoxic mechanisms of OPs, the ways in which this happens is largely unexplored and is the focus of this study.

Diazinon is a particular OP that is still widely used agriculturally in the U.S. and abroad, even after its ban for residential use in 2004 (EPA, 2011). Those living in close proximity to crops sprayed with such pesticides are at risk for increased exposure and subsequent health effects (ATSDR, 2008). The literature indicates that diazinon (DZ) and its oxygen-metabolite, diazoxon (DZO), are developmental neurotoxicants, but the mechanisms by which they exert these effects are unclear. Developmental effects are evident in studies of long-term effects of late gestational and neonatal exposures to DZ: early exposure to diazinon affected learning and memory (Levin et al., 2008; Roegge et al., 2006; Timofeeva et al., 2008), as well as neural cell development and synaptic function (Slotkin et al., 2008) in adolescent rodents. Most studies

attempting to explain mechanisms of DZ and DZO neurotoxicity have been completed in cell lines (Axelrad et al., 2003; Flaskos et al., 2007; Sidiropoulou et al., 2009a), primarily focused on direct damage to neurons. The present study highlights the effects of DZ and DZO on astrocyte function and their ability to foster neuronal development, using primary cell cultures of cortical astrocytes and hippocampal neurons to explore a mechanism of DZ/DZO neurotoxicity.

Previous work in our lab demonstrated that other environmental contaminants (i.e. manganese) shown to induce oxidative stress, as well as known oxidants (hydrogen peroxide (H_2O_2) and 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ)) exert their neurotoxic effects by impairing astrocyte-neuronal interactions (Giordano et al., 2009b). Astrocytes, a particularly abundant type of glial cell in the brain, are increasingly recognized as having essential roles in the function and development of the brain (Benarroch, 2005; Guizzetti et al., 2008; He and Sun, 2007). Additionally, astrocytes tend to be more resistant to oxidative stress than other neural cell types, likely due to the fact that they contain higher levels of endogenous antioxidants, such as glutathione, than neuronal cells (Giordano et al., 2006; Thorburne and Juurlink, 1996). In many instances, astrocytes are protective of neuronal damage due to exogenous agents (Calkins et al., 2010; Giordano et al., 2009a).

For these reasons, damage to astrocytes and impairment of their normal functioning is considered detrimental to neuronal cell functioning and neurodevelopment. Here we investigate the potential for DZ and DZO to impair the ability of cortical astrocytes to foster neurite outgrowth in primary hippocampal neurons. Specifically, we the examined the ability for these compounds to elicit oxidative stress in astrocytes, and subsequently, how such processes impair neurite outgrowth, as well as modulate levels of the pro-neuritogenic extracellular matrix protein, fibronectin.

2.2 Materials and Methods

Materials. Neurobasal-A medium, DMEM medium, fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), GlutaMAX, anti-mouse Alexa fluor-488 secondary antibody, Hoechst 33342, 2,7′-dichlorofluorescin diacetate (H_2DCF-DA), SuperSignal West Pico Chemiluminescent Substrate (Pierce), papain, and gentamicin were from Invitrogen (Carlsbad, CA). Diazinon (DZ; 99.4%) and diazinon-O-analog (diazoxon; DZO; 98%) were from Chem-Service (West Chester, PA). Poly-D-lysine, antibodies: peroxidase-conjugated anti-mouse IgG, mouse anti-beta-actin, horseradish peroxidase-conjugated anti-rabbit IgG, rabbit anti-fibronectin, rabbit anti-map-2, mouse anti-tau, goat serum, dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), and N-t-butyl-alpha-phenylnitrone (PBN) were from Sigma-Aldrich (St. Louis, MO). Protease inhibitors were from Roche Diagnostics (Indianapolis, IN). Mouse β-III-tubulin antibody was from Millipore (Billerica, MA). Melatonin was from EMD Chemicals (Rockland, MA). Purified human fibronectin (FN) was from BD Biosciences (Bedford, MA).

Preparation of fetal rat cortical astrocytes. Primary cultures of cortical astrocytes were prepared as previously described (Guizzetti and Costa, 1996). Cultures were randomly checked for their purity (>95%) by immunofluorescence using an antibody against Glial Fibrillar Acidic Protein (GFAP). After at least 12 days in culture, astrocytes were plated in 24-well plates for astrocyte–neuron co-culture experiments ($2x10^6$ cells/well), on glass coverslips (2.5×10^5 cells/coverslip) for immunocytochemistry, or in 100 mm dishes for Western blot analysis (2×10^6 cells/dish).

Astrocyte cell treatments. Four days in culture after plating, astrocytes were rinsed twice with PBS and placed in serum-free media (DMEM-BSA (0.1%)) for an additional 24 h. After 24 h, astrocytes were treated with the appropriate treatments: DZ, DZO, H_2O_2, or vehicle control (DMSO). DMSO concentrations in the treatment solutions never exceed 0.1%.

Antioxidant treatment. Where appropriate, after 24 h of serum deprivation, cells were treated with antioxidants melatonin (200 μM) or N-t-butyl-alpha-phenylnitrone (PBN; 100 μM) for 3 h.

After this time, cells were washed twice with PBS and treated with the respective chemicals in DMEM-BSA (0.1%) for 24 h unless otherwise stated.

Measurement of cell viability. Astrocyte viability was measured by the MTT assay, where 50 µL of MTT reagent (5 mg/mL) was added to each well after 24 h treatment with DZ or DZO. After 15 min. at 37 ºC, the media was removed and the formazan reaction product was dissolved in 250 µL DMSO. Absorbance was read at 562 nm and results were expressed as mean percentage of viable cells relative to untreated controls.

Preparation of fetal rat hippocampal neurons. Hippocampal neurons from embryonic day 21 (post-natal day 0; E21/PND0) rat fetuses were prepared as described (Brewer et al., 1993; Guizzetti et al., 2008; VanDeMark et al., 2008). For quantitative analysis of neurite outgrowth, neurons were plated on glass coverslips (2×10^4 cells/coverslip), previously coated overnight with 100 µg/mL poly-D-lysine at 37 °C.

Astrocyte-neuronal co-cultures. This model provides a way to understand astrocyte-neuronal interactions in an *in vitro* system that more accurately reflects *in vivo* processes (Giordano et al., 2009a). Hippocampal neurons were prepared as described and plated on glass coverslips to which 4 paraffin beads were previously affixed. After 1 h incubation in Neurobasal A/FBS (10%) medium to allow neurons to attach, the glass coverslips were inverted onto 24-well plates containing astrocytes, as described by Viviani et al. (1998).

Rat cortical astrocytes were previously treated with vehicle control, DZ, or DZO (0-10 µM) for 24 hr. After 24 h, the treatment is washed out and replaced with fresh "serum-free" media (DMEM-BSA (0.1%)). Freshly isolated hippocampal neurons are then incubated with these astrocytes for 48 h. The neurons are plated on coverslips containing paraffin wax beads that prevent their touching the astrocyte monolayer while allowing them to share the same media that includes subsequent growth factors and secreted proteins. After 48 h, the neurons are removed from the plates containing the astrocytes, flipped over, and washed twice with warmed HBSS. The neurons are then fixed or 15 min. at 37 °C with 4% paraformaldehyde (PFA).

For experiments evaluating the effect of exogenous FN on DZ/DZO-induced inhibition of neurite outgrowth, 10 µg/mL FN was added to the co-culture system (where appropriate) when

the neurons were placed with the astrocytes for the 48 h incubation. Stock solutions of FN were prepared as per manufacturer's recommendations and stored in 1 mg/mL aliquots at -20 °C.

Quantitative Morphological Analysis of Neurite Outgrowth. For co-culture experiments, neurons were fixed in 4% PFA after 48 h in culture with the astrocytes. Neurons were labeled with an anti-β-III-tubulin isoform antibody followed by a fluorescein-conjugated secondary antibody (Alexa-488); the nuclei were stained with Hoechst 33342. Only pyramidal neurons with three or more neurites, not touching any other cells/neurites were included for quantitative analysis. At least 30 cells per treatment were analyzed for each experiment.

Measurement of Reactive Oxygen Species. Reactive oxygen species (ROS) formation was determined by fluorescence using 2,7′-dichlorofluorescin diacetate ($DCFH_2$-DA). Upon entering cells $DCFH_2$-DA is de-esterified to $DCFH_2$, which is then oxidized by ROS to form the fluorescent 2,7′-dichlorofluorescein (DCF). For astrocytes, cells were incubated for 30 min. with 10 μM $DCFH_2$-DA in Locke's solution. Afterwards, the probe was washed out and the astrocytes are treated with the appropriate chemical dilutions for the desired time point(s). After treatments (at 37 °C), the test solution was removed, and 0.1 M KH_2PO_4 0.5% Triton X-100 (pH 7.2) was added. Cells were then scraped from the dish and the extract centrifuged (10 min at 12,000 rpm). The supernatant was collected and the fluorescence was immediately read using a Perkin-Elmer spectrofluorimeter (excitation 488 nm, emission 525 nm).

Western blot analysis. Measurements of fibronectin protein levels in astrocyte lysate and in medium by Western blot were carried out as previously described (Guizzetti et al., 2008). Cells were scraped in 200 μL of lysis buffer (0.2% SDS, 1mM PMSF, 1 mL 10x Cell Signaling lysis buffer (Cell Signaling Technology, Inc., Beverly, MA), and a protease inhibitor EDTA-free mixture), sonicated, and stored at -20 ºC. Proteins were quantified by the BCA method, separated by electrophoresis, and transferred to PVDF membranes.

Membranes were blocked for 2-3 hours at room temperature in 5% milk/Tris-buffered saline plus 0.1% Tween-20 (TBST) buffer. Membranes were then incubated with primary antibodies as follows: rabbit anti-fibronectin (1:1000) in 3% BSA/TBST for 1 h at room temperature. Membranes are then washed three times in TBST and incubated for 1 h at room

temperature with anti-rabbit horse radish peroxidase (HRP)-labeled antibody (1:1000) in 3% BSA/TBST. Membranes from cell lysate proteins were re-probed for beta-actin as a loading control: mouse anti-β-actin (1:10,000) in 3% BSA/TBST for 30 min., followed by anti-mouse HRP-linked antiobody (1:1000) in 3% BSA/TBST for 30 min., all at room temperature. After being washed with TBST, membranes are visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Optical density of each band was quantified using ImageJ software (National Institutes of Health).

Confocal microscopy analysis of fibronectin levels. Astrocytes were plated on glass coverslips previously coated with 100 µg/mL poly-D-Lysine. Astrocytes were cultured and treated as in all other experiments. After 24 h treatment with 10 µM DZ or DZO, astrocytes were washed and fixed in 4% paraformaldehyde for 5 min. at room temperature. The cells were then blocked in 10% goat serum in PBS for 1 h at room temperature. Astrocytes were labeled with an anti-fibronectin antibody (1:50) in 3% BSA/PBS, followed by fluorescein-conjugated secondary antibody (Alexa-594; 1:75 in 3% BSA/PBS). Nuclei were stained with Hoechst 33342.

Extracellular fibronectin levels on astrocytes were assessed by taking images of 10 random fields per treatment per experiment with an Olympus Fluoview-1000 confocal microscope. Each image consists of 21 z-directional slices with a 0.5 µm step-size. Integrated fluorescence intensity was measured using MetaMorph software integrated morphometry analysis tools. The sum of integrated optical density over all planes for each image was taken; integrated intensity was normalized by cell number. A total of 10 random images per treatment were taken and analyzed for each experiment where treatments were completed in triplicate wells. Extracellular fibronectin levels are expressed as mean integrated intensity relative to untreated control.

Acetylcholinesterase (AChE) Measurements. AChE levels in astrocytes were measured as previously described (Li et al., 2000) in a microtiter plate assay based on the method of Ellman *et al.* (Ellman et al., 1961). Cell lysates were collected in a 0.1 M sodium phosphate buffer (pH 8.0). For duplicate assays, 50 µL of cell lysates was combined with 150 µL of the assay buffer containing 0.1 M sodium phosphate and 0.1 mM of DTNB (5,5'-dithiobis-2-nitrobenzoic acid). The kinetic assay was initiated by addition of acetylthiocholine (final concentration: 1 mM) and

the reaction was continuously monitored for 10 min. at room temperature. Absorbance was read at 412 nm in a Beckman DU-70 spectrophotometer. The amount of 5-thio-2-nitrobenzoate formed was calculated using an extinction coefficient of 13 600 M^{-1}/cm. AChE activity was expressed as nmol/min/mg protein.

Statistical Analysis. For neurite outgrowth measurements, the following parameters were measured for each cell using Metamorph software: longest neurite length (including the length of any branches originating from the neurite); length of minor neurites; and total number of neurites/cell. About 30 cells per treatment were analyzed in each experiment. Final data summaries are displayed as the mean ± SEM for 90-120 cells per treatment derived from at least 3 independent experiments, unless otherwise indicated.

For Western blot analysis, ROS measurements, and extracellular fibronectin levels, at least 3 separate experiments were performed where all samples were completed in duplicate or triplicate. Results are reported as the mean percent of the control ± SEM of results from at least 3 experiments. Statistical significance for all analyses (unless otherwise indicated) was evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison post-hoc test. Western blot data were analyzed using the non-parametric analysis of variance Kruskal-Wallis test followed Dunn's post-test. $P < 0.05$ is considered significant. All data were analyzed using GraphPad Prism software.

2.3 Results

Acetylcholinesterase (AChE; EC 3.1.1.7) activity in astrocytes was measured after 24 h treatment with 1 or 10 µM DZ or DZO. DZO caused higher inhibition of AChE activity than DZ, as is expected for the oxon form of the parent compound. Astrocytes treated with 1 µM DZ exhibited AChE activity levels similar to untreated controls (less than 4% inhibition), whereas 10 µM DZ decreased AChE activity by 18%, compared to untreated astrocytes. AChE activity in astrocytes was decreased about 25% relative to control levels after 24 h exposure to either 1 or 10 µM DZO (Fig. 2.1). Mean AChE activity (± SEM) in untreated control astrocytes was 3.09 ± 0.33 nmol/min/mg protein.

Reactive oxygen species (ROS) formation was measured in astrocytes treated with various concentrations of DZ or DZO (0.1, 1, and 10 µM). Astrocytes treated with 10 µM DZ or DZO for 1 h resulted in a statistically significant 2-fold increase in ROS, comparable to the effect of treatment with 20 µM H_2O_2 (Fig. 2.2a). Astrocytes treated with 10 µM DZ or DZO for various time points (0, 15, 30, 60, 360 min.) showed peak ROS formation at 1 h (Fig. 2.2b). No cytotoxicity to the astrocytes was observed at any concentration tested (Table 2.1).

Knowing that these compounds elicit a significant increase in ROS production in astrocytes, and that neurons depend on astrocytes' proper functioning for their growth and development, it was thought that these alterations in astrocyte function may subsequently affect neuronal development. The effect of DZ- and DZO-treated astrocytes on neurite outgrowth of primary hippocampal neurons was measured to assess this relationship. Astrocytes previously treated with 10 µM DZ or DZO for 24 h were cultured together with newly prepared primary hippocampal neurons for 48 h. After 48 h, the neurons were fixed and several parameters of neurite outgrowth were measured for each treatment: longest neurite length, minor neurite length, and number of neurites/cell. There was a significant 50% decrease in longest neurite length in hippocampal neurons when cultured with astrocytes previously treated with 10 µM DZ (Fig. 2.3a). The data also suggest a decrease in minor neurite length in neurons incubated with astrocytes previously treated with concentrations as low as 0.1 µM DZ (Fig. 2.3b). No differences in the number of neurites per cell were observed for any DZ treatment concentration (Fig. 2.3c).

Neurons cultured with astrocytes that were previously treated with either 1 or 10 µM DZO demonstrate a 35% and 60% decrease in longest neurite length, respectively (Fig. 2.4a). Similar to the results with DZ, astrocytes treated with 0.1-10 µM DZO also resulted in a decrease in minor neurite length in neurons cultured with these cells (Fig. 2.4b). For both DZ and DZO, the changes in minor neurite length are much smaller in magnitude than those observed for the longest neurite length. No differences in number of neurites per cell were seen as a result of astrocytes treated with DZO (Fig. 2.4c).

Figure 2.5 further illustrates the effect of DZ and DZO on neurite outgrowth: neurons cultured with astrocytes previously treated with either 10 µM DZ or DZO display starkly stunted neurite lengths compared to the untreated control (Fig. 2.5c and d). This effect is comparable to that of neurons cultured with astrocytes that were previously treated with a known oxidant, H_2O_2 (Fig. 2.5b). In a separate experiment, neurons incubated with astrocytes previously exposed to DZ or DZO were fixed and labeled with antibodies to the axon-specific marker, Tau, and dendrite marker, MAP-2 (microtubule-associated protein 2). These data confirmed that the longest neurite is the axon of the neurons (Fig. 2.6). The identity of axons and dendrites has been previously verified in the same co-culture system in this manner (Guizzetti et al., 2008; VanDeMark et al., 2009).

To further investigate the role of oxidative stress in the mechanism of impaired astrocyte-ability to foster neurite outgrowth by DZ and DZO, a set of experiments was carried out in which astrocytes were pre-treated with antioxidants melatonin (200 µM) or N-t-butyl-alpha-phenylnitrone (PBN; 100 µM) for 3 h. After 3 h, the antioxidants were washed out and the astrocytes were treated as previous: with either 10 µM DZ or DZO for 24 h, followed by 48 h co-culture incubation with newly prepared hippocampal neurons. Pre-treatment with either antioxidant completely prevents the decrease in longest neurite length caused by astrocyte treatment with 10 µM DZ or DZO (Fig. 2.7 a,d). Antioxidant pre-treatment does not appear to prevent the decreases observed in the minor neurite outgrowth (Fig. 2.7 b,e). Astrocytes pre-treated treated with melatonin alone do not exhibit effects on any parameter of neurite outgrowth, as compared to the untreated control (Fig. 2.7 a-c). Astrocytes pre-treated with PBN alone also do not have any effect on neurite outgrowth; the decrease in minor neurite length shown in Figure 2.7b is considered an abnormality since this effect was not observed in a separate, independent experiment where astrocytes pre-treated with PBN did not result in any

differences in neurite lengths (longest or minor) compared to the untreated control (data not shown).

In efforts to explain *how* DZ and DZO were adversely affecting astrocyte function, and subsequently neuritogenesis, alterations to the production and secretion of certain extracellular matrix (ECM) proteins in astrocytes was investigated. Western blot analysis was used to quantify relative intracellular protein levels of the pro-neuritogenic fibronectin (FN) in astrocytes treated with or without 10 µM DZ or DZO. Figure 2.8 illustrates that 10 µM DZ or 10 µM DZO cause a 26% and 33% decrease in FN levels in astrocytes, respectively. Additionally, astrocytes pre-treated with antioxidants melatonin or PBN successfully prevent these decreases in FN (Fig. 2.8).

Confocal microscopy and immunocytochemistry were used to confirm that DZ and DZO decrease FN protein levels in astrocytes. Relative integrated intensity measurements demonstrate a marked decrease in FN bound to the extracellular surface of the astrocytes as a result of 10 µM DZ or DZO exposure, as compared to an untreated control (Fig. 2.9a). DZ and DZO (10 µM) cause a 30% and 40% decrease in extracellular FN levels on astrocytes, respectively (Fig. 2.9b).

Exogenous FN was added to the co-culture system in the presence of 10 µM DZ or DZO to confirm the involvement of FN on the effect of these compounds on astrocytes' ability to foster neurite growth. Purified FN (final concentration: 10 µg/mL) was added to the astrocyte-neuron culture system at the time when the newly prepared neurons were added to the plates containing the astrocytes. The astrocytes and neurons were then cultured together in the presence of FN (where appropriate) for 48 h as in the previous experiments. Adding FN completely prevents the inhibition of longest neurite length normally observed in neurons cultured with astrocytes previously treated with 10 µM DZ or DZO (Fig. 2.10a). No protective effect of exogenous FN was seen for the decreases in minor neurite length (Fig. 2.10b). In these set of experiments (N = 3), there were significant differences in the number of neurites per cell for neurons cultured with astrocytes exposed to 10 uM DZ or DZO, as compared to untreated controls. Such results are not seen in any other experiments and should be considered with that in mind.

Taken together, these results demonstrate that DZ and DZO can impair astrocytes' ability to foster neurite outgrowth of hippocampal neurons, as evidenced by stunted neurite outgrowth in neurons cultured in the presence of previously-treated astrocytes. These compounds also affect the production and extracellular expression of the pro-neuritogenic ECM protein, fibronectin.

Both the intracellular and extracellular levels of FN in astrocytes were decreased by DZ and DZO treatment. Additionally, adding FN to the co-culture system rescues this effect, where the inhibition of neurite outgrowth caused by astrocytes previously treated with DZ or DZO was no longer seen in the presence of FN. This suggests an essential role for the ECM protein fibronectin in the impaired neuritogenesis that results from astrocytes exposed to DZ or DZO. Lastly, these results provide strong evidence demonstrating that DZ and DZO increase oxidative stress in astrocytes, which subsequently modulates neurite outgrowth in hippocampal neurons, as well as the neurite-promoting fibronectin.

2.4 Discussion

The main finding in this study demonstrates the ability of the OP DZ and its oxygen metabolite, DZO, to interfere with a particular aspect of glial-neuronal interactions: the ability for astrocytes to promote neuritogenesis in developing hippocampal neurons. Astrocytes are increasingly recognized as important contributors to central nervous system function and development. In addition to playing important roles in maintaining ion homeostasis, the blood brain barrier, and regulating glutamate recycling from the synapse (He and Sun, 2007; Nedergaard et al., 2003), astrocytes have more recently been shown to have integral roles in neuronal differentiation, neuritogenesis (Giordano et al., 2011; Guizzetti et al., 2008; Guizzetti et al., 2010), and synaptogenesis (Christopherson et al., 2005).

Our lab has previously shown that certain agents, including manganese and ethanol, affect astrocyte function, and indirectly inhibit neurite outgrowth (Giordano et al., 2009b; Guizzetti et al., 2010; VanDeMark et al., 2008). Many OPs, such as DZ and DZO, are known neurotoxicants, and may elicit their neurotoxic effects by adversely affecting astrocytes in the brain. While work has been done investigating the effects of DZ and DZO on neurons (Flaskos et al., 2007; Sidiropoulou et al., 2009a; Slotkin et al., 2008), this study demonstrates that DZ and DZO inhibit neurite outgrowth by impairing astrocyte function and their ability to foster neuronal differentiation.

Astrocytes exposed to either DZ or DZO resulted in a significant reduction in neurite lengths of the longest and minor neurites of hippocampal neurons. These effects were not governed by cytotoxicity to the astrocytes as there was none. The identity of the longest neurites and minor neurites were confirmed to be the tau-positive axons and MAP-2-positive dendrites, respectively, in a separate experiment. These results are consistent with work from Guizzetti et al. (2008) and Giordano et al. (2009): both concluded the same in an identical co-culture system.

The inhibitory effect of DZ and DZO on the longest neurite outgrowth appears to be mediated by oxidative stress, as suggested by the concentration-dependent increase in ROS production in astrocytes, as well as the ability for two different antioxidants, melatonin and PBN, to completely prevent inhibition of the longest neurite outgrowth. Additionally, the inhibitory effects of DZ and DZO are comparable to that of a known oxidant, H_2O_2, which similarly inhibited astrocytes' ability to foster neurite outgrowth in hippocampal neurons. Interestingly,

the inhibitory effects of DZ and DZO on minor neurite length were not prevented by antioxidants, potentially suggesting diverse mechanisms of inhibition between axons and dendrites. Reports of differing effects of another OP, chlorpyrifos, on axonal and dendritic growth are documented in the literature (Howard et al., 2005). It should be noted, however, that the effects on minor neurite outgrowth were less dramatic and less consistent than those on longest neurite length. Further study is required to determine if these divergent effects are truly a feature of DZ and DZO neurotoxicity.

These results in this study are supported by the fact that various OPs, including the neurotoxic chlorpyrifos, as well as diazinon specifically, have been shown to increase oxidative stress *in vitro* in PC12 neuronal cells (Crumpton et al., 2000; Lee et al., 2012; Slotkin and Seidler, 2009) and primary cerebellar granule cells (Giordano et al., 2007). Importantly, there is also evidence demonstrating that OPs cause oxidative stress in the brain *in vivo*: lactational exposure to malathion caused oxidative stress the brain, plasma, and erythrocytes of rat pups (Selmi et al., 2012); diazinon increased lipid peroxidation and other markers of oxidative stress in the brain, heart, and spleen of Wistar and Norway rats (Jafari et al., 2012; Yilmaz et al., 2012), and in the brains of a freshwater fish, *Oreochromis niloticus* (Uner et al., 2006).

Future work on the specific sources of DZ and DZO-induced ROS would complement the results of this study. Evidence exists demonstrating the ability for OPs to impair various aspects of mitochondrial function, including mitochondrial respiration and energy production (Karami-Mohajeri and Abdollahi, 2013; Massicotte et al., 2005). Mitochondria are both a producer and a target of ROS and reactive nitrogen species (RNS): damage to the respiratory chain uncouples the processes that utilize free radicals, which results in even further free radical generation and a detrimental cycle for cellular health and survival (Cardinali et al., 2013; Genova et al., 2004; Raha and Robinson, 2000). For these reasons, exploring mitochondrial integrity and function in astrocytes exposed to DZ and DZO may further explain how these compounds increase oxidative stress, which ultimately results in neurite outgrowth inhibition in hippocampal neurons.

Astrocytes primarily mediate their effects on neuritogenesis and neuronal development by secreting various factors, including extracellular matrix (ECM) proteins, growth factors, and "glio-transmitters" (e.g. $_{D}$-serine, ATP, and glutamate) (Volterra and Meldolesi, 2005). Previous work by Moore and colleagues (2009) used a proteomic approach to identify various factors

secreted by astrocytes stimulated with the pro-neuritogenic cholinergic agonist carbachol. Several extracellular matrix (ECM) proteins were identified as those involved in promoting neuronal differentiation, including FN and laminin (Moore et al., 2009). FN is indicated in playing a role in cell adhesion, cell migration, and neurite outgrowth (Kiryushko et al., 2004; Matthiessen et al., 1989; Tom et al., 2004). Highlighting its role in neuronal development, it has been shown that inhibition of FN by function-blocking antibodies strongly inhibits neurite outgrowth, as shown in primary neurons (Guizzetti et al., 2008) as well as in hippocampal slices (Giordano et al., 2011).

Here, we show that DZ and DZO decrease intracellular and extracellular-bound protein levels of FN in astrocytes. Interestingly, the same antioxidants (melatonin and PBN) that successfully prevent the inhibition of neurite outgrowth in hippocampal neurons also prevent the decreases in FN levels in astrocytes caused by DZ and DZO. The decreases in FN lysate levels found here are comparable to those found in our prior work that demonstrated the ability of Mn to decrease FN levels, as well as the success of antioxidants in preventing this decrease in cortical rat astrocytes (Giordano, et al., 2009). It is important to note that a known oxidant, H_2O_2, also caused similar decreases in intracellular protein levels of FN in astrocytes. Additionally, we found that the addition of purified FN to the media of the astrocyte-neuronal co-culture system completely attenuates the effects of DZ and DZO on astrocytes' ability to foster neurite outgrowth in hippocampal neurons. Taken together, these data strongly implicate astrocyte-derived FN in the role of DZ and DZO neurotoxicity.

The regulation of FN by oxidative stress is not clear. FN appears to be modulated by a variety of factors, both endogenous and exogenous. Ethanol in rat C6 glioma cells (Ren et al., 2000), kainic acid in hippocampal astrocytes (Mahler et al., 1997), interferon-gamma in primary cortical astrocytes (DiProspero et al., 1997), as well as ascorbate (vitamin C) in human skin fibroblasts (Peterszegi et al., 2002) were found to decrease levels of FN. Ethanol and kaininic acid, in particular, have been shown to elicit oxidative stress (Giordano et al., 2006; Montoliu et al., 1995), and may potentially modulate FN in this way as well.

DZ and DZO may be indirectly modulating FN by affecting various other growth factors or cytokines that have been shown to alter levels of the protein. Factors such as epidermal growth factor, transforming growth factor-β (TGF- β) 1 and 2, platelet derived growth factor, basic fibroblast growth factor, and interleukin-6 (IL-6) have been shown to alter FN in the brains

or neural cell types of rodents (Gris et al., 2007; Mahler et al., 1997; Martinez and Gomes, 2002; Pasinetti et al., 1993). Evidence in the literature suggests the potential for other neurotoxic OPs (e.g. chlorpyrifos, malathion) to induce neuro-inflammation, in some cases specifically upregulating levels of aforementioned inflammatory agents, including interferon-gamma and IL-6 (Banks and Lein, 2012; Mense et al., 2006; Rodgers and Xiong, 1997). The role of inflammation in the neurotoxicity of DZ/DZO remains unexplored and could be involved in the mechanism of impaired neurite outgrowth by modulation of FN in astrocytes.

Matrix metalloproteinase (MMP) enzymes are another possible modulator of FN under conditions of elevated oxidative stress. MMPs comprise a family of zinc-dependent enzymes that play an important role in ECM turnover and remodeling, as well as in other physiologic processes in the CNS, including tissue morphogenesis, wound-healing, neurite outgrowth, and neuro-inflammation (Yong et al., 2001). MMP-2 and -9, in particular, can bind and degrade FN (Wang and Lai, 2013; Watanabe et al., 2000; Woessner, 1991). These same MMPs are also upregulated in response to oxidative stress in the brain (Lin et al., 2012; Skowronska et al., 2012). Lin and colleagues (2012) specifically demonstrate that ROS increase MMP-9 in astrocytes and rat brain tissue. DZ and DZO may increase members of the MMP family by increasing oxidative stress, and subsequently modulate FN in this manner; further study is needed to explore this possible mechanism.

The finding that the parent compound, DZ, elicits oxidative stress and inhibits astrocyte-mediated neurite outgrowth indicates that it can cause neurotoxic effects on its own. It is important to note that neurite outgrowth was inhibited as a result of co-incubation with astrocytes treated with an order of magnitude lower concentration of DZO. There is a possibility, then, that contamination of the parent compound by small amounts of the oxon, or biotransformation of DZ to DZO in the astrocytes could have contributed to the effect of DZ on ROS formation and inhibition of neurite outgrowth.

Using acetylcholinesterase (AChE) inhibition as a proxy for DZ biotransformation to DZO in astrocytes, however, may suggest otherwise. Unlike the oxon form of OPs, the parent compound does not affect AChE activity directly; thus, inhibition of AChE activity in the astrocytes is thought to occur by the presence of DZO, either by biotransformation or oxon contamination of the parent compound. Astrocytes treated with DZ exhibited a slight decrease in AChE activity, suggesting that there may be a small amount of oxon found in the DZ-treated

astrocytes. DZO exposure caused slightly greater AChE inhibition in astrocytes at the concentrations tested (25%). However, AChE inhibition is not likely to be the key mediator in this process for two reasons: first, the effects of DZ and DZO on astrocytes-mediated inhibition of neurite outgrowth are completely prevented by antioxidants. Seeing as antioxidants should have no bearing on AChE activity, the effects of these compounds on neurite outgrowth would likely not have been prevented by antioxidants if these mechanisms were governed by AChE inhibition. Second, the parent compound increases ROS production in astrocytes and causes astrocyte-mediated inhibition of neurite outgrowth in a similar manner to the oxon, without the same extent of AChE inhibition.

In summary, these findings indicate that through a mechanism of oxidative stress, DZ and DZO are able to inhibit neurite outgrowth, in part by decreasing FN levels in astrocytes. Given the ubiquitous use of OPs in conjunction with the suspected involvement of oxidative stress in various diseases, these findings support further investigation into OP-induced oxidative stress on neurotoxic mechanisms of these compounds.

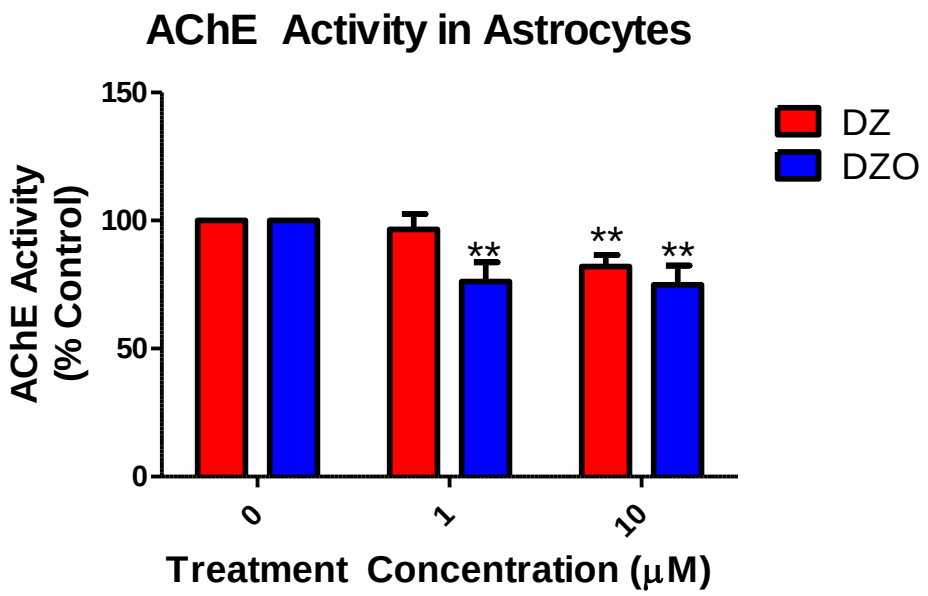
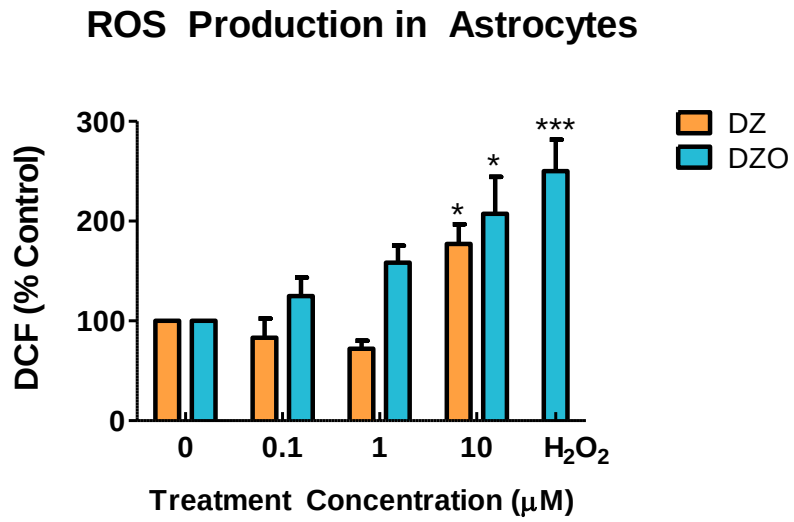


Figure 2.1. AChE Activity in Astrocytes. Astrocytes were exposed to 1 or 10 µM DZ or DZO for 24 h, after which lysates were collected and AChE activity was measured using a modified Ellman assay. Mean AChE activity (± SEM) in untreated control astrocytes was 3.09 ± 0.33 nmol/min/mg protein. Results are expressed as mean percent control of untreated astrocytes ± SEM gathered from three independent experiments. **P<0.01 Statistically different than untreated control (One-way ANOVA followed by Bonferroni's post-hoc test).

Treatment Concentration (μM)	**DZ** Viability (% Control)	**DZO** Viability (% Control)
0	100	100
1	101.19 ± 7.95	100.82 ± 6.48
5	104.35 ± 5.07	97.55 ± 1.75
10	106.04 ± 8.04	91.43 ± 2.22
25	104.35 ± 6.78	98.19 ± 3.28
50	114.54 ± 8.12	99.18 ± 2.06

Table 2.1. Cell viability of DZ or DZO-treated astrocytes. Astrocytes were treated with varying concentrations of either DZ or DZO. Cell viability of astrocytes was not compromised by any concentration of DZ or DZO tested. These data are the result of at three separate experiments where astrocytes where treated in triplicate. Results are expressed as mean percent control ± SEM.

(a)



(b)

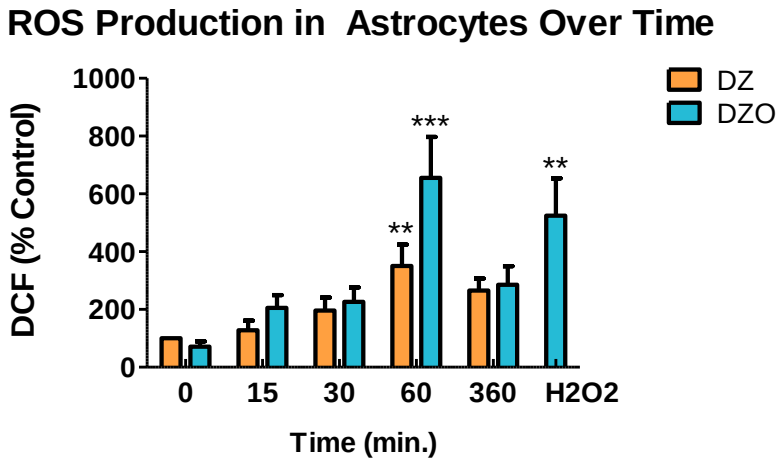


Figure 2.2. DZ and DZO increase oxidative stress in astrocytes. Intracellular ROS production in DZ- and DZO-treated astrocytes over 1 hour (a), or 10 µM DZ or DZO for indicated time points (b). H_2O_2 is 20 µM. Results are expressed as percent untreated astrocytes and represent the mean ± SEM of three separate experiments. *P<0.05, **P<0.01, ***P<0.001 Statistically different from respective untreated controls (One-way ANOVA and Bonferroni's post-hoc test).

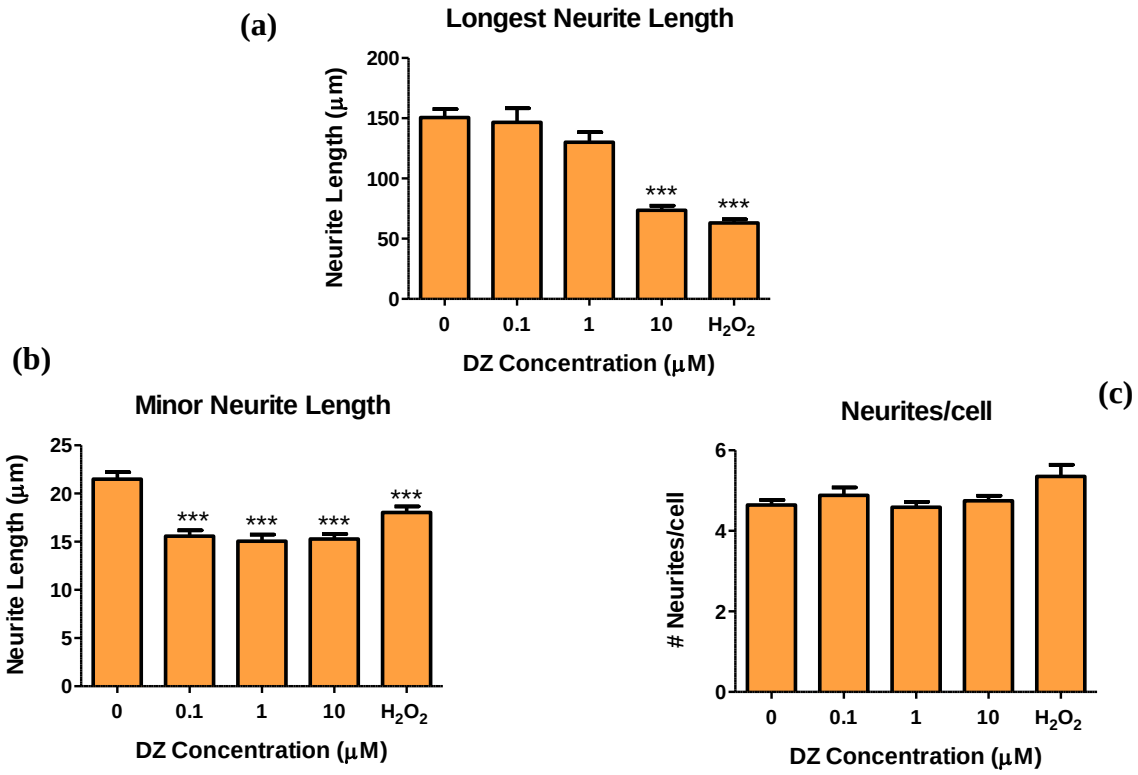


Figure 2.3. DZ inhibits astrocytes' ability to foster neurite outgrowth in hippocampal neurons. Astrocytes were treated with DZ for 24 hours, washed out, and cultured with freshly prepared hippocampal neurons for an additional 48 hours. After 48 hours, neurons were fixed, stained, and analyzed for longest neurite length, minor neurite length, and # neurites/cell. These data are the result of 90-120 cells derived from at least three different experiments. 20 µM H_2O_2 exists as a positive control. **P<0.01 Statistically different from untreated control (One-way ANOVA and Bonferroni's post-hoc test).

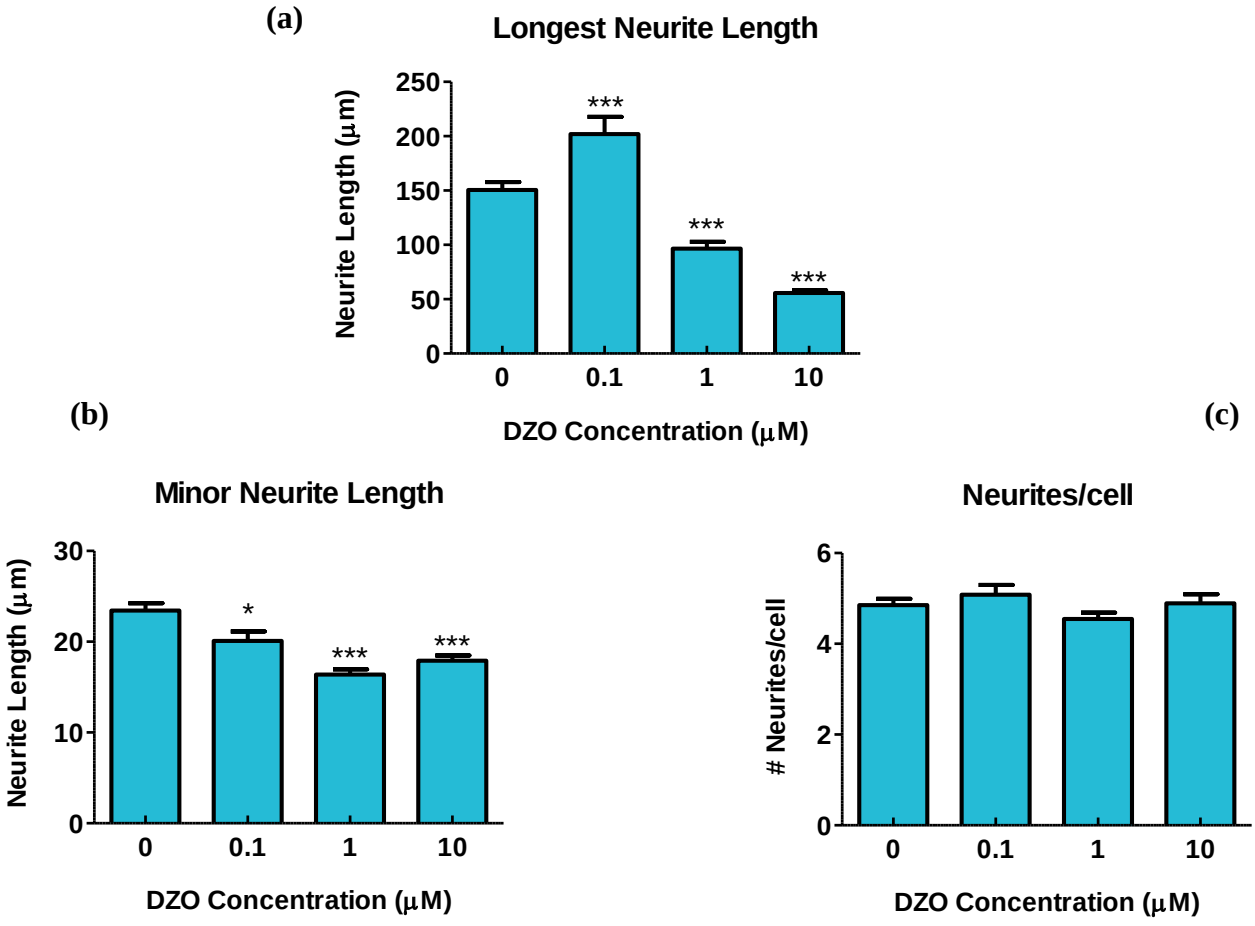


Figure 2.4. DZO inhibits astrocytes' ability to foster neurite outgrowth in hippocampal neurons. Astrocytes were treated with DZO for 24 hours, washed out, and cultured with freshly prepared hippocampal neurons for an additional 48 hours. After 48 hours, neurons were fixed, stained, and analyzed for longest neurite length, minor neurite length, and # neurites/cell. These data are the result of 90-120 cells derived from at least three independent experiments. **P<0.01 Statistically different from untreated control (One-way ANOVA and Bonferroni's post-hoc test).

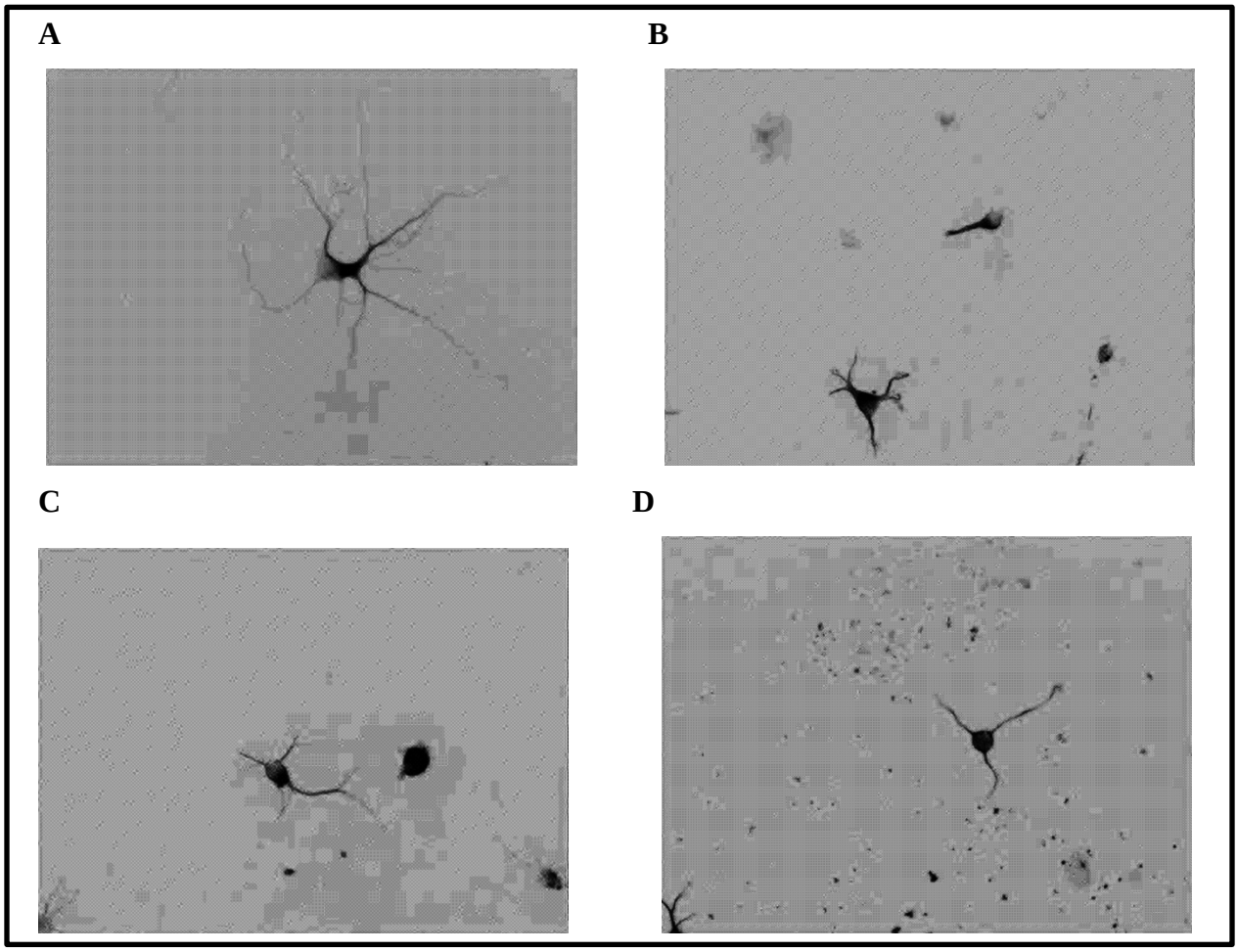


Figure 2.5. DZ and DZO-treated astrocytes inhibit neurite outgrowth in hippocampal neurons. Neurite outgrowth of hippocampal neurons co-incubated with astrocytes previously treated with DZ or DZO is significantly inhibited. Images display representative neurons co-incubated with astrocytes previously treated with (A) 0 µM, (B) 20 µM H_2O_2, (C) 10 µM DZ, or (D) 10 µM DZO.

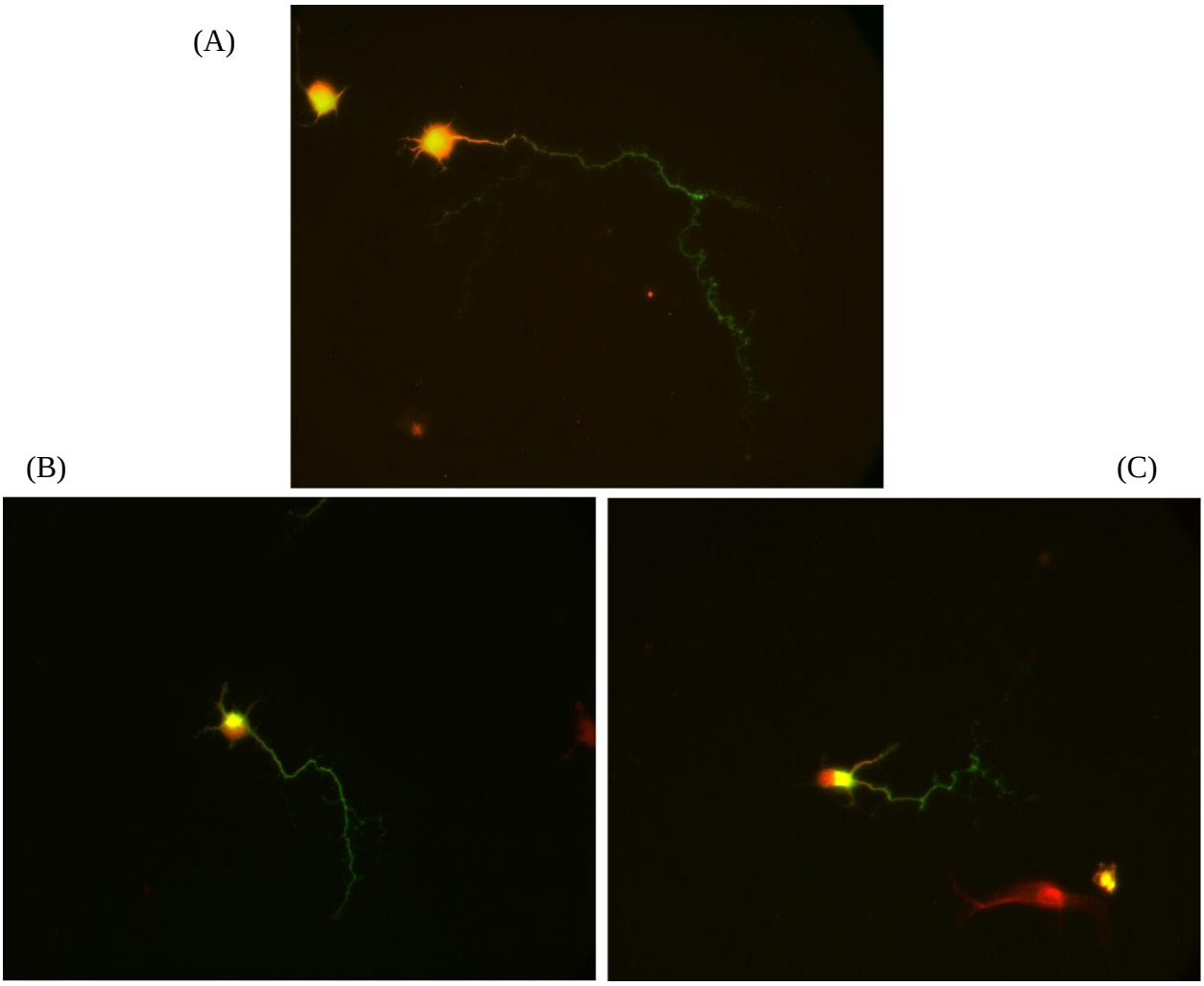


Figure 2.6. Axon- and dendrite-specific markers Tau and MAP-2 verify identity of longest and minor neurites. Images display representative neurons that were co-incubated with astrocytes previously treated with either (A) 0 µM, (B) 10 µM DZ, or (C) 10 µM DZO. Green = anti-tau (axons). Red/yellow = anti-MAP-2 (dendrites).

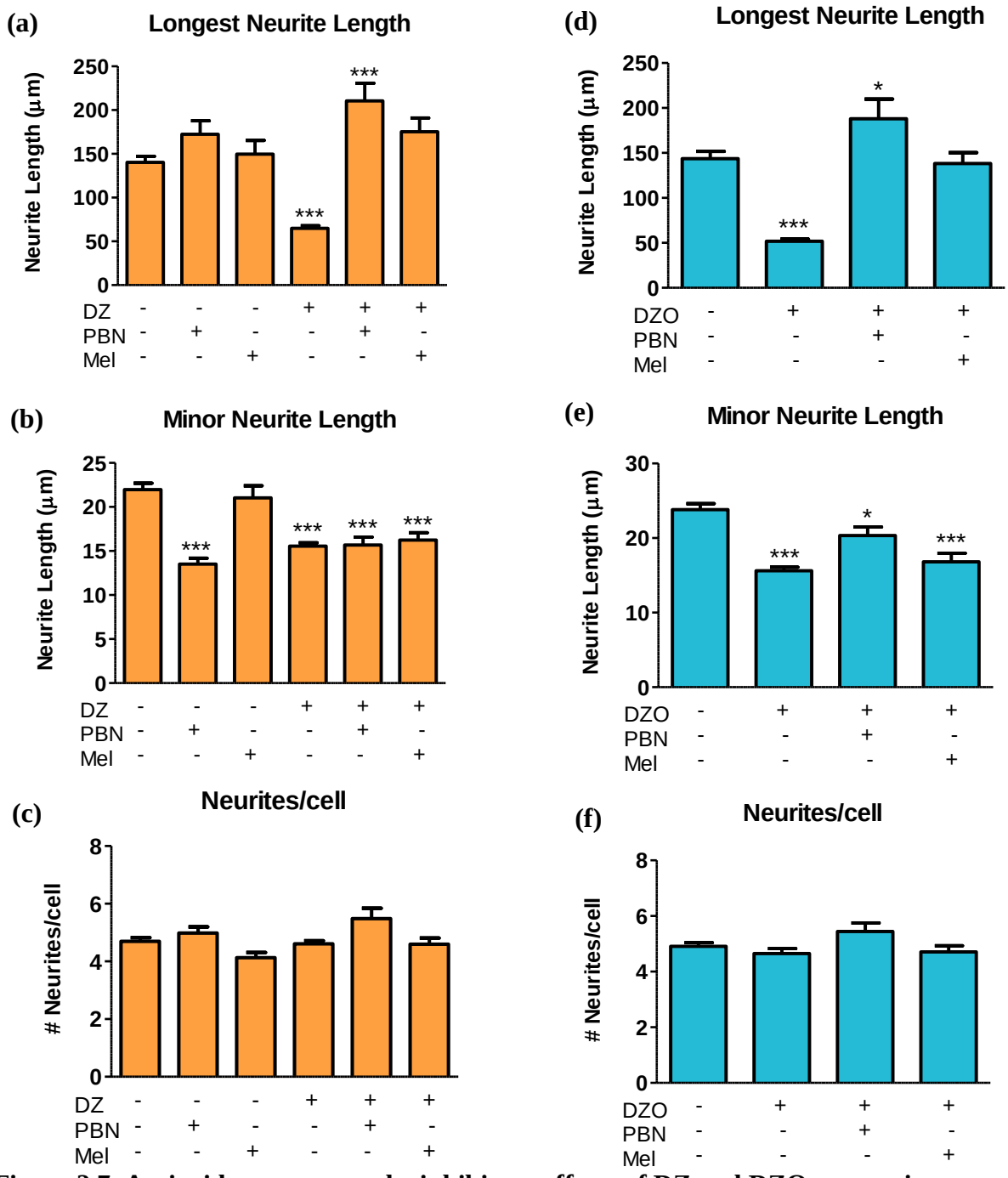


Figure 2.7. Antioxidants prevent the inhibitory effects of DZ and DZO on neurite outgrowth. Astrocytes were pre-treated with 200 µM melatonin (Mel) or 100 µM PBN for 3 hours prior to wash-out and treatment with 10 µM DZ or DZO for 24 hours. Data illustrating antioxidant pre-treatment are the result of at least 60 cells taken from two independent experiments. 20 µM H_2O_2 exists as a positive control. *P<0.05 ***P<0.001 Statistically different than untreated control (One-way ANOVA and Bonferroni's post-hoc test).

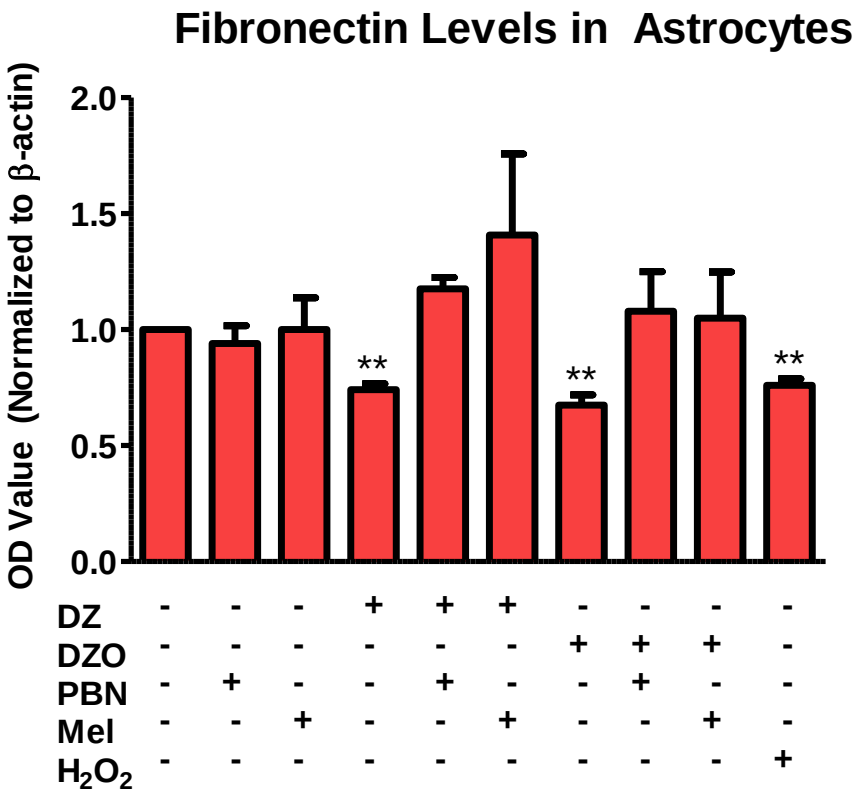
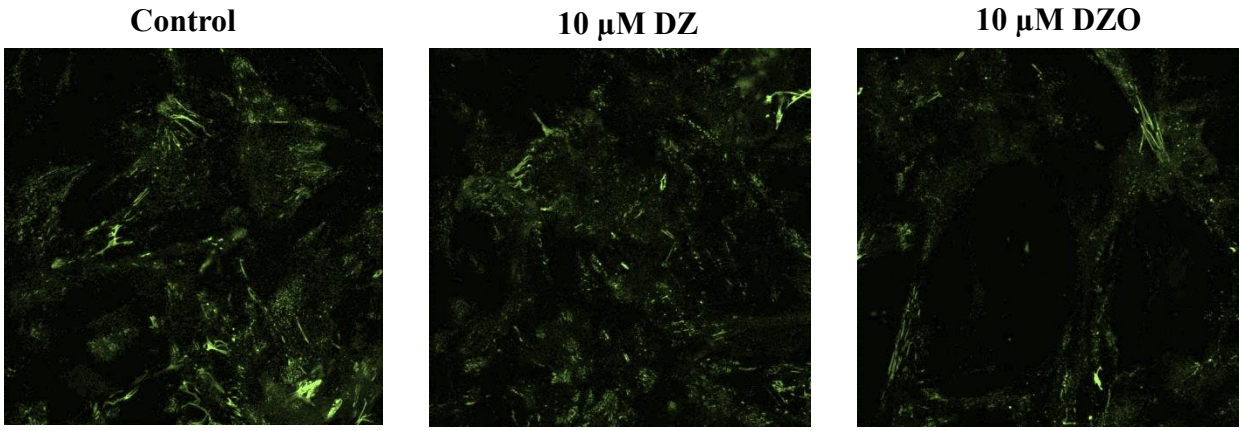


Figure 2.8. DZ and DZO decrease fibronectin lysate levels in astrocytes. Astrocytes were pre-treated or not with antioxidants melatonin (200 µM) or PBN (100 µM) for 3 h before washout and 24 h treatment with 10 µM DZ or DZO. 20 µM H_2O_2 exists as a positive control. DZ and DZO cause a 30% decrease in lysate fibronectin levels in astrocytes; this decrease is prevented by antioxidant pre-treatment. These data are the result of at least three independent experiments in which astrocytes were treated in duplicate. **P<0.01 Statistically different from control (Kruskal-Wallis followed by Dunn's post-hoc test).

(a)



(b)

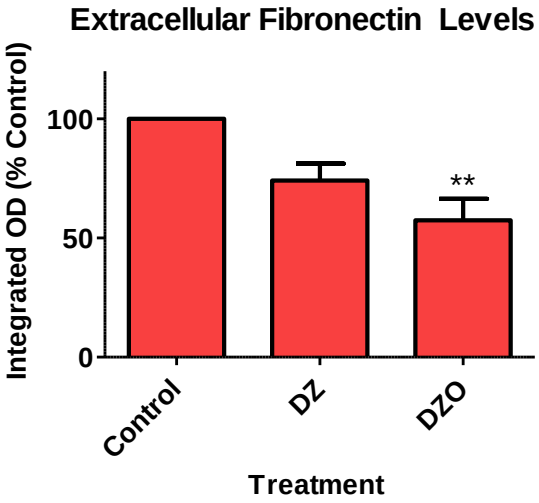


Figure 2.9. DZ and DZO decrease extracellular expression of fibronectin on astrocytes. Extracellular-bound fibronectin expression on astrocytes was imaged with confocal microscopy. Integrated intensity was measured and normalized by cell number. 10 µM DZ and 10 µM DZO decrease extracellular expression of fibronectin on astrocytes: (a) Representative confocal microscopy images of extracellular fibronectin expression on astrocytes; (b) quantification of the integrated intensity relative to control. Results are expressed as mean percent control ± SEM of data from three independent experiments. **P<0.01 Statistically different from control (Kruskal-Wallis followed by Dunn's post-hoc test).

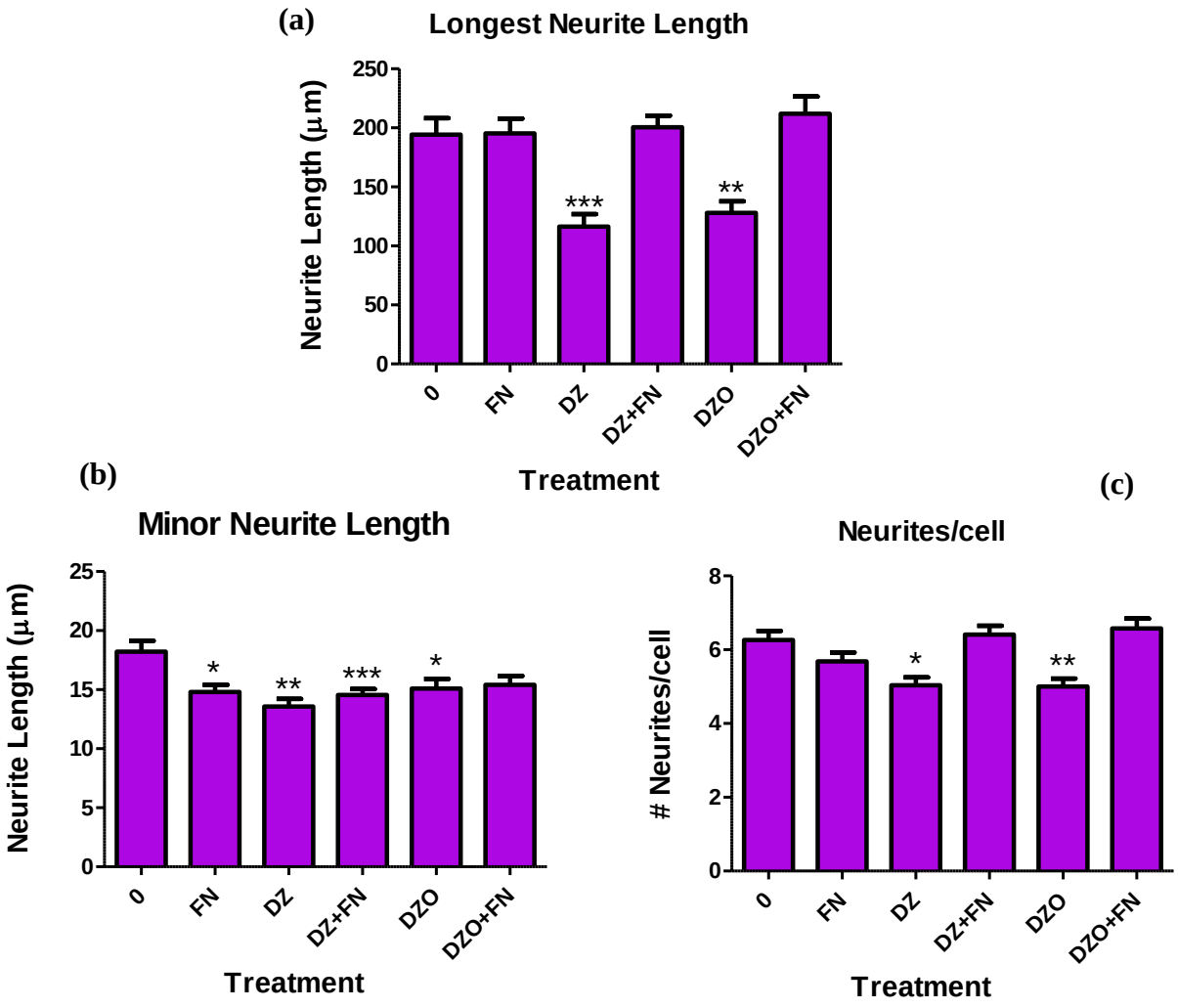


Figure 2.10. Fibronectin prevents inhibition of neurite outgrowth caused by DZ and DZO. Purified fibronectin (final concentration: 10 µg/mL) was added to the media of the astrocyte-neuronal co-culture system when newly-prepared neurons were placed with previously-treated astrocytes. These data are the result of 90-100 cells derived from at least three independent experiments. Results are expressed as mean ± SEM for all parameters measured. *P<0.05, **P<0.01, ***P<0.001 Statistically different from control (One-way ANOVA, followed by Bonferroni's post-hoc test).

Chapter 3

ASTROCYTES PROTECT AGAINST DIAZINON- AND DIAZOXON-INDUCED INHIBITION OF NEURITE OUTGROWTH BY REGULATING NEURONAL GLUTATHIONE

3.1 Introduction

Diazinon (DZ) and its active metabolite, diazoxon (DZO), are members of a ubiquitously-used class of insecticides known as organophosphorus insecticides (OPs) (EPA, 2011). Children in communities in close proximity to crops where these insecticides are sprayed are exposed regularly to a variety of OPs, and are at increased risk for adverse neurological effects as a result. Recent studies link such exposures to various neurobehavioral deficits, such as ADHD and lowered I.Q. (Bouchard et al., 2010; Eskenazi et al., 2007; Rauh et al., 2011; Rohlman et al., 2011). While acute effects of OP toxicity primarily results from acetylcholinesterase (AChE) inhibition and subsequent cholinergic overstimulation, increasing evidence demonstrates that these compounds can exert a multitude of other effects, including alterations in signal transduction, DNA synthesis, and increases in oxidative stress (Adigun et al., 2010; Guizzetti et al., 2005; Lukaszewicz-Hussain, 2010; Slotkin et al., 2006).

In addition to confirmed child exposure, the literature suggests DZ and DZO are developmental neurotoxicants: chlorpyrifos and several other OPs have been shown to differentially affect the developing brain as young rats are more sensitive to the acute effects of AChE inhibition caused by OP exposure than adults (Pope and Liu, 1997; Won et al., 2001). Long-term effects of late gestational and neonatal exposures to OPs have also been demonstrated: early exposure to chlorpyrifos and diazinon affected learning and memory (Icenogle et al., 2004; Levin et al., 2008; Roegge et al., 2006), as well as neural cell development and synaptic function (Slotkin et al., 2008) in adolescent rodents. Some studies demonstrate neurotoxic effects at levels separate from AChE inhibition (Rush et al., 2010; Sidiropoulou et al., 2009a; Yang et al., 2008), further supporting the role of alternative neurotoxic mechanisms of these compounds.

Several studies have pointed to oxidative stress as a potential mechanism of OP neurotoxicity (Giordano et al., 2007; Lukaszewicz-Hussain, 2010; Slotkin et al., 2005). The overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) results in cellular oxidative stress. This ultimately leads to deleterious effects on various macromolecules, including DNA, lipids, and proteins (Valko et al., 2007). Furthermore, oxidative stress is increasingly implicated in a variety of diseases, including several neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Barnham et al., 2004), as well as to neurodevelopmental disorders, including autism and schizophrenia (Chauhan et al., 2012; Do et al., 2009; Tang et al., 2013).

Cellular defenses against oxidative stress are plentiful: enzymes such as superoxide dismutases, catalase, and glutathione peroxidases, and the nonenzymatic agents which include glutathione, ascorbic acid (Vitamin C), a-tocopherol (Vitamin E), and flavonoids (Valko et al., 2007). Even so, these defense systems can be overwhelmed in times of acute and/or chronic stress, rendering the cell defenseless against free radicals and oxidative species. The brain is particularly vulnerable to oxidative stress due to its high oxygen consumption, oxidizable lipid content (i.e. polyunsaturated fatty acids), as well as relatively low levels of endogenous antioxidants (Barnham et al., 2004; Matés, 2000). Levels of antioxidants in the brain differ by region and cell type. Glial cells, for example, have a higher glutathione content than neuronal cells; ascorbate, however, appears to predominate in neurons (Rice and Russo-Menna, 1997).

Glutathione (GSH; ɣ-glutamyl-cysteinyl-glycine) is an abundant tripeptide, cellular thiol that is one of the most prominent antioxidants in the CNS (Lu, 2013). GSH is potent defender against ROS due its ability to non-enzymatically scavenge free radicals, as well as its role as a cofactor for glutathione peroxidases and glutathione transferases against reactive aldehyde and peroxide accumulation within the cell (Dringen, 2000). The dysregulation of GSH redox cycling, as well as genetic deficiencies in GSH-related enzymes have been shown to adversely affect neurodevelopment and play a large part in various neurodegenerative diseases (Ballatori et al., 2009; Sian et al., 1994). Furthermore, GSH has been shown to modulate neurotoxicity that results from several environmental chemicals, including the flame retardants, polybrominated diphenyl ethers (PBDEs), domoic acid, and certain OPs (Giordano et al., 2007; 2008; 2006).

This study serves to evaluate the ability for the OP DZ and its primary metabolite, DZO, to induce oxidative stress and inhibit neurite outgrowth in primary hippocampal neurons.

Additionally, the role of primary cortical astrocytes and astrocyte-derived GSH is explored in the mechanism of DZ- and DZO-induced neurotoxicity.

3.2 Materials and Methods

Materials. All materials were the same as those stated in Chapter 2, section 2.2, with the following additions: glutathione ethyl-ester (GSHee), L-Buthionine-(S,R)-Sulfoximine (BSO), reduced L-glutathione (GSH), tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), and naphthalene dicarboxaldehyde (NDA) were from Sigma-Aldrich (St. Louis, MO).

Preparation of fetal rat hippocampal neurons. Primary cultures of hippocampal neurons were prepared as previously described in Chapter 2, section 2.2. Here, neurons were freshly prepared and plated on poly-D-lysine-coated glass coverslips for 2 h in Neurobasal A-FBS (10%) media to allow sufficient attachment. After 2 h, the neurons were washed once in HBSS and the media was replaced with astrocyte-conditioned media (ACM). Treatments occurred 24 h after this.

Hippocampal neuron treatments. Neurons were freshly prepared and plated on poly-D-lysine-coated glass coverslips for 2 h in Neurobasal A-FBS (10%) media to allow sufficient attachment. After 2 h, the neurons were washed once in HBSS and the media was replaced with astrocyte-conditioned media (ACM). After 24 h, neurons were treated with varying concentrations of DZ and DZO (0, 0.1, 1, 10 µM) for 24 h; the total time in culture was 48 h. After this time, the neurons are washed twice with HBSS and fixed in 4% PFA for 15 min. at 37 C.

For experiments where antioxidants were used, neurons were pre-treated with either melatonin (200 µM), N-t-butyl-alpha-phenylnitrone (PBN; 100 µM), or glutathione ethyl-ester (2.5 mM) for 3 h prior to DZ or DZO treatment. After 3 h, the antioxidants were washed out twice with HBSS, fresh ACM was replaced, and neurons were treated for 24 h with 10 µM DZ or DZO.

Astrocyte-Conditioned Media (ACM) Preparation. Primary cultures of cortical astrocytes were prepared as previously described (Guizzetti and Costa, 1996). After 14 days in culture (with regular media changes), flasks of confluent astrocytes were washed twice with PBS and replaced with "serum-free" DMEM-BSA (0.1%) media for 24 h. This media was now considered astrocyte-conditioned. The ACM was collected from the flasks and centrifuged at 1,000 rpm for 10 min. at room temperature to pellet any cells or debris. This ACM was then used as the culture

media for the rest of the incubation period for all neurite outgrowth experiments in neurons. ACM is used for the remainder of the experiment to better replicate *in vivo* conditions where astrocytes promote a healthy environment for neuronal growth and development through secreting various growth factors, proteins, and neurotransmitters.

Measurement of cell viability. Neuron viability was measured by the MTT assay, where 50 µL of MTT reagent (5 mg/mL) was added to each well after 24 h treatment with DZ or DZO. After 3 h at 37 ºC, the media was removed and the formazan reaction product was dissolved in 250 µL DMSO. Absorbance was read at 562 nm and results were expressed as mean percentage of viable cells relative to untreated controls.

Measurement of GSH levels. Neurons were plated in 6-well plates ($1x10^6$ cells/well) and conditioned as previously described. After 24 h in ACM, designated neurons were treated for 3 h with glutathione ethyl-ester (GSHee; 2.5 mM). After 3 h, all neurons were washed twice in HBSS and fresh ACM was replaced. At this time, neurons were either left alone or astrocytes plated on 6-well inserts were added to the wells. Astrocytes were plated on poly-D-lysine-coated inserts and cultured for 4 days. After 4 days, they were serum-deprived for 24 h in DMEM-BSA (0.1%) media. These inserts containing astrocytes were then added to wells containing hippocampal neurons, where appropriate, in order to assess the effects of astrocytes on neuronal glutathione content.

Total intracellular glutathione (GSH) levels were measured as previously described (Giordano et al., 2008). Briefly, neurons were homogenized in Locke's buffer and an aliquot was taken to measure the protein concentration while a second aliquot was diluted (1:1) with 10% 5-sulfosalicylic acid (SSA). The SSA fraction was centrifuged at 12,000 rpm for 5 min at 4 C and the supernatant was used for GSH determination. Aliquots from the SSA fraction were added to a 96-well plate and pH was adjusted to 7.0 with 0.2 M N-ethylmorpholine/0.02 M KOH. Oxidized glutathione was reduced by adding 10 µl of 10 mM tris (2-carboxyethyl)-phosphine hydrochloride (TCEP) for 15 min at room temperature. The pH was then adjusted to 12.5 using 0.5 N NaOH before adding naphthalene dicarboxaldehyde (NDA; 10 mM for 30 min). Finally, the samples were analyzed on a spectrofluorometric plate reader (Ex 472 nm and Em 528 nm). The total amount of GSH in the sample was expressed as nmol/mg protein determined from a

standard curve obtained by plotting known amounts of GSH incubated in the same experimental conditions versus fluorescence.

Astrocyte-neuronal co-cultures. To assess the potential for astrocytes to protect neurons from DZ- and DZO-induced inhibition of neurite outgrowth, an indirect co-culture model was used. This model provides a way to understand astrocyte-neuronal interactions in an in vitro system that more accurately reflects the in vivo processes.

Hippocampal neurons were prepared as described and plated on glass coverslips to which 4 paraffin beads were previously affixed. The neurons are plated on coverslips containing paraffin wax beads that prevent their touching the astrocyte monolayer while allowing them to share the same media that includes subsequent growth factors and secreted proteins. After 2 h incubation in Neurobasal A/FBS (10%) medium to allow neurons to attach, neurons were washed twice in HBSS and ACM was added, as previously described. After 24 h, the glass coverslips containing the neurons were inverted onto 24-well plates containing astrocytes, as described by Viviani et al. (1998).

This astrocyte-neuronal co-culture was treated with 10 µM DZ or DZO or vehicle control (0.1 % DMSO) for 24 h. Neurons alone (not in the presence of astrocytes) were also treated with 10 µM DZ or DZO to assess the effect of the presence of astrocytes on the inhibitory effect of DZ/DZO on neurite outgrowth. Where appropriate, astrocytes were previously treated with L-Buthionine-(S,R)-Sulfoximine (BSO; 25 µM) for 24 h to deplete GSH.

Quantitative Morphological Analysis of Neurite Outgrowth. Primary hippocampal neurons previously prepared from E21 rat fetuses were plated on glass coverslips. After 24 h treatment, neurons were fixed in 4% paraformaldehyde (PFA). Neurons were labeled with an anti-β-III-tubulin isoform antibody followed by a fluorescein-conjugated secondary antibody (Alexa-488); the nuclei were stained with Hoechst 33342. Only pyramidal neurons with three or more neurites, not touching any other cells/neurites were included for quantitative analysis. At least 30 cells per treatment were analyzed for each experiment.

Measurement of Reactive Oxygen Species. Reactive oxygen species (ROS) formation was determined by fluorescence using 2,7′-dichlorofluorescin diacetate ($DCFH_2$-DA). Upon entering

cells $DCFH_2$-DA is de-esterified to $DCFH_2$, which is then oxidized by ROS to form the fluorescent 2,7′-dichlorofluorescein (DCF).

Neurons were plated in a black-welled 96-well plate ($2x10^4$ in 100 µL/well) previously coated overnight with 100 ug/mL poly-D-Lysine. Neurons were plated for 2 h in Neurobasal A-FBS (10%) media, washed, and switched to ACM as previously described. After 48 h in culture, cells were incubated for 30 min. with 10 µM $DCFH_2$-DA in HBSS. Afterwards, the probe was washed out and the neurons were treated with the appropriate chemical dilutions for the desired time point(s). After treatment, neurons were washed once in HBSS and the plate was read on a Perkin-Elmer spectrofluorimeter (excitation 488 nm, emission 530 nm).

Acetylcholinesterase (AChE) Measurements. AChE levels in neurons were measured as previous (Li et al., 2000) in a microtiter plate assay based on the method of Ellman *et al.* (Ellman et al., 1961). Hippocampal neurons were plated in 6-well plates ($1.2x10^6$ per well) in Neurobasal-A-FBS (10%) for 2 h, after which they were washed 1x with HBSS and switched to ACM. After 24 h, neurons were treated with DZ or DZO (1 or 10 µM) for an additional 24 h. Cell lysates were collected in a 0.1 M sodium phosphate buffer (pH 8.0). For duplicate assays, 50 µL of cell lysates was combined with 150 µL of the assay buffer containing 0.1 M sodium phosphate and 0.1 mM of DTNB (5,5'-dithiobis-2-nitrobenzoic acid). The kinetic assay was initiated by addition of acetylthiocholine (final concentration: 1 mM) and the reaction was continuously monitored for 10 min. at room temperature. Absorbance was read at 412 nm in a Beckman DU-70 spectrophotometer. The amount of 5-thio-2-nitrobenzoate formed was calculated using an extinction coefficient of 13 600 M^{-1}/cm. AChE activity was calculated as nmol/min/mg protein and presented as percent untreated control ± SEM of results from three independent experiments.

Statistical Analysis. For neurite outgrowth measurements, the following parameters were measured for each cell using Metamorph software: longest neurite length (including the length of any branches originating from the neurite); length of minor neurites; and total number of neurites/cell. About 30 cells per treatment were analyzed in each experiment. Final data summaries are displayed as the mean ± SEM for 90-120 cells per treatment derived from at least 3 independent experiments, unless otherwise indicated.

For ROS, GSH, and AChE measurements, at least 3 separate experiments were performed where all samples were completed in duplicate or triplicate. Results are reported as the mean percent of the control ± SEM of results from at least 3 experiments. Statistical significance for all analyses is evaluated as indicated: by one-way ANOVA followed by Bonferroni's post-hoc test, by the non-parametric ANOVA Kruskal-Wallis followed by Dunn's post-hoc test, or with Student's t-test using GraphPad Prism software.

3.3 Results

AChE activity in hippocampal neurons was measured after 24 h incubation with 1 or 10 µM DZ or DZO. DZO caused higher inhibition of AChE activity than DZ, as is expected by the oxon form of the parent compound. Neurons treated with 1 or 10 µM DZ for 24 h resulted in a 20% and 43% decrease in AChE activity, respectively, relative to untreated control levels. Neurons exposed to 1 or 10 µM DZO for 24 h resulted in a 55% and 85% decrease in AChE activity, respectively (Fig. 3.1). Mean AChE activity (± SEM) in untreated control neurons was 3.77 ± 0.55 nmol/min/mg protein.

Primary hippocampal neurons were exposed to varying concentrations of DZ or DZO (0.1-10 µM) for 24 h in ACM and neurite outgrowth was measured. Both DZ and DZO caused a concentration-dependent decrease in longest neurite length of hippocampal neurons, with significant inhibition of longest neurite outgrowth from 0.5, 1, and 10 µM DZ or DZO (Fig. 3.2 and 3.3). While no differences in minor neurite length were observed as a result of DZ exposure (Fig. 3.2b), there is a trend towards decreased minor neurite length in hippocampal neurons exposed to DZO. Minor neurite length of neurons exposed to 0.5-10 µM DZO were significantly decreased compared to those of untreated controls (Fig. 3.3b). No differences in number of neurites per cell compared to untreated controls were seen in neurons exposed to either DZ or DZO (Fig. 3.2c and 3.3c). Viability was measured with the MTT assay, confirming that neuron cell-viability is not compromised between DZ/DZO and untreated controls (Table 3.1).

Reactive oxygen species (ROS) production in primary hippocampal neurons was measured by fluorescence using 2,7′-dichlorofluorescin diacetate ($DCFH_2$-DA). Both DZ and DZO (1 and 10 µM) caused an increase in ROS production in hippocampal neurons. 10 µM DZ and DZO caused over a two-fold increase in ROS production compared to an untreated control, as measured by DCF fluorescence (Fig.3.4). Additionally, results in Table 3.1 demonstrate that oxidative stress processes are directly involved in the inhibition of neurite outgrowth caused by DZ and DZO. Neurons that were pre-treated for 3 h with various antioxidants did not exhibit decreased neurite outgrowth as a result of DZ or DZO exposure. Pre-treatment with melatonin (200 µM), N-t-butyl-alpha-phenylnitrone (PBN; 100 µM), and glutathione ethyl-ester (2.5 mM) completely prevented the inhibition of neurite outgrowth caused by 10 µM DZ or DZO. There were no differences between neurons pre-treated with these antioxidants (with or without DZ/DZO) and untreated controls (Table 3.2).

In a situation more closely resembling *in vivo* conditions, the potential for astrocytes to protect against DZ- and DZO-induced inhibition of neurite outgrowth at lower concentrations was explored. The results in Figure 3.5 show that the presence of astrocytes successfully prevents the inhibition of neurite outgrowth caused by DZ or DZO. Neurons were treated with 10 µM DZ or DZO as previously, either with or without astrocytes. There were no differences observed in the longest neurite length of neurons treated with DZ or DZO in the presence of astrocytes compared to untreated controls. As previously shown, 10 µM DZ and DZO significantly inhibited neurite outgrowth of the longest neurites in neurons that were treated in the absence of astrocytes (Fig. 3.5).

Both DZ and DZO appear to elicit these effects on neurite outgrowth as a result of increasing oxidative stress in the neurons, as evidenced by increased ROS production (Fig. 3.4) as well as the ability for various antioxidants to prevent these decreases in neurite growth (Table 3.2). Since astrocytes have a greater antioxidant capacity than neurons, it was hypothesized that astrocytes prevent against inhibition of neurite outgrowth by protecting against the oxidative conditions produced by DZ and DZO exposure. One of the ways astrocytes have a greater ability to combat oxidative stress than neurons lies in the fact that they have higher glutathione content. The presence of astrocytes significantly increased GSH levels in hippocampal neurons; similar effects were observed in neurons pre-treated with 2.5 mM GSH-ethyl ester (Fig. 3.6).

To further implicate the role of GSH in this protective effect of astrocytes on DZ- and DZO-induced inhibition of neurite outgrowth, the effect of astrocytic GSH depletion was examined. Astrocyte GSH was depleted by pre-treating astrocytes with 25 µM L-buthionine sulfoximine (BSO) for 24 h before culturing them with the neurons. BSO specifically inhibits γ-glutamylcysteine synthetase (EC 6.3.2.2; also known as glutamate cysteine ligase, GCL) the enzyme responsible for the first step of GSH biosynthesis (Anderson, 1998). The results in Figure 3.8 reveals that, with their antioxidant capability compromised, astrocytes were not able to confer protection to neurons against DZ- and DZO-induced inhibition of neurite outgrowth as they had previously. Neurons cultured with BSO-treated astrocytes and exposed to 10 µM DZ or DZO for 24 h resulted in a 38% and 30% decrease in longest neurite length, respectively (Fig. 3.7). This is in contrast to no differences in neurite length in neurons treated with DZ or DZO in the presence of astrocytes not previously treated with BSO (Fig. 3.5).

3.4 Discussion

The main findings in this study are that DZ and DZO inhibit neurite outgrowth in primary hippocampal neurons, and these inhibitory effects are prevented by the presence of astrocytes. Additionally, these compounds are shown to elicit oxidative stress in these hippocampal neurons, and the inhibitory effects on neurite outgrowth are successfully prevented by antioxidants. Lastly, this study demonstrates that astrocyte-derived GSH, a cellular thiol with abundant antioxidant capability, is responsible for this protective effect of astrocytes on neuronal growth. Depletion of GSH eradicates astrocyte ability to protect against DZ/DZO-impaired neuritogenesis; this has implications for populations exposed to multiple oxidative compounds, and/or with genetic polymorphisms in the many genes involved in GSH synthesis and regulation.

The ability for these OPs to inhibit neurite outgrowth is consistent with the literature in that there are various studies which shown that DZ and DZO can impair inhibit neurite outgrowth in neuronal (N2a) cell lines (Axelrad et al., 2003; Flaskos et al., 2007; Sidiropoulou et al., 2009a). Limited published data exist, however, demonstrating such effects in primary cells. While cell lines are valuable tools in science research, they often react differently to substances compared to *in vivo* responses; primary cells better reflect the human system and relevant functionality. Furthermore, the role of astrocytes, as well as the involvement of GSH, in the mechanism of DZ and DZO neurotoxicity has not been extensively studied.

Both the parent compound and the oxon metabolite caused inhibition of neurite outgrowth, with significant inhibition occurring at concentrations as low as 0.5 µM of either compound. These results are supported by the literature, where authors have found that 1 and 10 µM DZ and DZO inhibit axon-like processes in the N2a neuroblastoma cell line (Flaskos et al., 2007; Sidiropoulou et al., 2009a). Chlorpyrifos (CPF) is used as a positive control as it is a different OP that has been extensively studied and shown to inhibit neurite outgrowth at relatively low concentrations (Eaton et al., 2008; Sachana et al., 2001; Yang et al., 2008). Exposure to 5 µM CPF resulted in a significant inhibition of neurite outgrowth in this model, comparable to that caused by 10 µM DZ or DZO.

The role of oxidative stress in these neurotoxic effects of DZ/DZO on inhibiting neurite outgrowth in hippocampal neurons was further assessed by measuring ROS production in neurons, as well as the potential for antioxidants to prevent the decrease in neurite length caused

by DZ and DZO. Both DZ and DZO do, indeed, increase ROS production in hippocampal neurons. These findings agree with other studies demonstrating the ability for these compounds, as well as other OPs, to elicit oxidative stress in neuronal cells (Giordano et al., 2007; Slotkin and Seidler, 2009) and in the brains of exposed rodents (Jafari et al., 2012; Yilmaz et al., 2012). Additionally, three antioxidants (melatonin, PBN, and glutathione ethyl ester (GSHee)) successfully prevented inhibition of the longest neurite outgrowth caused by DZ and DZO. PBN and GSHee have both been shown to prevent increases in ROS production and lipid peroxidation caused by OPs (including DZ and DZO) in cerebellar granule neurons (Giordano et al., 2007). Taken together, these data support the fact that both DZ and DZO increase oxidative stress in hippocampal neurons and this ultimately contributes to inhibition of neurite outgrowth in these cells.

The fact the neurite outgrowth inhibition occurs at concentrations as low as 0.5 μM DZ/DZO suggests that the direct effects of DZ and DZO on neurite outgrowth of hippocampal neurons are more sensitive than the indirect effects of these compounds on impairing astrocytes' ability to foster neuronal growth (see results from Chapter 2). The latter only resulted in a statistically significant inhibition of neurite outgrowth in neurons cultured with astrocytes previously exposed to a higher concentration of DZ or DZO (10 μM). However, there is limited evidence in the literature that shows astrocyte-conditioned media (ACM) protects against this inhibition of neurite outgrowth by DZ (Harris et al., 2009a). Additionally, other studies have shown a protective effect of astrocytes on various types of neuronal injury. Some examples include astrocytes protecting against cerebellar granule neuron toxicity by polybrominated diphenyl ethers (PBDEs) (Giordano et al., 2009a); from oxidative stress and cytoxicity of ethanol (Watts et al., 2005); and against fetal cortical neuron toxicity by the pesticides rotenone and paraquat (Rathinam et al., 2012). Such evidence implies that, in a situation in which neurons and astrocytes function together in close proximity (i.e. *in vivo*), astrocytes can protect neurons from toxicity by various compounds. To further investigate this valuable role of astrocytes, the ability for astrocytes to protect against DZ- and DZO-induced inhibition of neurite outgrowth was evaluated.

The presence of astrocytes during the 24 h treatment with either DZ or DZO completely attenuated the formally observed neurite outgrowth inhibition caused by both compounds. It can be noted that the protective effect of ACM alone is not observed in this model, which utilizes

ACM from primary cortical astrocytes. In this study, experiments are conducted with primary hippocampal neurons, which are potentially more sensitive to insult than the N2a neuroblastoma cell line used by Harris et al. (2009a); the media alone may not prove sufficient in preventing toxicity in our system.

Astrocytes have been recognized for their roles in supporting the growth and development of neurons; they do so by secreting a variety of factors (e.g. proteins and growth factors), as well as by regulating various homeostatic mechanisms in the brain (e.g. glucose and neurotransmitter levels). It is also known that astrocytes have a higher content of the thiol-redox-capable peptide, GSH, than their neuronal counterparts (Rice and Russo-Menna, 1997). GSH is a crucial player in a multitude of cellular processes, ranging from cell differentiation to apoptosis, as well as a vital role in cellular defense against oxidative stress and insult (Ballatori et al., 2009).

In the context of glial-neuronal interactions, astrocytes can increase GSH levels in neurons by providing the necessary precursors for GSH biosynthesis. Upon release from astrocytes, GSH is metabolized by ɣ-glutamyl transpeptidase (GGT) to the di-peptide cysteinylglycine, from which cysteine is released by an ectopeptidase found on the surface of neuronal membranes. These precursors are then taken up by neurons through transporters and utilized for GSH synthesis within the cell (Dringen et al., 1999; Hirrlinger et al., 2002b; Sagara et al., 1993). In this way, astrocytes can bolster neuronal antioxidant defenses that may otherwise be depleted under conditions of high oxidative stress. Indeed, the findings in this study demonstrate that the presence of astrocytes increase intracellular GSH in hippocampal neurons almost 2-fold. This effect is similar to that of increasing GSH content in neurons by way of exogenous supplementation with the cell-permeable ethyl-ester form of glutathione (GSHee).

Astrocytes were depleted of GSH using BSO (L-buthionine sulfoximine), an agent that potently inhibits the enzyme that catalyzes the first and rate-limiting step of GSH biosynthesis, glutamate cysteine ligase (GCL) (Anderson, 1998; Berger et al., 1994). BSO is broadly shown in the literature to significantly reduce GSH levels in various *in vitro* models, including primary astrocyte cultures (Gabryel et al., 2005; Maryon et al., 2013; Masubuchi et al., 2011). Previous work from Giordano and colleagues confirmed that BSO (25 µM for 24 h) significantly decreases total GSH in primary cortical astrocytes, from 17.4 ± 0.9 to 5.3 ±0.3 nmol/mg protein (Giordano et al., 2009b). Confirming the role of GSH in this process, astrocytes depleted of the

thiol-peptide were no longer able to confer protection to neurons under conditions of simultaneous exposure to DZ or DZO. Taken together, these data strongly indicate that oxidative stress induced by DZ and DZO affects GSH function, and can alter the ability for astrocytes to regulate neurite outgrowth in neurons by doing so.

The findings in this study are supported by various studies demonstrating the involvement of GSH in the neurotoxicity of OPs and other contaminants. In these studies, neuronal cells from *Gclm* (+/+) and *Gclm* (-/-) mice were used; the latter mice lack the modifier subunit of GCL. In the absence of GCLM, the efficiency of the catalytic subunit of GCL (GCLC) is significantly impaired, resulting in 80-90% lower levels of GSH than that found in *Gclm* (+/+) animals (Giordano et al., 2008; 2006; McConnachie et al., 2007; Yang et al., 2002). Toxicity from the OPs chlorpyrifos (CPF) and DZ, as well as their oxon metabolites (CPFO and DZO), was enhanced in cerebellar granule neurons (CGN) from *Gclm* (-/-) mice (Giordano et al., 2007). Similarly, a polybrominated diphenyl ether (PBDE) mixture (DE-71) also caused enhanced toxicity in CGNs and other neuronal cells from *Gclm* (-/-) mice, due to their low GSH content (Giordano et al., 2008).

Additionally, the ability for astrocytes to protect neurons via astrocyte-derived GSH has also been demonstrated using these *Gclm* (+/+) and *Gclm* (-/-) mice: astrocytes from *Gclm* (+/+) animals were very protective against DE-71 toxicity to CGNs from *Gclm* (-/-) mice. This effect is in contrast to the greatly lowered protection conferred by astrocytes from *Gclm* (-/-) animals (Giordano et al., 2009a). The fact that astrocytes from *Gclm* (-/-) mice provided some degree of protection against DE-71 toxicity highlights the existence of alternative mechanisms of astrocyte-mediated protection of neurons. With respect to GSH, GGT has been shown to transfer the ɣ-glutamyl moiety of GSH to extracellular cystine, which forms ɣ-glutamylcystine. This can be taken up into cells, reduced to ɣ-glutamylcysteine, and used by GSH synthethase (GS) to synthesize GSH, thus bypassing the need for GCL (Chinta et al., 2006). This compensatory mechanism for GSH synthesis is an alternative way in which astrocytes, as well as other cells, may increase neuronal GSH (albeit, much less drastically) in the absence of appropriate GCL activity.

Inappropriate alterations in GSH levels, synthesis and catabolism rates, and/or oxidation states have been linked to the etiology of a host of human diseases, including cancer and the neurodegenerative Parkinson's and Alzheimer's disease (Ballatori et al., 2009; Hirrlinger et al.,

2002a; Sian et al., 1994; Viña et al., 2004). In general, glutathione deficiency or shifts in the GSH/GSSG (glutathione disulfide) ratio results in increased susceptibility to oxidative stress and the subsequent damage this causes (Ballatori et al., 2009). For this reason, the finding that without GSH, astrocytes can no longer protect against neurite outgrowth inhibition highlights the implication for developmental neurotoxicity of individuals exposed to these OPs in addition to other ROS-inducing neurotoxic compounds, and/or those chronically exposed. These exposures would likely also deplete astrocytes of GSH and thus eliminate their protective functions against impaired neuritogenesis. Populations with deficiencies in GSH function as a result of defects in proteins that control GSH synthesis and regulation (e.g. GCL and GS) may also be at increased risk of neurotoxicity due to these compounds. Several genetic polymorphisms in GCL have been described and implicated in adverse health effects, including some in the *Gclm* gene that are associated with low levels of GSH (Dalton et al., 2004; Nakamura et al., 2002).

One of the reasons for the higher GSH content in astrocytes is attributed to nuclear factor erythroid-2-related factor 2 (Nrf-2)-driven gene activation that is preferential in astrocytes over neurons (Kraft et al., 2004; Lee et al., 2003). The synchronized increase in astrocytic GSH production and release is an essential component in the observed neuronal protection by Nrf2-activation in astrocytes, especially in response to oxidative stress (Calkins et al., 2010; Lee et al., 2003; Vargas and Johnson, 2009). Astrocytes devoid of Nrf2 (obtained from $Nrf2^{-/-}$ mice) not only have lower GSH levels than those with normal Nrf2 function, but are unable to induce Nrf2-dependent gene transcription and increase GSH after stimulation with the Nrf2-inducing agent, *tert*-butylhydroquinone (tBHQ) (Lee et al., 2003).

Seeing as Nrf2-dependent gene transcription controls a variety of vital antioxidant genes and pathways, including those involved in GSH production, efforts to upregulate Nrf2 in the exposed populations may be of interest from a public health perspective. There is evidence that certain dietary components (e.g. flavonoids, sulfur-containing vegetables) can increase GSH, as well as induce Nrf2-dependent transcription of such antioxidant-responsive genes (Bousova and Skalova, 2012; Tateishi et al., 1974; Vomhof-DeKrey and Picklo, 2012; Yanaka et al., 2009). For these reasons, some might suggest that appropriate dietary planning for potentially exposed populations, such as families in close proximity to crops sprayed with OPs, may be a useful start in protecting against the oxidative effects of these compounds. However, protecting people from

exposure in the first place by instituting proper controls on the use of pesticides would be a much more effective way of preventing the adverse effects of pesticide exposure.

It is possible that astrocytes defend against neuronal insult through other mechanisms that do not include GSH. Since DZ and DZO increase ROS production in neurons, other astrocyte-derived, thiol-containing proteins may be active in this mechanism of protection. Thioredoxins and peroxiredoxins are two notable proteins of this type that are also capable of detoxifying free radicals and oxidative species through their redox capabilities (Dickinson and Chang, 2011; Kudin et al., 2012). Alternatively, astrocytes may protect neurons—at least in part—by directly sequestering the compounds or their produced free radicals. In this way, the compounds would be detoxified, or radicals neutralized, before they ever come in contact with the neurons. Nevertheless, here we provide robust evidence that supports GSH as an essential factor in this role of astrocyte-mediated protection against DZ and DZO-induced inhibition of neurite outgrowth: astrocytes cannot prevent the inhibitory effects of DZ and DZO on neurite outgrowth in the absence of GSH.

Both DZ and DZO caused some decrease in acetylcholinesterase (AChE) activity in hippocampal neurons, with DZO causing predictably higher levels of inhibition than the parent compound. Such significant inhibition of AChE activity by the parent compound was not expected due to the fact that the parent compound does not inhibit AChE directly. It is thought that any inhibition of AChE in the neurons is a result of the presence of DZO, either by biotransformation of DZ in the cell, and/or by contamination with DZO impurities in the DZ chemical. It is important to note, however, that inhibition of neurite outgrowth in these neurons occurred after exposure to DZ at concentrations as low as 0.5 µM. Neurons treated with a higher concentration of 1 µM did not exhibit a significant decrease in AChE activity, and therefore the neurite outgrowth impairments seen in this study is not solely governed by AChE inhibition. Additionally, antioxidant factors successfully prevented the inhibition of neurite outgrowth in this model, further suggesting that there are other, non-cholinergic mechanisms at play.

It is worth considering AChE activity at the cellular level due to the fact that localized metabolism of OP compounds to the oxon in the brain is thought to occur. In studies with CPF in rats, for example, researchers observed decreased AChE inhibition and CPF-oxon levels in the brain after inhibition of a specific cytochrome P-450 (CYP) enzyme (CYP2B); no detectable changes were observed in serum CPF levels or AChE activity (Khokhar and Tyndale, 2012).

Local metabolism of these compounds in the brain may increase their neurotoxic potential in the absence of systemic effects, and is an area in need of further study (Khokhar and Tyndale, 2012; Miksys and Tyndale, 2013).

In conclusion, this study demonstrates that the widely-used OP DZ and its primary metabolite, DZO, directly impair neuritogenesis in hippocampal neurons. Interestingly, the presence of astrocytes prevents the observed inhibition of neurite outgrowth. This novel finding was found to be governed by astrocyte-derived GSH: astrocytes were able to increase GSH in hippocampal neurons, and without it, the astrocytes could no longer confer protection against DZ- and DZO-induced inhibition of neurite outgrowth. These results continue to highlight both the neurotrophic functions of astrocytes as well as the importance of strong antioxidant defenses in the brain, which rely heavily on properly functioning astrocytes.

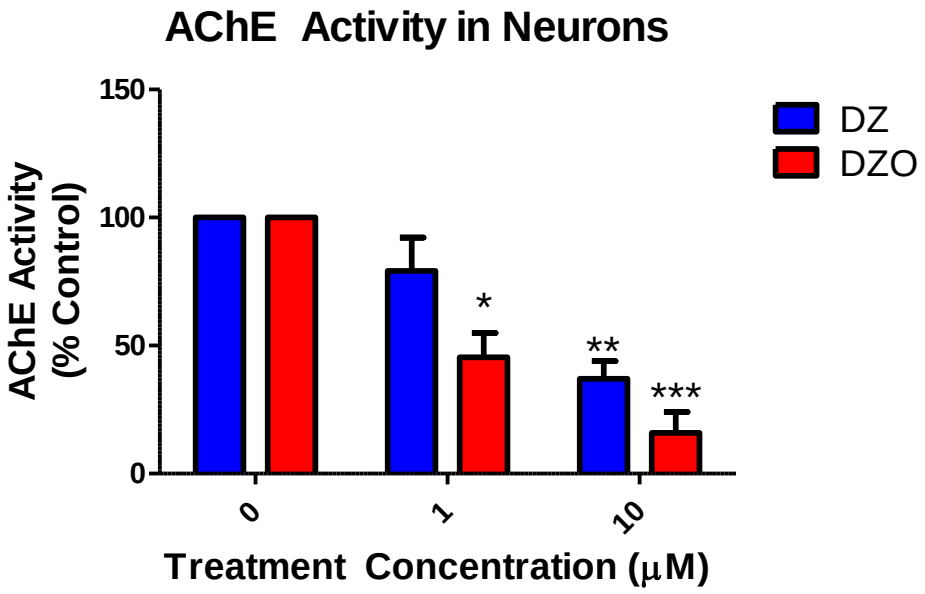


Figure 3.1. AChE activity in primary hippocampal neurons exposed to DZ or DZO. Newly-harvested hippocampal neurons are plated in Neurobasal/FBS (10%) for 2 h before they are switched to astrocyte-conditioned media (ACM) for an additional 24 h. Astrocytes are then treated for 24 h with 1 or 10 μM DZ or DZO. These data are the result of three independent experiments. Mean AChE activity (± SEM) in untreated control neurons was 3.77 ± 0.55 nmol/min/mg protein. Results are presented as mean percent control AChE activity relative to untreated control neurons ± SEM. *P<0.05, **P<0.01, ***P<0.001 Statistically different than untreated controls (One-way ANOVA followed by Bonferroni's post-hoc test).

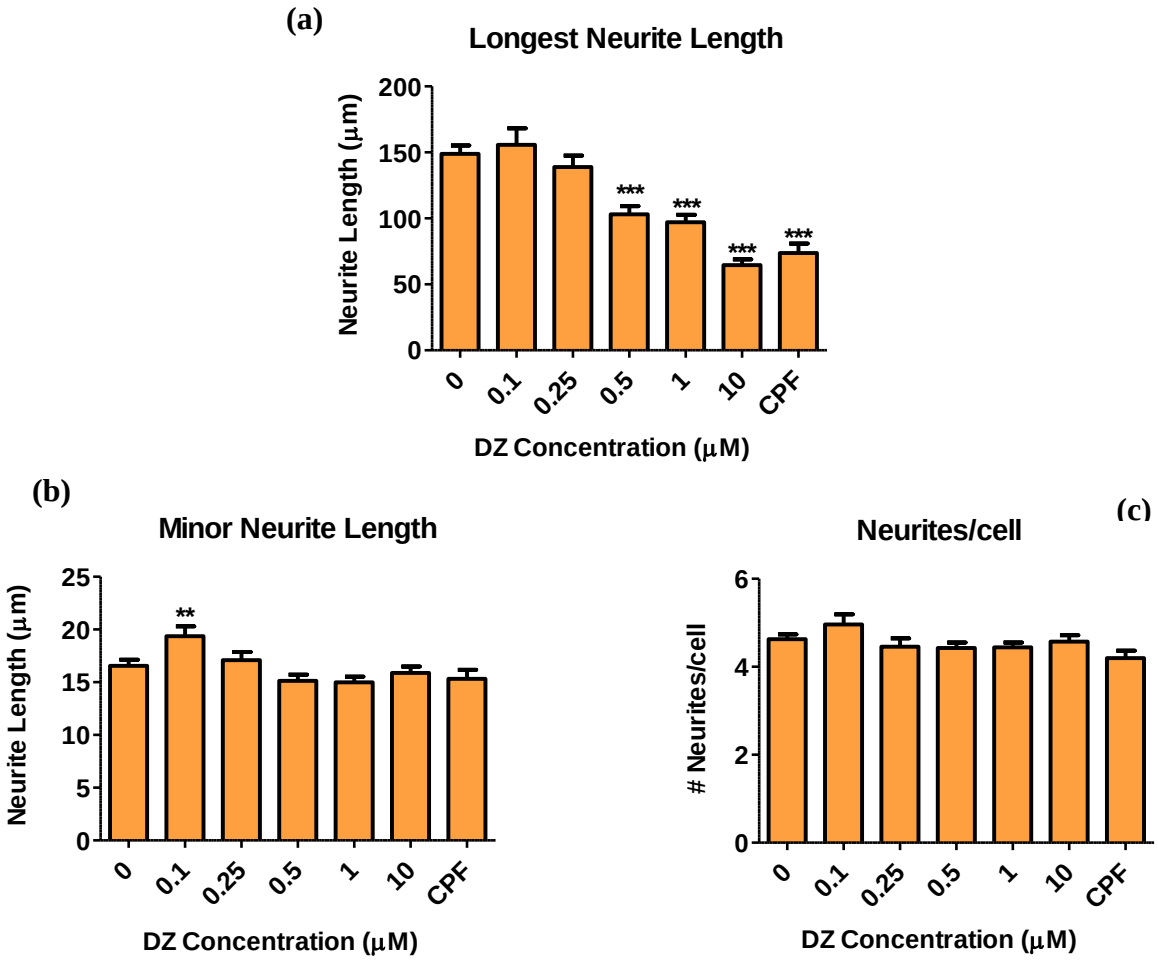


Figure 3.2. DZ inhibits neurite outgrowth in hippocampal neurons. Hippocampal neurons are plated in Neurobasal/FBS (10%) media for 2 h before being washed and switched to ACM for 24 h. The neurons are treated with varying concentrations of DZ for 24 h. Chlorpyrifos (CPF; 5 µM) is used as a positive control. Results are expressed as mean ± SEM of 90-120 cells derived from at least three independent experiments. **P<0.01 Statistically different from control (One-way ANOVA, Bonferroni's post-hoc test).

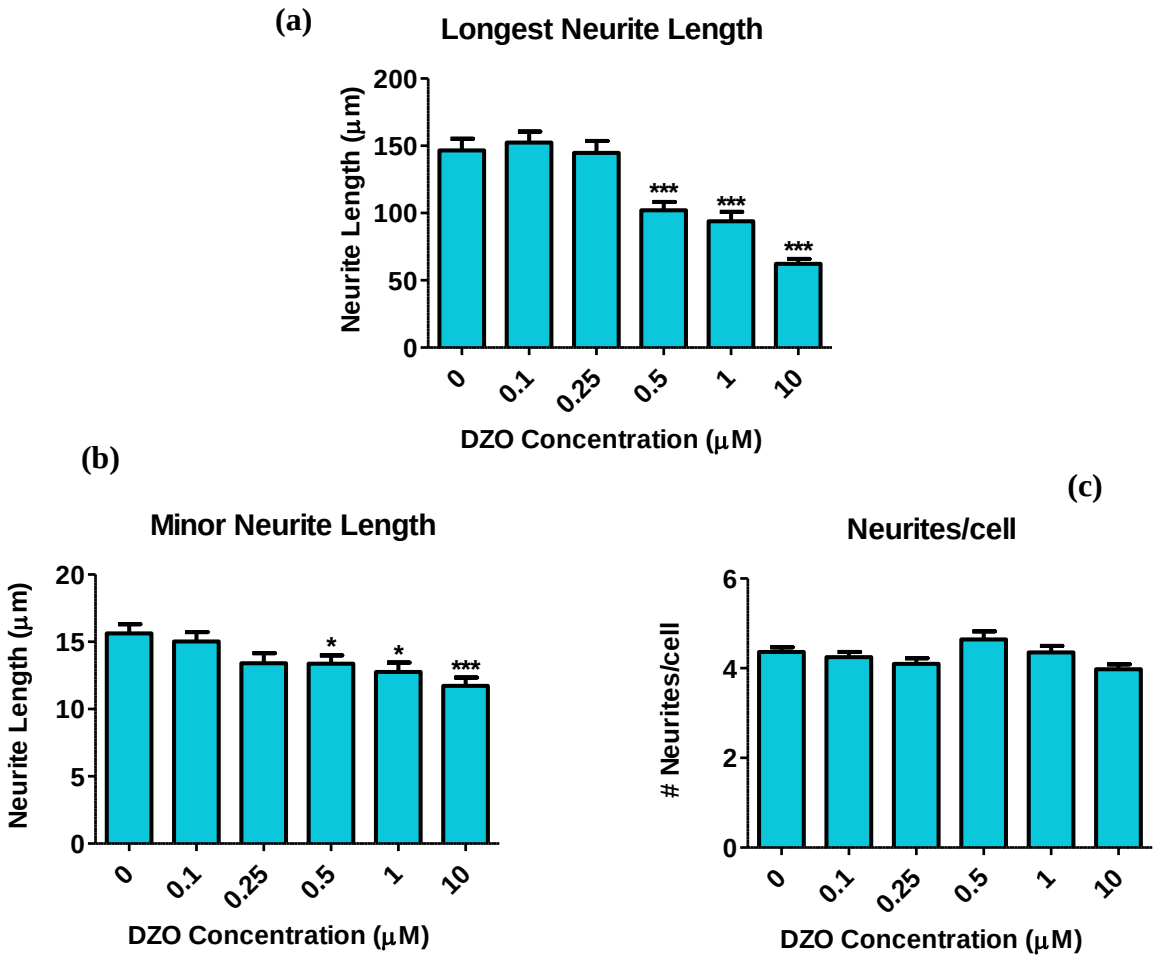


Figure 3.3. DZO inhibits neurite outgrowth in primary hippocampal neurons. Hippocampal neurons are plated in Neurobasal/FBS (10%) media for 2 h before being washed and switched to ACM for 24 h. The neurons are treated with varying concentrations of DZO for 24 h. Results are expressed as mean ± SEM of 90-120 cells derived from at least three independent experiments. **P<0.01 Statistically different from control (One-way ANOVA, Bonferroni's post-hoc test).

Treatment Concentration (μM)	**DZ** Viability (% Control)	**DZO** Viability (% Control)
0	100	100
1	108.93 ± 6.14	103.94 ± 2.82
10	100.14 ± 2.81	104.83 ± 0.81

Table 3.1. DZ and DZO do not compromise cell viability of hippocampal neurons.
Hippocampal neurons are plated in Neurobasal A/FBS (10%) media for 2 h before being washed and switched to ACM for 24 h. The neurons are treated with varying concentrations of DZ or DZO for 24 h, after which cell viability is assessed by the MTT assay. Results are expressed as mean percent control ± SEM of data from at least three independent experiments in which treatments were performed in triplicate.

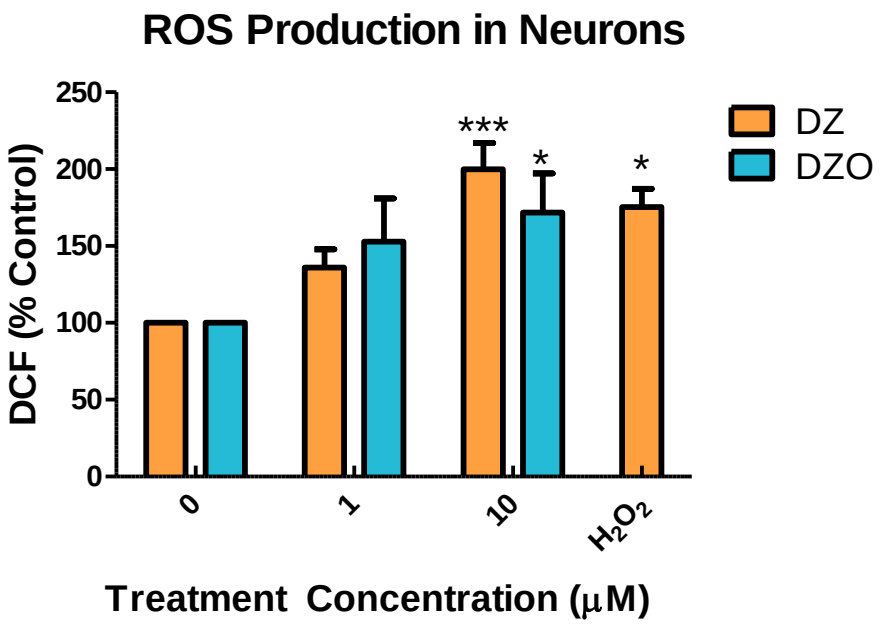


Figure 3.4. DZ and DZO increase ROS production in hippocampal neurons. Hippocampal neurons are plated in Neurobasal/FBS (10%) media for 2 h before being washed and switched to ACM for 47 h. The neurons are probed with 10 µM H2DCF-DA for 30 min. The probe is washed out and neurons are treated with DZ or DZO for 1 h. Results are expressed as mean ± SEM of data obtained from at least three independent experiments in which treatments were performed in triplicate. **P<0.01, ***P<0.001 Statistically different from control (One-way ANOVA, Bonferroni's post-hoc test).

Treatment	Longest Neurite Length (µm)	Minor Neurite Length (µm)	Neurites/cell
Control	142.37 ± 5.84	17.12 ± 0.64	4.55 ± 0.11
Mel	132.20 ± 10.79	18.05 ± 1.17	4.34 ± 0.17
PBN	116.50 ± 6.547	16.85 ± 0.75	4.64 ± 0.16
GSH	131.40 ± 16.18	15.04 ± 1.19	5.48 ± 0.55
10 DZ	68.97 ± 5.923***	14.45 ± 0.66	4.30 ± 0.12
DZ + Mel	122.20 ± 10.15	17.29 ± 0.99	4.50 ± 0.17
DZ + PBN	105.00 ± 9.645	17.17 ± 0.94	4.80 ± 0.22
DZ + GSH	157.77 ± 12.15	16.35 ± 1.07	4.49 ± 0.19
10 DZO	86.79 ± 5.894***	12.54 ± 0.60***	4.20 ± 0.13
DZO + Mel	133.10 ± 9.417	13.84 ± 0.68*	4.44 ± 0.16
DZO + PBN	121.80 ± 7.779	14.51 ± 0.65*	4.57 ± 0.18
DZO + GSH	163.87 ± 13.57	13.77 ± 1.20	5.20 ± 0.32

Table 3.2. Antioxidants prevent inhibition of neurite outgrowth caused by DZ and DZO in primary hippocampal neurons. After 24 h in ACM, neurons were pre-treated for 3 h with either melatonin (Mel; 200 µM), PBN (100 µM), or glutathione ethyl-ester (GSH; 2.5 mM). After 3 h, antioxidants were washed out and neurons were treated for 24 h with 10 µM DZ or DZO. Results are expressed as mean ± SEM of 60-90 cells obtained from three independent experiments. *P<0.05, **P<0.01, ***P<0.001 Statistically different from control (One-way ANOVA, Bonferroni's post-hoc test).

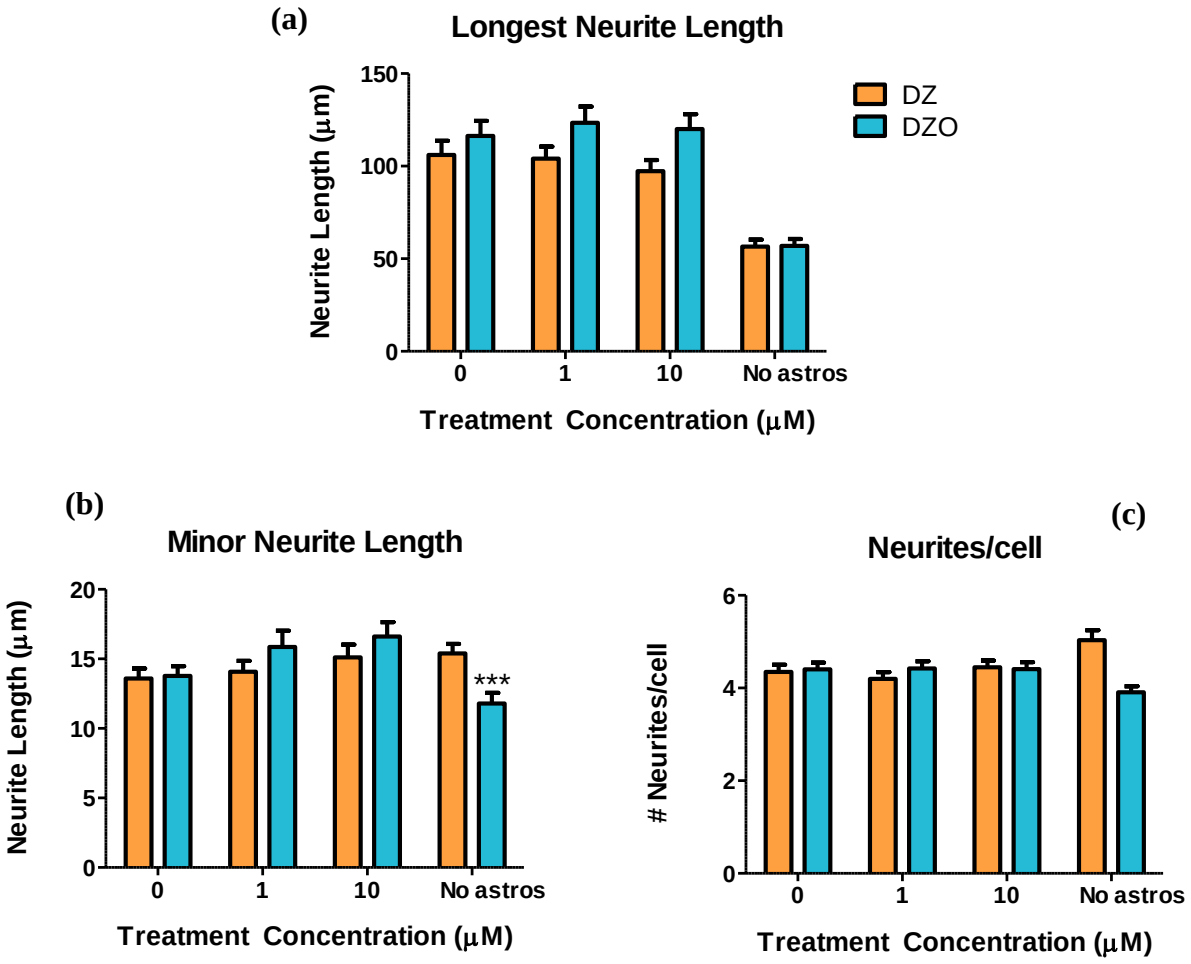


Figure 3.5. Astrocytes protect against DZ- and DZO-induced inhibition of neurite outgrowth. After 24 h in ACM, neurons were placed into astrocyte-containing wells and treated with DZ or DZO. Results are expressed as mean ± SEM of at least 90 cells obtained from at least three independent experiments. **P<0.01 Statistically different from control (One-way ANOVA, Bonferroni's post-hoc test).

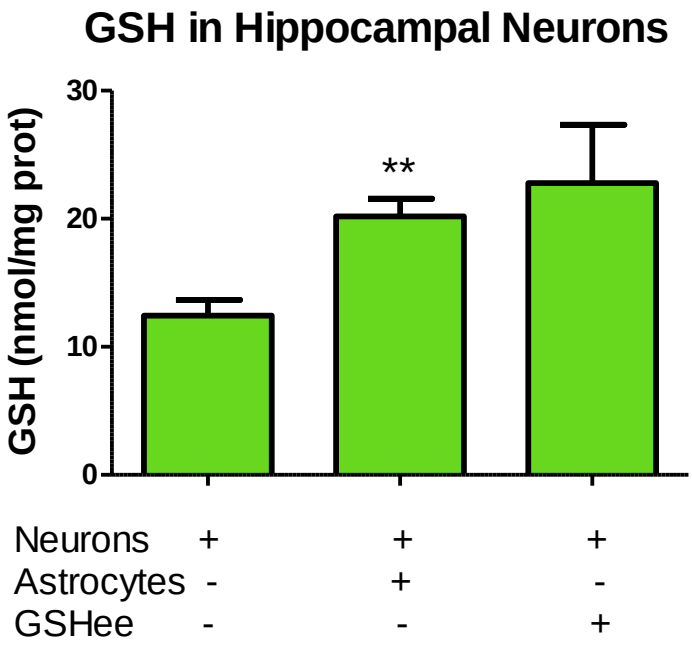


Figure 3.6. Astrocytes increase intracellular GSH levels in hippocampal neurons. After 24 h in ACM, neurons were either: left alone in ACM, placed with astrocyte-containing inserts, or pre-treated with GSHee (2.5 mM). After 24 h, neurons were washed and collected and assayed for total intracellular GSH. Results are expressed as GSH (nmol/mg protein) ± SEM, obtained from three independent experiments. **P<0.01 Statistically different from neurons alone (Student's t-test).

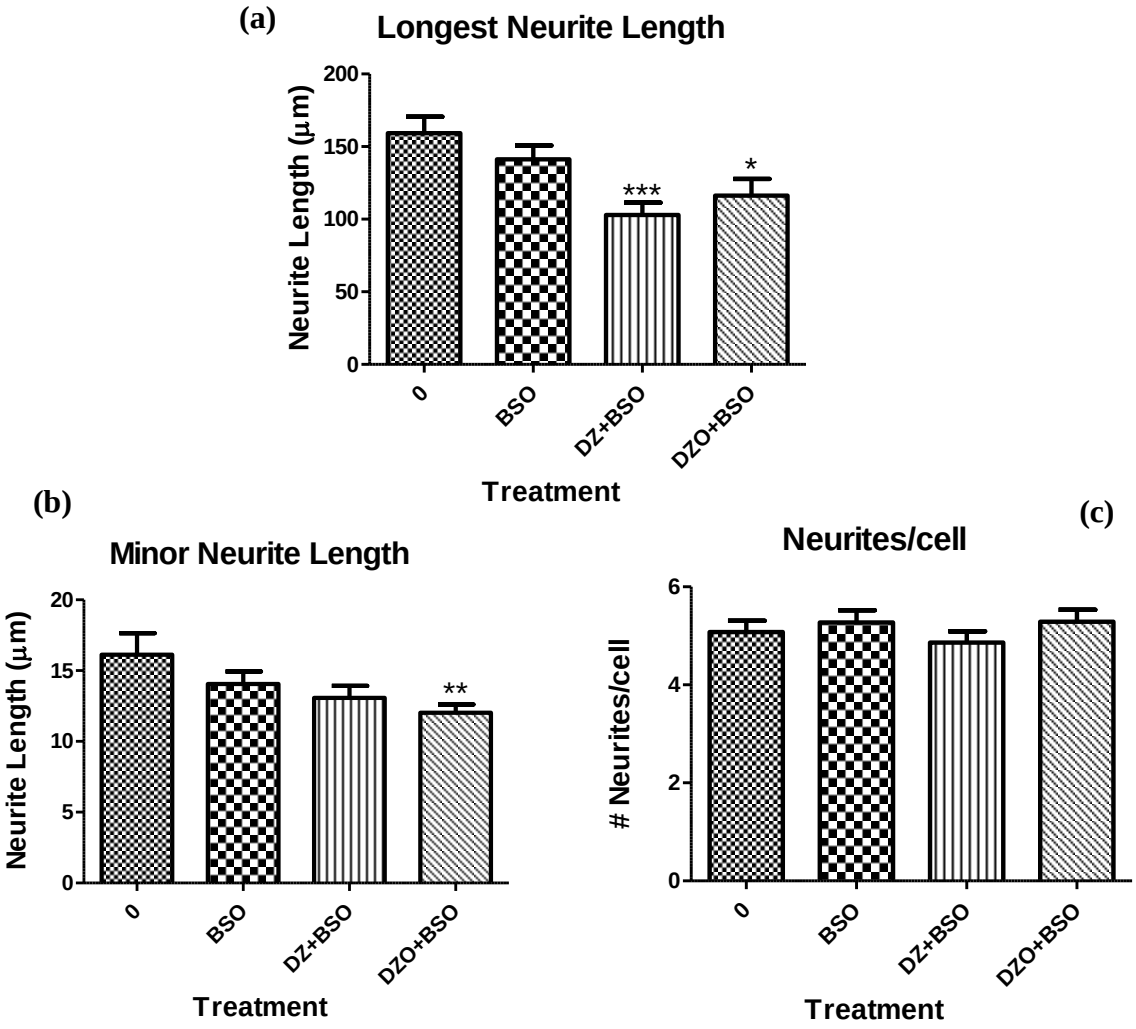


Figure 3.7. Astrocytes depleted of GSH do not protect against DZ- and DZO-induced inhibition of neurite outgrowth. After 24 h in ACM, neurons were placed with astrocytes previously treated with BSO for 24 h. Astrocytes were depleted of GSH by previous treatment with 25 µM BSO for 24 h. Results are expressed as mean ± SEM of at least 90 cells obtained from three independent experiments. (One-way ANOVA and Bonferroni's post-hoc test).

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

Organophosphorus insecticides (OPs) are a ubiquitous class of environmental contaminants to which humans are regularly exposed. Concern has grown over their neurotoxic effects and, specifically, their ability to adversely affect neurodevelopment (Costa, 2006; Eaton et al., 2008). In conjunction with existing literature, this study further demonstrates the developmental neurotoxic effects of such compounds, including diazinon (DZ) and its oxygen metabolite, diazoxon (DZO). More interestingly, though, the work discussed here presents several novel findings: the ability for DZ and DZO to adversely affect astrocyte functioning and impair neurite outgrowth in this manner; the role of oxidative stress in modulating neurite outgrowth, as well as protein levels of a vital neurite-promoting protein, fibronectin; and the role of astrocyte-derived glutathione in modulating DZ and DZO-induced developmental neurotoxicity.

In this study, the effects of DZ and DZO on hippocampal neuronal growth were evaluated. The hippocampus is a part of the brain responsible for learning and memory functions, including the ability to store long-term memories. Epidemiology studies in humans have linked early OP exposure to neurodevelopmental deficits like ADHD and lowered I.Q. (Bouchard et al., 2010; Rauh et al., 2011; Rohlman et al., 2011), both of which include learning deficits and, plausibly, hippocampal function. While these associations have been made, the mechanisms by which these deficiencies occur remains unclear.

For the first time, we show that DZ and DZO can impair astrocyte function and subsequently impair neurite outgrowth by doing so. This finding offers a novel mechanism for OP neurotoxicity; previous studies have primarily focused on the ability for these compounds to directly affect neuron growth and function (Flaskos et al., 2007; Sidiropoulou et al., 2009a; Slotkin et al., 2008). Furthermore, we demonstrate that these effects on neurite outgrowth are modulated by the ability for DZ and DZO to elicit oxidative stress in astrocytes. Interestingly, this DZ/DZO-induced oxidative stress also affects levels of the pro-neuritogenic protein, fibronectin, which is produced and secreted by astrocytes. These findings underscore the

interconnected nature of cellular communication in the brain, and highlight its importance in investigating mechanisms of environmental neurotoxicants.

This model provides a useful and unique tool to better mimic an *in vivo* scenario while maintaining the control and usefulness of *in vitro* models. In this way, this model can be used to explore mechanisms of a variety of developmental neurotoxicants whose mechanisms are not well understood. The *in vitro* model used in this project utilizes a co-culture model of two different cell types that interact with and affect each other, while simultaneously allowing for the separate modulation of each cell type. Here we used astrocytes and neuronal cells because of their vital roles in neurodevelopment. The interactions between other cell-types can be explored with this model, as well. Other glial cell types, for example, that are increasingly recognized as having dynamic roles in brain function and disease. Microglia, the resident macrophages of the CNS, have been shown to play important roles in neuro-inflammation and disease (Röhl and Sievers, 2005; Rostworowski et al., 1997). Similarly, effects on oligodendrocytes, the myelin-forming glial cells of the brain, are of interest in neurodegenerative diseases like multiple sclerosis (Baumann and Pham-Dinh, 2001). Potential effects of neurotoxicants on these cells have only just begun to be explored.

The 'Rodney Dangerfield' of the neurological world, astrocytes didn't always get the respect they deserved for their vital role in brain function and development. Here, we underscore their importance in modulating neuronal growth and development. While we demonstrate that DZ and DZO directly impair neuritogenesis in hippocampal neurons, the presence of astrocytes in this model prevents the observed inhibition of neurite outgrowth due to the OPs. Additionally, astrocyte-derived glutathione (GSH) was determined to be an essential factor in this protective effect of astrocytes against DZ- and DZO-induced inhibition of neurite outgrowth. These results not only continue to highlight the neurotrophic functions of astrocytes, but also the importance of fully-functioning antioxidant defense systems; in the brain, a strong armor against ROS relies heavily on properly functioning astrocytes. These defense systems, such as that of GSH and its related enzymes, are vital in protecting against cellular oxidative stress that results from various environmental stressors. When these fortifications are depleted or deficient, the cell is particularly vulnerable to oxidative stress and the damage it may cause to anything from DNA synthesis to morphological impairments. These deficiencies may come in the form of previous

and/or chronic insult, as well as genetic abnormalities, such as genetic polymorphisms in antioxidant defense genes (i.e. GCL).

To better address the cumulative effects of various oxidative stress-eliciting compounds, future studies should focus on chronic exposure, or exposure to mixtures of compounds. In light of evidence demonstrating an increased vulnerability to the ubiquitous effects of increased oxidative stress, it is likely that an initial exposure to certain compounds could adversely affect the body's response to further insult by subsequent exposures. It is worth noting that little is known about the toxicity to mixtures of these compounds—which is often how we are exposed to them in reality. For example, the work on individual compounds and their effects (such as that done here on diazinon) is valuable and could be used in models created to better understand the effects of a mixture of OPs. Further study is also warranted to assess the neurotoxic effects of OPs in conjunction with other contaminants to which we are co-exposed: dietary exposure to the potential endocrine-disrupting phthalates and plasticizers, for example, or to neurotoxic flame-retardants (i.e. PBDEs) in household dust.

From a public health perspective, the work here provides strong evidence pointing to the developmental neurotoxicity of compounds that are pervasive in the environment. The parent compound (DZ), as well as the oxon metabolite (DZO) both exert neurotoxic effects governed by oxidative stress. The oxon form has long been the focus of past neurotoxicology studies on OPs since it has a much greater ability to inhibit AChE and cause acute toxicity in this manner (Costa, 2006). The work presented here demonstrates the ability for the parent compound, in the absence of significant AChE inhibition, to inhibit neurite outgrowth and elicit oxidative stress in important brain cell types (neurons and astrocytes). These effects occur at levels that more closely reflect relevant human exposures; Buratti and colleagues have determined that OP concentrations of 10 μM and below are more appropriate for extrapolation to *in vivo* conditions (Buratti et al., 2002; 2003).

The results of this work also show that DZO impairs astrocyte ability to foster neurite outgrowth at lower levels than that of the parent compound, albeit at levels that slightly inhibit AChE in astrocytes. The effects of the oxon metabolite remains relevant since recent evidence shows that we are directly exposed to DZO in the environment (Armstrong et al., 2013). Additionally, newer work suggests that localized CYP-mediated metabolism contributes to the neurotoxic potential of these compounds (Khokhar and Tyndale, 2012; Miksys and Tyndale,

2013). The latter information becomes important when looking at the fact that, in this study, both the parent compound and the oxon form significantly inhibit AChE in hippocampal neurons when exposed directly to the compounds. The ability for the parent OP compound to be metabolized to the oxon form in the brain can be altered by genetics, as well as other environmental exposures, and has been shown to modulate the neurotoxicity associated with these compounds (Khokhar and Tyndale, 2012). Future work that elucidates and incorporates such information will help to improve physiologically-based pharmacokinetic models (PBPK) of these compounds, ultimately providing invaluable information for assessing OP safety and toxicity.

The work completed for this dissertation will, hopefully, contribute towards improved regulation on the global use of OPs. Continuing to demonstrate the developmental neurotoxic potential of OPs also provides additional impetus to develop safer alternatives to these compounds and to pesticide use in general.

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