Mechanisms of Neuronal Death Induced by Environmental Toxicants in Murine Cortical Culture

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MECHANISMS OF NEURONAL DEATH INDUCED BY ENVIRONMENTAL TOXICANTS
IN MURINE CORTICAL CULTURE

By

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ABSTRACT

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This study was directed at examining the neurotoxic mechanisms of several classes of environmental toxicants implicated in neurodegenerative disease. Primary cortical cultures were exposed to organophosphorus pesticides, heavy metals and the cyanobacterial toxin, beta-N-methylamino-L-alanine (BMAA). Several components relating to neuronal injury were assessed in each study and novel aspects are described.

The main action of organophosphorous insecticides is generally believed to be the inhibition of acetylcholinesterase. However, these compounds are now recognized to inhibit many other enzymes and cause neuronal death through a variety of mechanisms. I found that exposure to chlorpyrifos or diazinon caused concentration-dependent neurotoxicity that could not be attributed to acetylcholinesterase inhibition. Chlorpyrifos exposure increased extracellular glutamate and induced a diffuse nuclear staining characteristic of necrosis; the toxicity was sensitive to ionotropic glutamate receptor antagonists. Diazinon toxicity was blocked by caspase inhibitors. Additionally, diazinon induced punctuate chromatin staining characteristic of apoptosis. These results represent two distinct, novel mechanisms of organophosphorous neurotoxicity.

Heavy metals are ubiquitous in the environment and are of significant health concern worldwide. Exposure to lead, iron, mercurials (inorganic mercury, methylmercury, or thimerosal, i.e. ethylmercury) or other heavy metals is implicated as a risk factor for neurodegenerative disease. I found that the toxicity of these metals may be enhanced when interacting with chelators used to treat metal intoxication. As well, my studies describe a new role for mercury-induced oxidative stress as a cytoprotective signal to enhance glutathione levels. My data also suggests an obligate role for MRP1 in the detoxification of methylmercury.

Neurodegenerative diseases likely involve complex interactions between genetic predisposition and multiple environmental factors. My final study tested the interaction of the methylmercury and BMAA. Importantly, concentrations of BMAA that caused no toxicity by themselves potentiated methyl mercury toxicity. BMAA plus methylmercury, at concentrations that had no effect by themselves, depleted cellular glutathione. The combined toxicity was attenuated by glutathione monoethyl ester, and the free radical scavenger, trolox, but not by the NMDA receptor antagonist, MK-801. The results indicate a synergistic neurotoxic interaction targeting the cellular redox state. This finding may have implications for neurodegenerative disease caused by environmental toxicant exposure.

ACKNOWLEDGMENTS

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CHAPTER I

GENERAL INTRODUCTION

General Introduction

Given the prevalence of toxicants such as mercury and pesticides in our environment human exposure to these chemicals is ubiquitous. Such exposures can cause damage to the developing and mature nervous system. It has been hypothesized that damage from neurotoxicant exposure may account for subsequent onset and/or development of neurodegenerative diseases such as Alzheimer's Disease (AD), amyotrophic lateral sclerosis (ALS) and Parkinson's Disease (PD). Of specific interest to this thesis, exposures to mercury, organophosphorous pesticides (OPs) and/or a cyanobacterial toxin known as beta-N-methylamino-L-alanine (BMAA), have each been implicated in development of one or more of the aforementioned neurodegenerative diseases. The aim of this thesis is to identify mechanisms of toxicity effecting primary cortical cells for several environmental toxicants in order to better understand how these toxicants may trigger the onset of neurodegenerative disease. Further, upon recognizing that many toxicants have overlapping cellular targets in the primary cortical neurons and glia, the aim expands to demonstrate synergistic toxicity exerted by combined insults.

The data presented here identify that mercury compounds cause a redox imbalance concurrent with a loss of the major antioxidant to the central nervous system (i.e. glutathione) which is in agreement with previous reports. Exposure to BMAA also led to a decrease in glutathione (GSH). However, OP pesticides did not disrupt GSH levels. These data are of particular relevance because of the historical implications of oxidative stress and disruptions of glutathione observed in patients afflicted with neurodegenerative disease(s). The present studies provide novel information describing how mercury and BMAA disrupt GSH cycling between cortical astrocytes and neurons. The following sections discuss these data and the implications in detail, as well as the synergistic toxicity observed when subtoxic methylmercury and BMAA insults are combined. There is considerable evidence to suggest that certain populations (e.g. fish consumers) are likely to be exposed to both methylmercury and BMAA. However, the greater

value of these data is to validate further investigation of combined toxicity. While the present data strongly support a role for redox disruptions and glutathione deficiency in the development of neurodegenerative diseases following exposure to mercury and/or BMAA, it is important and interesting to note that evidence from the enclosed studies of OPs suggests a distinct mechanism that does not involve glutathione depletion.

Environmental Toxins

Pesticides

Organophosphorous (OP) insecticides are the most commonly used pesticides in worldwide agriculture and industry. OP pesticides are favored over their organochlorine predecessors, such as dichlorodiphenyltrichloroethane (commonly known as DDT), because OP compounds are more potent and they are designed to chemically degrade more rapidly when in soil and groundwater or when exposed to direct sunlight (for review: Eaton et al., 2008). Despite this rapid degradation, however, significant quantities are often detected in food and water from rural sources (Ivey, 1979; Fenske et al., 2002). Chlorpyrifos (CPF, sold under the tradenames LORSBAN and DURSBAN) and diazinon (DZN, sold under the tradenames Spectracide and Alfatox) are among the most widely used for their greater stability and persistence (Mileson et al., 1998). It is estimated that CPF itself is responsible for tens of thousands of deaths each year throughout the world (Gunnell et al., 2007), though it should be noted that the majority of these deaths are by self-intoxication meant to cause suicide (Eddleston et al., 2005). Also, CPF has been documented as less likely to cause OP-induced delayed polyneuropathy (OPIDP, Richardson et al., 1993). However, because OP pesticides are lethal, their sale for residential use has been banned in the US. Despite this regulation, there remain significant stores sold prior to the ban, and many people continue to be exposed (Eskenazi et al., 1999; Whyatt and Barr, 2001; Whyatt et al., 2007; Adgate et al., 2009).

The concern for the acute neurotoxicity of these pesticides is largely due to the designed mechanism of action of OP agents. These pesticides act much like their well-known weaponized partner compounds such as sarin, VX and tabun which are more potent. The primary action of OP compounds, including CPF and DZN is to irreversibly inhibit acetylcholinesterase (AChE) enzymes throughout the nervous system (Mileson et al., 1998). Such inhibition leads to accumulation of the neurotransmitter, acetylcholine (ACh), and subsequent overstimulation of cholinergic receptors at synapses in insects and humans, as well as at the neuromuscular junction. Such overstimulation, in humans, is clearly responsible for the cholinergic symptoms observed with acute exposure; these include seizures, pupil & bronchial constriction, convulsions and muscular fasciculations. Ultimately, these muscle spasms lead to cessation of diaphragmatic movement causing death by respiratory failure (Mileson et al., 1998; Giordano et al., 2007). Importantly, and of great interest to this thesis, is that OP agents are known to inhibit many serine hydrolase enzymes (Casida and Quistad, 2005) and exert toxicity through several mechanisms (Li and Casida, 1998). Also noteworthy, is that OP pesticides such as CPF and DZN do not produce significant inhibition of AChE until after being metabolized to their -oxon forms, chlorpyrifos-oxon (CPO) and diazinon-oxon (DZO), respectively (Sams et al., 2004; Foxenberg et al., 2007). Despite this, CPF and DZN have been demonstrated to be neurotoxic without forming their –oxons (Jett et al., 1999). This is significant because there is wide variability of detectable CPO in the blood of individuals exposed to CPF; the variability is likely due to polymorphisms that have been identified in activating and deactivating enzymes that convert CPF to CPO, and is relevant to many other OP pesticides (Eyer et al., 2009). For many years now, the central hypothesis for even low-level, chronic OP exposure leading to neurological dysfunctions has been aimed at understanding the anticholinesterase activity of these compounds, however, interest has now shifted to investigating the neurotoxic mechanisms independent of AChE inhibition (Garcia et al., 2001; Costa, 2006).

Chlorpyrifos and other OPs have been shown to induce production of reactive oxygen species (ROS) leading to oxidative stress. Acute, chronic and even developmental exposures to OPs have been clearly demonstrated to cause oxidative stress in animals, humans and *in vitro* studies (Banerjee et al., 2001; Abou-Donia et al., 2003; Kovacic, 2003). Also, non-lethal OP exposure can decrease the antioxidant capacity (e.g. expression of antioxidant-related enzymes) in rat blood, liver and lungs, and in human brain (Akhgari et al., 2003; Bebe and Panemangalore, 2003; Sharma et al., 2005). Measures of lipid peroxidation have also indicated an oxidative mechanism of OP-induced toxicity (Verma and Srivastava, 2001; Oncu et al., 2002).

Also supporting an oxidative stress mechanism is evidence of protection against OP toxicity with glutathione or antioxidant supplementation with an exogenous antioxidant, N-tert-butyl-alpha-phenylnitrone (PBN), or catalase (Gultekin et al., 2001; Gupta et al., 2001). In cerebellar granular neurons (CGNs) derived from mice lacking a glutathione-synthesizing enzyme, gamma-glutamate cysteinyl-synthetase (GCL), exposure to CPF or DZN caused heightened oxidative stress, lipid peroxidation and neurotoxicity over CGNs taken from wild-type mice (Giordano et al., 2007). This study also provided evidence for increased disulfide (i.e. oxidized) glutathione levels without any change to total glutathione. All of these effects were blocked by supplementation with exogenous, cell-permeant glutathione ethylester or catalase in the media. Interestingly, the ACh receptor antagonists, atropine and mecamylamine, lacked protection, and the ACh receptor agonist, carbachol, was not toxic. Taken together, these data suggest that the toxicity of these OPs does not involve a cholinergic mechanism.

OPs are toxic to a multitude of cell types including non-excitable cells in brain and other tissues. OPs induce apoptosis in oligodendrocyte precursors, cytotoxic T lymphocytes and cell lines including PC12 and C6 cells (Qiao et al., 2001; Caughlan et al., 2004; Saulsbury et al., 2009). CPF-induced cellular effects include an inhibition of cAMP production by adenylyl cyclase (AC) and an elevation of phosphorylated-CREB (pCREB, Huff et al., 1994; Schuh et al., 2002) . Elevated pCREB can be induced by CPF, CPO and the non-toxic CPF metabolite, 3,5,6-

trichloropyridinol (TCP). Since TCP increases pCREB and blocks cAMP production, a cholinergic mechanism is again, unlikely to be involved. Decreased AC activity is thought to be responsible for additional inhibition of DNA synthesis and alterations to protein expression that have been reported (Garcia et al., 2001). These genomic effects may be of particular interest when considering neurodevelopmental maladies induced by OP exposure.

Embryonic and neonatal exposure to CPF is known to produce neurobehavioral abnormalities as well as locomotor, coordinate and cognitive deficits (Guillette et al., 1998; Terry et al., 2003). Childhood exposure induces neurobehavioral changes as well (Ricceri et al., 2006). Initial studies into the mechanisms of OP-induced developmental effects found that OP agents inhibit neural replication, differentiation and can stunt neurite outgrowth (Li and Casida, 1998; Das and Barone, 1999). CPF is also toxic to oligodendrocytes and their precursors and decreases myelin associated glycoprotein expression in these cells (Garcia et al., 2002). Reduced myelination may account for many of the neurotransmission defects and the improper circuit connectivity observed later in animals exposed to OPs during early periods of development. Gross morphology alterations also result from perinatal OP exposures (Campbell et al., 1997; Roy et al., 2004). Further alterations to expression of genes fundamentally involved in neurotransmission have been detected by genetic microarray studies (Slotkin et al., 2006; Slotkin et al., 2010). These findings include downregulation of cholinergic receptors and mixed changes to glutamatergic signaling proteins (i.e. NMDA and AMPA receptors, among others). Many of these effects are suggested to be independent of AChE inhibition. The potential mechanisms of OP neurotoxicity are not mutually exclusive and are likely to act in concert to produce neurological deficits.

Heavy Metals

Heavy metals are ubiquitous in the environment and are thus of significant concern. Acute exposures to high levels of Pb, Hg, Fe, Mn or other heavy metals are known to cause neurological damage and death. Additionally, heavy metal exposure perinatally or during childhood is well-documented to cause severe deleterious neurological effects on the developing nervous system. Further, chronic exposures to heavy metals, be they dietary, environmental (i.e. inhalation of pollutants) or occupational, have been implicated as major risk factors for neurodegenerative diseases (Mutter et al., 2004; Monnet-Tschudi et al., 2006; Praline et al., 2007). The following sections will introduce the historical and present knowledge surrounding several heavy metals of specific concern; incidence of exposure, neurodegenerative implications and cytotoxic mechanisms will be described primarily for mercurials. Following these discussions, the practice of chelation therapy as well as its implications for human health will be introduced and potential reasons for concern in this field will be addressed.

Mercury

Mercury is perhaps the most frequently encountered of the heavy metals because of its multiple routes of human exposure. The most common forms of concern for humans are elemental mercury (Hg), ethylmercury (EtHg) and methylmercury (MeHg). Elemental mercury is usually inhaled as mercury vapor either in an occupational setting or having been released from amalgam dental restorations (Patterson et al., 1985; Bates et al., 2004). Once the vapor is inhaled, the mercury easily passes into the blood stream and is rapidly ionized to form divalent (i.e. inorganic) mercury (Bjorkman et al., 2007). The inorganic mercury then accumulates in the kidneys, liver and brain tissues (Hursh et al., 1976). Ethylmercury is also presented to humans in the antifungal agent and bacteriocide, thimerosal. Thimerosal has been and continues to be used

to preserve consumer products such as nasal sprays, dermal topical agents (e.g. tattoo inks), ophthalmic and otic products and is infamous for its inclusion in multi-use vials of vaccines (Elferink, 1999; Burbacher et al., 2005). Administration of ethylmercury-containing vaccines has been of specific health concern for two main reasons. First, direct intramuscular injection of ethylmercury gives the toxicant privileged access to the blood stream where it can readily cross the blood-brain barrier (BBB) (Mahaffey et al., 2004; Burbacher et al., 2005). Second, many vaccinations are administered perinatally and during the early years of childhood, allowing ethylmercury to potentially exert damaging effects on the developing nervous system. The latter concern led to a prolonged series of studies debating whether thimerosal exposure increased the risk of, or perhaps directly triggered autism in vaccinated children (Mutter et al., 2004). This hypothesis has since been rejected by numerous epidemiological studies, and has been highly criticized for dissuading many concerned parents from providing their children with appropriate vaccinations (for review: Geier et al., 2007).

For numerous reasons, the most concerning form of mercury is methylmercury (MeHg). MeHg is produced by aquatic bacterial methylation of elemental or inorganic mercury (Clarkson, 1997). The bacterial MeHg subsequently accumulates in fish and shellfish throughout the food web (Mahaffey et al., 2004). Many of these mercury-contaminated species are later consumed by humans. MeHg is readily absorbed in the GI tract and crosses the BBB easily (Kerper et al., 1992). While there is evidence that much of the MeHg is eliminated from the brain quickly, some of the MeHg is demethylated by astrocytes in the brain, leaving a mix of MeHg and inorganic mercury to accumulate with multiple dietary doses (Mahaffey et al., 2004). Studies in primates and case studies in humans have demonstrated that a single episode of dietary MeHg leaves relatively little organic mercury but significant amounts of inorganic mercury detectable in brain many months after the exposure (Friberg and Mottet, 1989; Burbacher et al., 2005; Bjorkman et al., 2007).

Acute toxicity of methylmercury is also of great concern due to its use in manufacturing and industry. Several incidents of widespread mercury poisoning due to industrial accidents have occurred in recent decades. Severe neurological damage and many lethalities were reported following a major release of methylmercury into Minamata Bay, Japan (Harada, 1995; Tsuda et al., 2009). Methylmercury-contaminated seed-grain farmed and consumed in Iraq produced similar reports in that population (Myers et al., 2000).

Interestingly, many decades of research aimed at understanding the mechanisms of mercury toxicity have yielded limited conclusive results. There are no ideal methods of treating mercury intoxication. The data presented in this thesis strongly suggest that augmenting the endogenous glutathione system while addressing some other aspects of mercury-induced toxicity should be further investigated.

Mercurial Toxicity Mechanisms

Mercurials have long been documented to damage the CNS in humans and several animal species. There have been many studies investigating the manner(s) by which mercury compromises neuronal health and function. Major findings for mercurials are several-fold. Acute and chronic mercury exposure can disrupt neuronal signaling through actions on various ion channels (e.g. L-type calcium channels; Atchison and Hare, 1994). Mercurials can cause swelling of astrocytes and compromise the integrity of the plasma membrane (Aschner, 2000). Intracellular release of calcium stores and zinc freed from proteins lead to cation dishomeostasis (Haase et al., 2009; Kawanai et al., 2009). Membrane transport of excitatory amino acids, such as glutamate, has also been reported to be disrupted by the presence of mercurials (Allen et al., 2001; Fonfria et al., 2005) Common pathways for mercury induced toxicity also include the induction of oxidative stress through increased production of reactive oxygen species (ROS) and mitochondrial dysfunction (Ali et al., 1992). Glutathione depletion also occurs and has been posited to result from the mercurial-induced oxidative stress (Yee and Choi, 1996; Sanfeliu et al.,

2001). Recently, the focus has been on these oxidative mechanisms and how they relate to protein oxidation/misfolding and neurodegenerative disease (Rooney, 2007).

The focus on mercurial disruption of redox status has naturally turned to investigating glutathione, the major endogenous antioxidant in the mammalian nervous system. Support for a role of glutathione is abundant. It has been suggested that lower glutathione content accounts for the increased sensitivity of cerebellar granule neurons (CGNs) relative to cortical cells (Kaur et al., 2007; Wang et al., 2009). In addition, glutathione precursors or exogenous glutathione are neuroprotective against mercurial insult when supplemented *in vitro* and have been reported to be effective in a rodent model (Fujiyama et al., 1994; Kaur et al., 2006; Toyama et al., 2007). These findings have largely been suggested to be due to glutathione acting as an antioxidant. However, some reports have suggested that glutathione directly conjugates with mercurials via disulfide bonds for which mercury has a great affinity (Rooney, 2007). Importantly, studies have suggested antioxidant supplementation to be neuroprotective as well (Gasso et al., 2001; Shanker and Aschner, 2003). However, as demonstrated and later discussed in this thesis, very few studies have demonstrated gross cyto-protection against mercurial insults with antioxidants despite the ability of these antioxidants to reduce mercurial-induced oxidative stress (Shanker and Aschner, 2003; Kaur et al., 2010). In fact, my work provides evidence that reducing an early mercurial-induced oxidative response can potentiate subsequent neuronal death. These data suggest that an early oxidative burst signals functional upregulation of a cytoprotective mechanism involving system x_c^-. This allows for enhanced uptake of the glutathione precursor, cystine, and allows for greater detoxification of mercury in the cell. Supporting this hypothesis is a recent study targeting a means of augmenting this system to provide cytoprotection against mercurial insult to hepatocytes and a neuronal cell line (Toyama et al., 2011).

Implications in Neurodegenerative Disease

There exists strong evidence for mercury in the etiology of several neurodegenerative diseases. Many case studies and several epidemiological studies have identified elevated blood levels of mercury in patients with AD, PD and ALS (Hock et al., 1998; Praline et al., 2007). Cerebrospinal fluid samples and post-mortem brain tissue analyses have also confirmed the presence of mercury among such patient populations (Perry and Hodges, 1999; Riedl and Honey, 2008). There is a positive correlation between total mercury exposure and risk for development of ALS (Monnet-Tschudi et al., 2006); ALS-like symptoms have been observed in people 3 years after acute exposure to mercury (Johnson and Atchison, 2009). Recently, a study in the a SOD1 G93A rat model of ALS found that chronic exposure to methylmercury hastens the onset and progression of gait abnormalities and hind-limb weakness in these animals (Johnson et al., 2011).

Lead

Lead exposure has also been implicated in AD, PD, and ALS. Environmental exposure to lead is widespread. Occupational or industrial use of the metal puts factory workers at high risk (Nagatsu and Sawada, 2005; Kokayi et al., 2006). Many homes contain lead-based paints on their walls and window frames. While presence of lead in homes is of particular concern for childhood exposure, often through ingestion, adults are also exposed to lead dust created by opening and closing of these lead painted windows. Inhalation was also hazardous in the days of leaded fuel supplies for automobiles and many consumer products make use of lead-containing components. Thus, human exposure to lead is quite common (for review: Monnet-Tschudi et al., 2006).

Lead is known to accumulate in bones, marrow, kidneys, liver and brain tissues (Kamel et al., 2008). Its developmental effects are thoroughly documented and exposure of children to high levels of lead has been greatly reduced. However, even low and moderate concentrations

are now being recognized as capable of producing subtle neurological deficits in children, and perhaps contributing as a risk factor for later development of neurological disease. Chronic, low-level exposure is considered a risk factor for AD as it can lead to increased amyloid-precursor protein (APP) expression later elevating levels of amyloid beta (Monnet-Tschudi et al., 2006). As seen with mercurials, lead has also been reported to cause oxidative stress and to induce reactive gliosis (an indicator of inflammatory response in the CNS) *in vitro* and *in vivo*.

Chelation

The current therapeutic strategy for treating heavy metal intoxication whereby body burden of these metals is elevated beyond safe levels, is to administer one of several chelator compounds that are designed to, bind, mobilize and eventually remove the exogenous metal(s) from the body. In allopathic medical practice, this is reasonably effective at reducing the body burden of the offending metal by choosing an appropriate chelator, though this is not without complications. That is, these chelators are known to have limited specificity for heavy metals and can themselves disrupt endogenous metal ion homeostasis (e.g. Zn+, Mg+, etc) causing toxicity, illness and even leading to injury to several organ systems (Flora and Pachauri, 2010). An additional concern with the use of chelation is that the desired mobilization can lead to redistribution of the heavy metal to tissues, such as brain, where damage can be more severe (Andersen and Aaseth, 2002; Andersen, 2004).

A more concerning use of chelators is that in the field of alternative medical practice for "chelation therapy." The principle concept behind the use of chelators is similar to that in allopathic applications in that chelation therapy is meant to remove toxic exogenous metals from the body that may be responsible for some health conditions (e.g. heart disease, AD, autism) (Barnes et al., 2008). However, the employment of these chelators is less descriminate; the patient need not have knowingly been intoxicated by any specific heavy metal and little

consideration is made for which chelating compound is used. The most common in this practice is EDTA. The danger with this strategy is that without knowing which metal(s) to target, these chelators can be damaging. Evidence in this thesis suggests that each chelator can alter the inherent neurotoxic properties of each heavy metal. In some cases the chelator successfully reduces the metal-induced neurotoxicity, but more often, the chelator may exacerbate the insult. Given that chelation therapy is used to treat upwards of 66,000 people in a continuously growing industry, potentially damaging effects may not be of small consequence (Barnes et al., 2008).

beta-N-methylamino-L-alanine

Initial interest in beta-N-methylamino-L-alanine (BMAA) as a neurotoxin came when it was isolated from cycad seeds regularly consumed by the endogenous Chamorro people of Guam (Steele and Guzman, 1987). These native residents of the Guam and those living throughout Western New Guinea have a higher incidence of ALS. The Guamanian form of this disease is actually described as amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) or locally as Lytico-Bodig. Afflicted individuals experience symptoms of ALS, PD and AD (Duncan, 1992; Cruz-Aguado and Shaw, 2009; Johnson and Atchison, 2009). The incidence of this disease was extremely high among the Chamorro people during the 1950's and 1960's. Many studies have aimed to identify the cause of this increased prevalence and several environmental neurotoxic hypotheses have emerged. Interestingly, all such theories put forth have involved dietary intake of a neurotoxic chemical, many of them shown to be present in the flour derived from cycad seeds (Duncan et al., 1992; Tabata et al., 2008). One well supported hypothesis is that insufficient dietary calcium and magnesium coupled with high intake of aluminum and manganese disrupts endogenous metal homeostasis and leads to subsequent neurodegeneration (Yoshimasu et al., 1980; Durlach et al., 1997). Cycasin, zinc, sterol glucosides are among the cycad-based neurotoxic chemical theories (Spencer et al., 1991; Duncan

et al., 1992; Tabata et al., 2008), but consumption of BMAA, the neurotoxic non-protein amino acid present in cyanobacteria within cycad seeds, is perhaps the most interesting and has garnered waves of interest through the decades of ALS-PDC research (Cox and Sacks, 2002; Duncan and Marini, 2006).

Strengthening the BMAA hypothesis, injection of BMAA directly into brains of monkeys induced motor deficits similar to PD (Spencer et al., 1987). However, the hypothesis lost momentum in 1990 when researchers realized that the cycad seeds were washed prior to consumption and that this washing was sufficient to eliminate much of the BMAA suggesting that unrealistic amounts of cycad flour would need to be consumed to reach toxic concentrations of BMAA (Duncan et al., 1990).

In 2003, another potential source of BMAA was reported. Fruit bats feed on cycad seeds which are symbiotically inhabited with cyanobacteria. BMAA bioaccumulates from the cyanobacteria into the cycad seeds, then further up the food chain into the fruit bats. Indeed, the fruit bats contain incredibly high levels of BMAA (Banack and Cox, 2003). Interestingly, Chamorros first began eating fruit bats as a regular component of their diet during the 1940's when Guam was occupied by Japanese troops (Banack et al., 2006). In subsequent decades, the fruit bat population dwindled correlating well with the timing of the subsequent decline in disease occurrence.

While this story is intriguing, there is limited evidence for a causal relationship between BMAA and ALS-PDC. Several animal model studies have been unable to demonstrate neurotoxicity even at high levels of intake (for review: Karamyan and Speth, 2008). The early studies in monkey (and subsequent findings in chicks) required incredibly high exposure levels. However, despite the conflicting results with BMAA as a neurotoxin in various models, some additional findings in recent years have provided rationale for considerable concern beyond explaining Guamanian ALS-PDC.

There are now many reports of cyanobacteria throughout the world that contain BMAA. Accordingly, several of the ecosystems harboring such bacteria have displayed the propensity of BMAA to bioaccumulate throughout the food web (Esterhuizen and Downing, 2008; Brand et al., 2010; Jonasson et al., 2010). This suggests that BMAA may be of concern for much broader population, potentially across the globe. Further support for such concern comes from studies that have reported detection of BMAA in brain samples from AD patients in Canada in addition to ALS-PDC patients of Guam (Murch et al., 2004b). Importantly, BMAA was detected in neither patients that had died of non-neurodegenerative disease, nor those that had died of the genetic neurodegenerative disease known as Huntington's Disease. However, subsequent studies performed by other research groups have failed to detect BMAA in these populations (Montine et al., 2005; Snyder et al., 2009), a fact that must be considered when assessing the BMAA hypothesis of ALS-PDC.

Mechanisms of BMAA Toxicity

BMAA has long been known to produce excitotoxic neuronal death in culture and *in vivo* models. Early studies in primates injected with BMAA demonstrated that BMAA could lead to neurological maladies similar to ALS and PD (Spencer et al., 1987). However, very high concentrations were required to produce damage. Our lab and others have also found that high concentrations into the low millimolar range of BMAA are required *in vitro* to cause significant acute toxicity. We have since characterized the excitotoxic and several other distinct mechanisms by which BMAA produces injury in cultured cortical neurons.

Specifically, electrophysiological studies from our lab have demonstrated that BMAA directly activates NMDA receptors as a glutamate analogue (Lobner et al., 2007). Further investigation has unveiled that BMAA can also induce glutamate release, cause activation of mGluRs, inhibit cystine uptake and decrease glutathione availability. Evidence suggesting these mechanisms contribute to BMAA-induced toxicity come from studies demonstrating that BMAA

inhibits system x_c^- mediated 14C-cystine uptake (i.e. Na-independent and CPG-sensitive) and that

BMAA drives glutamate release by this transporter. Additionally, BMAA toxicity is partially

sensitive to blockade by the NMDA receptor antagonist, MK-801. Once the primary NMDAR-

mediated component is blocked, administration of either the mGluR-5 antagonist, MPEP, or the

antioxidant, trolox can further block toxicity. Almost complete blockade of BMAA-induced

toxicity can be accomplished by combining all three agents, MK-801, MPEP and trolox (Liu et

al., 2009). These mechanisms are illustrated together in FIGURE 1.1.

Others have provided evidence for additional mechanisms of BMAA toxicity. In 2006,

Vyas et al., showed that BMAA can directly activate calcium-permeable AMPA/kainate channels

on spinal motor neurons (Rao et al., 2006). Another hypothesis is that BMAA may be

misincorporated into proteins while being synthesized. Such improper translation leads to an

unfolded protein response and eventually results in apoptotic cell death. While evidence for this

occurring with levodopa, this has not yet been published to occur with BMAA (Rodgers et al.,

2006). Further research is necessary to understand to what degree each mechanism contributes to

BMAA toxicity.
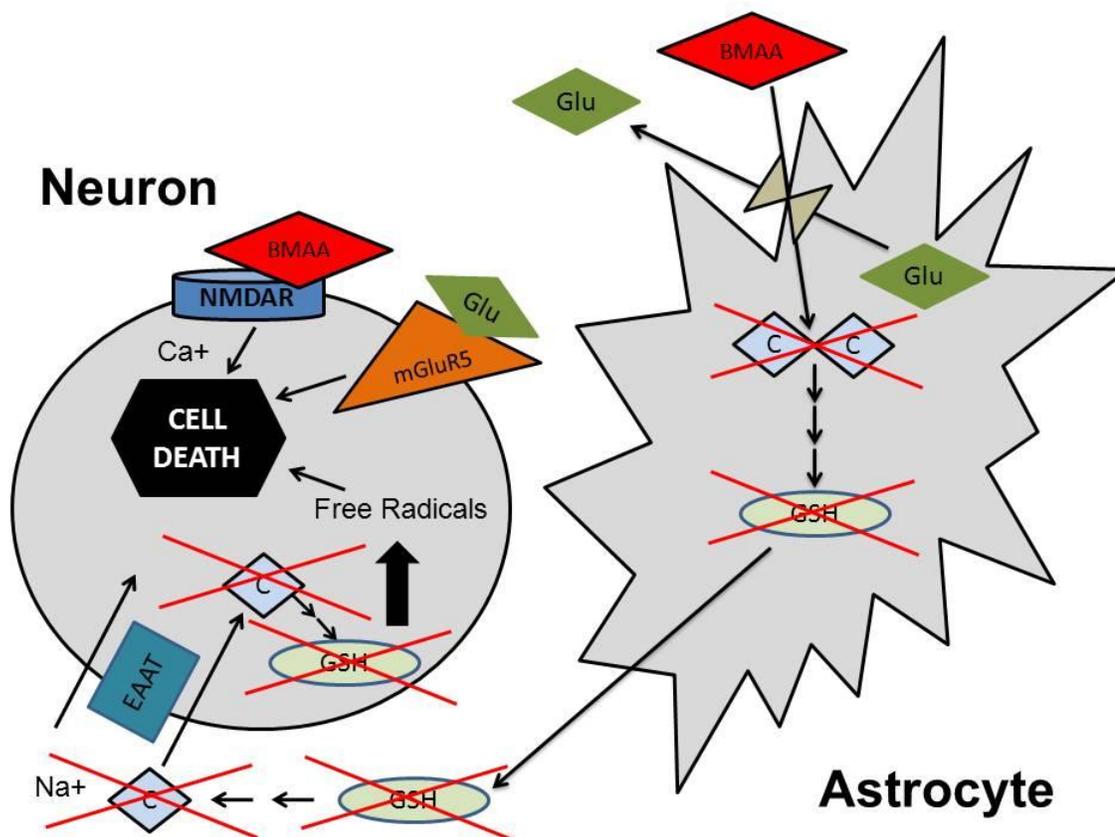
FIGURE 1.1. Mechanisms of BMAA neurotoxicity.

This illustration demonstrates the toxic effects of BMAA on primary cortical cultures. BMAA can directly activate NMDA receptors (NMDARs), or be transported via system x_c^- on astrocytes causing elevated glutamate release and decreased cystine uptake. The released glutamate (Glu) can then activate mGluR 5 receptors or NMDARs. Decreased astrocytic uptake of cystine (C-C) leads to a depletion of glutathione (GSH) and limited availability of GSH for neurons. With compromised glutathione cycling, the neurons become particularly vulnerable to free radicals (e.g. oxidative stress). Activation of the glutamatergic receptors leads to cation dishomeostasis and an imbalance in the redox status of the cells eventually leading to neuronal death.

Combined Environmental Insults

Considering the ubiquity of neurotoxicants in the environment, the risk of human exposure to multiple toxic agents is increasingly high. While much progress in understanding neurotoxic compounds in the environment has been made by studying these toxicants Cui isolation, this does not accurately represent the exposure conditions people are likely to encounter. Expecting any person to suffer exposure to only a single neurotoxic environmental factor is a drastic oversimplification. A more complicated scenario of concurrent or overlapping temporal exposures to multiple neurotoxicants is increasingly likely. Genetic variation may render some individuals particularly susceptible to damage by certain toxic insults. The complexity involved in studying multiple neurotoxicants and the possible interactions with individual genetic variation increases very quickly. However, it is imperative to consider that multiple risk factors likely converge to produce a disease state. Recent studies have begun targeting these interactions. My work describes a potential interaction of the environmental neurotoxicants methylmercury and BMAA.

Relevant Cell Physiology

Oxidative Stress and Glutathione

A common factor for all of the neurodegenerative diseases and neurotoxicants discussed in this thesis is the elevated production of reactive oxygen species (ROS) such as superoxide ($^{\cdot}O_2$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) (Di Monte et al., 2002; Cui et al., 2004). These oxidative insults can be generated from disruption of the mitochondrial electron transport chain or by induction of NADPH oxidase activity (Adam-Vizi, 2005; Brennan et al., 2009). There is much evidence, historically, for mitochondrial involvement. However, it has recently been posited that the data for mitochondria-generated free radicals are weak and may be

artifactual in light of the recent findings implicating NADPH oxidase as the primary source of damaging ROS (i.e. $^{.}O_2$). Because NADPH oxidase is localized to the plasma membrane, increased activity and production of $^{.}O_2$ can readily damage the plasma membrane (i.e. via lipid peroxidation) and redox sensitive proteins. Such damage can compromise membrane integrity leading to numerous deleterious effects (e.g. morphology changes, loss of chemical and ion gradients, cell swelling, and cell death). Protein oxidation can lead to improper cross-bridge formation and subsequent misfolding/malfunction resulting in further cellular dishomeostasis. Further investigation is necessary to definitively determine the contribution of each potential source of oxidative stress in each neuropathology, though cell death in many systems has been clearly documented as a result of severe or prolonged oxidative stress.

Normal cellular respiration and activity are known to produce ROS and the cell has endogenous mechanisms to detoxify these radicals. In the mammalian nervous system, glutathione (and related enzymes) is the primary antioxidant, though catalase and superoxide dismutase also contribute to the reduction of endogenous ROS produced. Glutathione (composed of glutamate, cysteine and glycine) is most widely recognized for its capacity as an antioxidant (AO); specifically, GSH detoxifies superoxide in a reaction catalyzed by glutathione peroxidase forming disulfide/oxidized glutathione (GSSG) and water. As ROS formation increases, the ratio of GSSG:GSH also increases. These reactions are accomplished by redox changes at the sulfhydryl group of the cysteine residue of GSH (Dringen and Hirrlinger, 2003). The rate of *de novo* synthesis of GSH is limited by the rate of cystine uptake (Sagara et al., 1993). Importantly, neurons of the CNS have limited capacity for cystine uptake, and are reliant primarily on astrocytes for the provision of (reduced) cysteine and cysteinylglycine (CysGly) for neuronal glutathione production (Dringen et al., 1999). Astrocytes express high levels of excitatory amino acid transporters also capable of importing cystine as well as a recently described cystine-glutamate antiporter known as system x_c^- (Dringen, 2000; Lewerenz et al., 2006). Once

cyst(e)ine enters the cell, it is combined with glutamate and then glycine in a multistep enzymatic process (Dringen et al., 2000).

Upon synthesis, GSH plays a role in a number of important cellular/physiological processes illustrated in FIGURE 1.2. The simplest possibility is direct export of GSH to the extracellular media, a process accomplished via the ATP-dependent multidrug resistant protein (MRP) 1 (Hirrlinger and Dringen, 2005; Minich et al., 2006). Intracellular GSH plays a role in the reduction of damaging ROS, forming GSSG (Shih et al., 2006). This GSSG is also a substrate for MRP1-mediated export. A third role for GSH is one that has historically been characterized in cancer research. GSH acts to detoxify xenobiotics, such as chemotherapeutics, often through sulfhydryl interaction catalyzed by a class of enzymes known as glutathione-S-transferases (GSTs; Johnson et al., 1993; Lu and Shervington, 2008). The glutathione conjugates (GSx) are also exported by MRP1 (de Jong et al., 2001).

In the extracellular space, glutathione-based molecules (i.e. GSH, GSSG and GSx) are each handled differently. GSH can remain as GSH or be broken down into its constituent amino acids or glutamate and the precursor cysteinylglycine. CysGly can be imported by astroycytes or neurons via gamma-glutamyltranspeptidase (Dringen et al., 1997). GSSG is reduced to its GSH components by an enzyme, GSH reductase. The GSx conjugates may or may not be broken down for recycling but may be mobilized to the renal system for elimination from the body (Franco et al., 2007b).

System x_c^-

The importance of regulating extracellular signaling molecules, particularly neurotransmitters and excitatory amino acids cannot be overstated. To this end, many enzymes and membrane-spanning transporters function to degrade or remove such signaling molecules from the synaptic, perisynaptic and extracellular spaces thereby facilitating precise cell-cell

communication. Of specific interest to neurotoxicity and degeneration is the regulation of the primary excitatory neurotransmitter, glutamate. Overstimulation of ionotropic and in some instances metabotropic glutamate receptors can lead to a calcium-mediated excitotoxicity (i.e. necrotic or apoptotic cell death) which involves oxidative stress. While sodium-dependent excitatory amino acid transporters (EAATs) are localized to astrocyte processes surrounding synapses to efficiently remove glutamate and prevent spillover into the extrasynaptic space, another important mechanism known as system x_c^- serves to regulate both glutamate and cystine concentration gradients by exchanging one intracellular glutamate for one extracellular cystine. As discussed previously, the imported cystine is primarily used for glutathione synthesis. The exported glutamate has been posited to play a role in activating perisynaptic type 2/3 metabotropic glutamate receptors on neurons thereby exerting inhibitory tone on neurotransmission at a particular synapse (Xi et al., 2002; Moran et al., 2005). Additionally, it is known that system x_c^- is primarily localized to extrasynaptic glial membranes with minimal expression in neurons (Lewerenz et al., 2006) and that AMPARs and NR2B-containing NMDARs are also expressed in extrasynaptic locations. Thus, system x_c^- may exert powerful influence over extracellular glutamate concentrations, synaptic transmission, and glutathione availability (Xi et al., 2002; Seib et al., 2011).

Importantly, modulation of system x_c^- activity can be damaging by both increased and decreased activity. Increased activity releases glutamate and subsequently activates glutamatergic receptors; inhibition of the antiporter decreases the amount of cystine imported and therefore decreases glutathione synthesis/availability which leads to an oxidative stress-mediated neuronal death (Murphy and Baraban, 1990; Chung et al., 2005). Since the antiporter has approximately the same affinity for import of cystine or glutamate, the direction of transport is largely determined by the concentration gradients of these amino acids. If extracellular glutamate is elevated, cystine uptake can be greatly diminished causing oxidative stress-mediate toxicity referred to as 'oxytosis' (Murphy and Baraban, 1990; Ratan et al., 1994). This effect was initially

characterized in immature cortical cultures not yet expressing ionic glutamate receptors limiting their sensitivity to excitotoxic stimulation by glutamate. However, it is likely that elevated glutamate concentrations act by these multiple mechanisms. Not surprisingly, provision of glutathione by astrocytes overexpressing system x_c^- can be protective against oxidative stress in neurons (Shih et al., 2006).

Diminished activity of system x_c^- has been hypothesized to contribute to schizophrenia as well as addiction (Baker et al., 2008; Kau et al., 2008; Gu et al., 2010). Glutamate released from activated microglia via system x_c^- was first shown to be excitotoxic to cerebellar granular neurons (CGNs) and oligodendrocytes (Piani and Fontana, 1994). It was shown that microglia also release ROS that can inhibit glutamate transporters on surrounding cells thereby prolonging and exacerbating the insult (Domercq et al., 2007). More recent research posited a role for increased expression of system x_c^- in reactive astrocytes increasing excitotoxicity in a culture model of hypoxia (Fogal et al., 2007; Jackman et al., 2010). Another interesting aspect of system x_c^- as a mediator of excitotoxic injury is found in the field of glioma research. Glial tumor cells express greatly elevated levels of xCT, the catalytic subunit (i.e. functional component) of the cystine-glutamate antiporter, and are capable of drastically elevating extracellular glutamate levels in the presence of physiological concentrations of cystine (Ye and Sontheimer, 1998). This elevated glutamate induces seizures and can lead to excitotoxic neuronal injury which promotes tumor growth by overcoming the spatial limitations imposed by the skull (Sontheimer, 2008). The increased ability of glioma cells to import cystine is also to their great advantage as it provides an abundance of the glutathione precursor which confers chemoresistance in these cells and protects against oxidative insults (Lu and Shervington, 2008).

There has been minimal investigation for a role of system x_c^- in the pathogenesis of neurodegenerative disease. Though, it has been implicated in neurodegenerative disease for its ability to elevate extracellular glutamate concentrations to excitotoxic levels (Piani and Fontana, 1994; Qin et al., 2006). In addition, diminished activity of the antiporter may decrease

glutathione available to reduce the oxidative stress as seen in several neurodegenerative diseases (Franco et al., 2007b). There is some indication it may be involved in the etiology of AD. xCT is suggested to be upregulated in brains of AD patients. Microglia of patients with Alzheimer's Disease express high levels of system x_c^-, particularly surrounding amyloid plaques, perhaps due to the inflammation associated with the disease. Rodents injected with amyloid beta or overexpressing mutant amyloid precursor protein (APP) also show this microglial characteristic (Qin et al., 2006). It is not clear whether the increased system x_c^- activity contributes to pathogenesis by elevating glutamate or if the upregulation serves to protect by providing cystine for glutathione synthesis. Administration of the antioxidants alpha-tocopherol and selegeline prolongs survival in AD patients and retards progression of cognitive impairments, though other antioxidant trials have not had these effects (Sano et al., 1997; Adair et al., 2001). N-acetylcysteine (NAC) is a sulfhydryl-containting direct antioxidant and serves as a cystine pro-drug capable of driving system x_c^- activity. NAC administration failed to significantly improve clinical AD measures in a study of probable AD patients (Adair et al., 2001). It is not clear whether a non-significant trend of beneficial effects was due to NAC's ability to drive cystine-glutamate exchange; further study is required.

Few studies have investigated involvement of system x_c^- in the pathogenesis of PD. Though, in a popular rodent PD model using 6-hydroxydopamine lesion of the nigrostriatal pathway, knockout of xCT protected substantia nigra neurons from the insult. The protection observed in the knockout animals was due to lowered extracellular glutamate concentrations in the striatum (Massie et al., 2008; Massie et al., 2011). This study suggests that increased system x_c^- activity due to inflammation characteristic of this neurodegenerative disease may contribute to pathogenesis. There have been no studies to assess function or expression of system x_c^- in parkinsonian tissues.

The story is similar for studies investigating the role of the antiporter in ALS. While there are no direct studies of system x_c^- in ALS model systems, there have been studies

investigating the effects of NAC supplementation in these models. Similar to the AD studies, it is not clear whether the effects of NAC can be attributed to increased drive of the antiporter. NAC reduces oxidative insult and prevents ROS-induced decreases in glutamate uptake in neuroblastoma cells expressing mutant G93A-SOD1, a gene associated with familial ALS (Beretta et al., 2003). There is conflicting *in vivo* evidence in G93A-SOD1 rodent models demonstrating no effect or delayed onset and prolonged survival with NAC administration (Jaarsma et al., 1998; Andreassen et al., 2000). In a clinical trial, no benefit was observed with NAC treatment, however, there was an indication that NAC effected ALS subpopulations differently. That is, patients whose symptoms had started in their limbs exhibited prolonged survival with NAC relative to those patients receiving placebo. Patients with a bulbar-onset of disease, which is known to be at least weakly benefited with the antiglutamatergic drug (i.e. antiexcitotoxic) riluzole, had decreased survival in this trial (Louwerse et al., 1995). This suggests that system x_c^- may play differential roles between these forms of ALS.

ROS can act as a signal to upregulate antioxidant-related cell components including system x_c^- and GSH synthesizing enzymes. The nuclear factor (erythroid derived-2)-like 2 antioxidant response element (NRF2-ARE) pathway is suggested to be responsible for this effect (Johnson et al., 2008; Vargas and Johnson, 2009). Endogenous and exogenous oxidative stress is known to upregulate mRNA and functional activity of xCT, the catalytic subunit of system x_c^- (Dun et al., 2006; Mysona et al., 2009). Also, cells with increased xCT expression are resistant to oxidative insult suggesting this upregulatory system is a protective mechanism that may be a viable therapeutic target in neurodegenerative diseases.
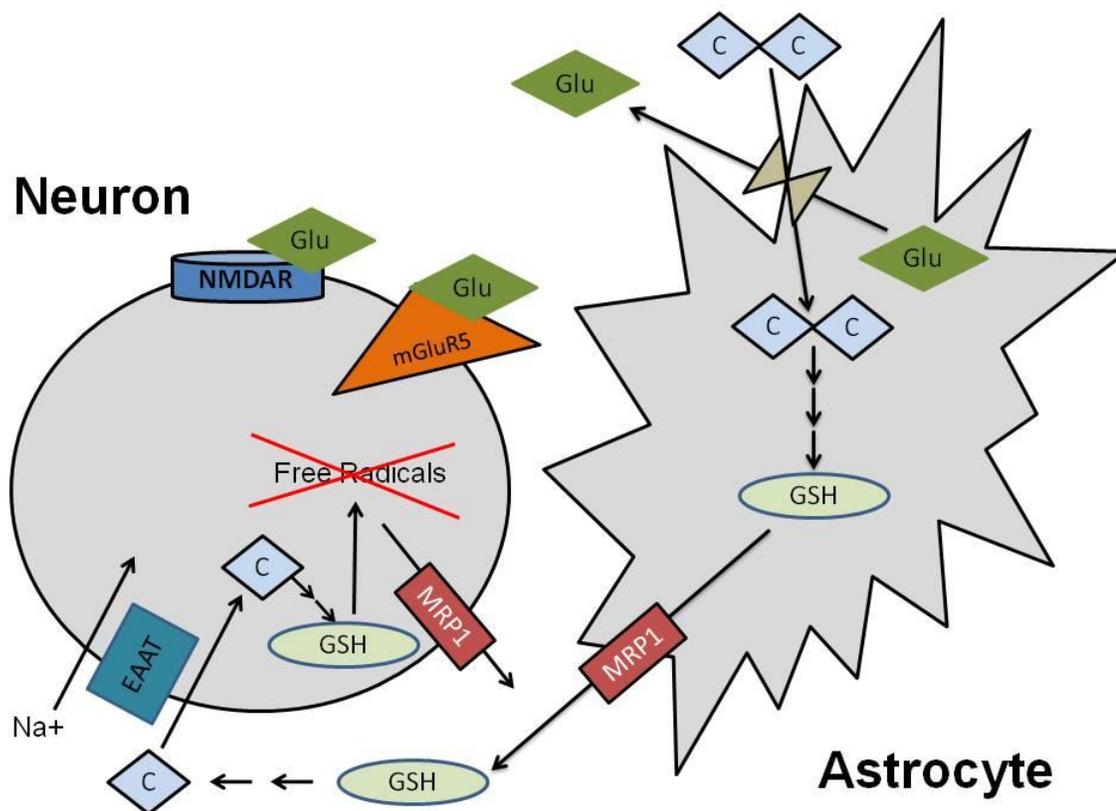
FIGURE 1.2. Glutathione cycling between astrocytes and neurons.

In the mammalian nervous system neurons rely on astrocytes for de novo synthesis of glutathione

and glutathione precursor availability. Astrocytes have enhanced ability to import cystine, the

rate-limiting substrate for de novo glutathione synthesis. In mixed cortical cultures cystine is all

but exclusively taken up by astrocytes via system x_c- whereby one extracellular molecule of

cystine is imported in exchange for one intracellular molecule of glutamate (Glu) being released.

The imported cystine is then reduced to two cysteine (C) molecules which are enzymatically

converted to glutathione (GSH). The newly synthesized glutathione can be utilized for a

multitude of tasks or released to the extracellular media. Upon export, the glutathione is

degraded by extracellular enzymes into its precursor molecules which can then be imported via

excitatory amino acid transporters (EAATs) and utilized by nearby neurons. While the uses for

glutathione are detailed in the text, the prominent attributes of glutathione include its ability to

reduce damaging free radicals to non-toxic substances and to detoxify the cell of exogenous

agents by conjugation and export via multidrug resistance proteins (MRPs). The provision of

glutathione precursors by astrocytes is crucial for neuronal health because of the limited capacity

of neurons to import cystine. Importantly, cystine-glutamate exchange via system x_c- not only

facilitates glutathione synthesis but also contributes to neuronal activity and health by regulating

glutamate levels in the extracellular/extrasynaptic media. The glutamate released by x_c- can

activate extrasynaptic NMDA receptors (NMDARs) and metabotropic glutamate receptors

(mGluRs). Figure abbreviations: C = cysteine, C-C = cystine, GSH = glutathione, GLU =

glutamate NMDAR = NMDA receptor, mGluR = metabotropic glutamate receptor, MRP1 =

multi-drug resistance protein 1, EAATs = excitatory amino acid transporters

General Methods

This brief section will introduce the most important aspects of the model system used for the work in this thesis. First is an overview of the cortical culture method used, the advantages and the limitations. Also described here is the assay for gross cell death by measuring the release of lactate dehydrogenase.

Cortical Culture

The cortical culture method used in these studies is based on a long-standing, well-characterized model. Specifically, cortical hemispheres are dissected from embryonic mice (gestational day 15), manually triturated, trypsinized and then plated on 24-well plates coated with poly-D-lysine and laminin in Eagle's minimum essential media containing 5% (v/v) heat-inactivated horse serum and 5% (v/v) fetal bovine serum, 2mM glutamine and glucose (total 21 mM). The cultures are then maintained in 5% CO_2 incubators at 37° C for ~2 weeks. The cultures are prepared at ~8 cortical hemispheres per plate and contain ~250,000 neurons with an additional ~100,000 glial cells growing to confluency over the weeks in culture. The process results in a system that recapitulates, in a simplified form, many key components of the intact cortex while allowing precise, relatively high-throughput manipulations of the local environment. Additionally, minimal change to the dissection and/or culturing method allows enrichment of one cell type over another. By adding the mitotic inhibitor, cytosine arabinocide, to the growth media 2-3 days after plating we can produce nearly-pure neuronal cultures. Delaying the dissection until postnatal day 1 produces pure-glial cultures as the neurons become sensitive to our culture conditions and die. While growing these cell types in relative isolation introduces some confounds by stepping further from the physiological setting, it allows certain inferences about neurons or glia to be made that would otherwise be masked.

Experiments in this system are generally performed on cultures at 13-15 days in vitro (DIV) for a variety of reasons. The delay of ~2 weeks from the day of dissection allows the cells to differentiate and mature; neurons grow and prune neurites and dendrites producing functional synapses and glia extend processes to encompass and support this neuronal/synaptic activity as they do *in vivo*. A photomicrograph of a typical mature culture exhibiting these features can be seen in FIGURE 1.3. Neuronal and glial expression of important signaling proteins more closely matches the in vivo profile after this 2 week incubation (e.g. Neurons express NMDA receptors at ~DIV 12).

Unfortunately, this system also has some inherent drawbacks. Considering that the cultures are only viable under these conditions for ~20 days, the ability to perform any long-term study is severely limited if not altogether preempted by this fact. Also, despite the formation of synapses that mimic intact neuronal networks, there is neither specific nor consistent circuitry formed in the cortical cultures used. Thus, conclusions regarding such pathways are better suited to cortical slice culture or other systems and cannot be drawn directly from studies in this culture system. Therefore, all of the studies in this thesis were performed to completion within DIV 13-15, with the majority being on the order of 6-24 hours duration from onset to data acquisition. These short exposure periods often require relatively high concentrations of the various pharmacological agents employed. By nature of the limitations of these methods, it must be assumed that acute, high-intensity exposure conditions somehow mimic the chronic pathophysiological conditions experienced in vivo so as to allow meaningful inferences and interpretations to be made from studies performed in this valuable system.
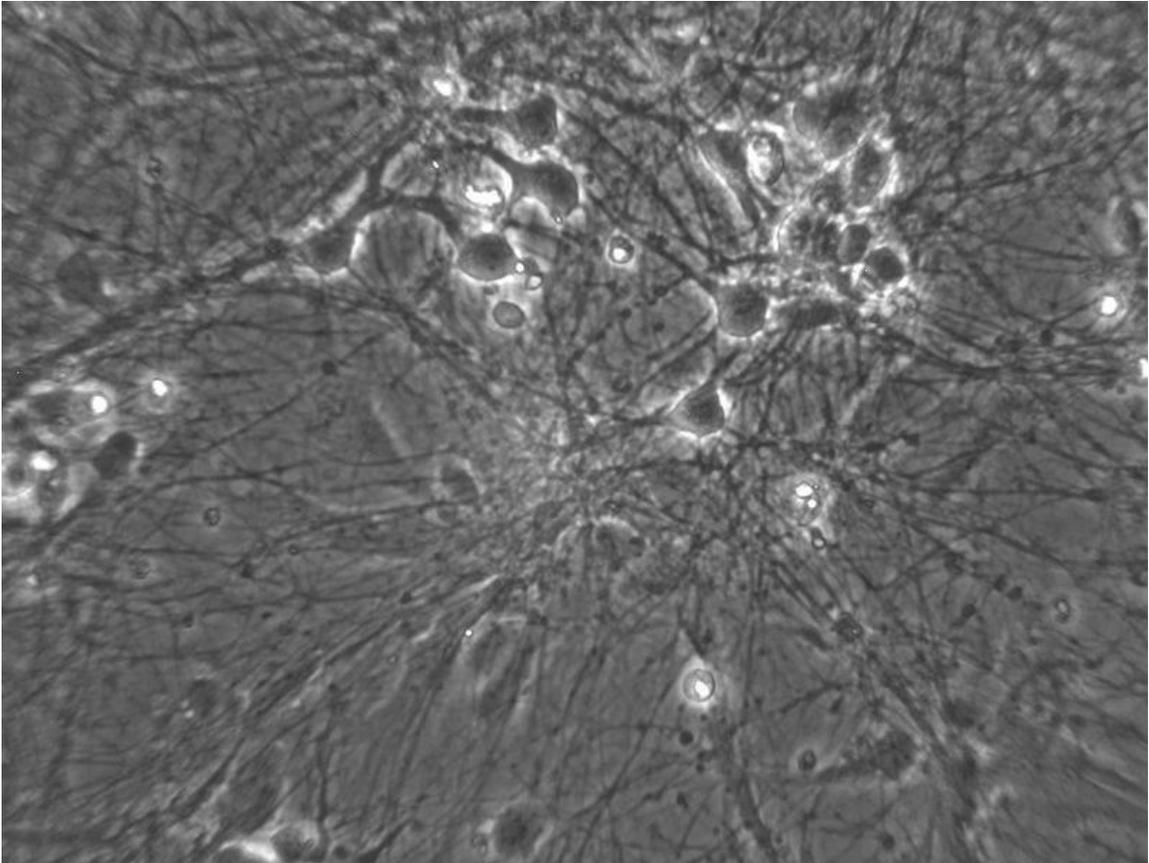
FIGURE 1.3. Light micrograph of cultured cortical cells.

This is a brightfield image of cultured cortical neurons (thin white arrows) and glia (thick white arrows) at DIV 14.

Measure of Cell Death

Many of the results presented in this thesis present quantification of the neurons that have died as a result of an insult. This quantification is based on a well-established protocol for measuring the amount of the cytosolic enzyme, lactate dehydrogenase (LDH), released to the extracellular media. Under normal culture conditions LDH is trapped within the cytosol by the intact plasma membrane of healthy neurons and glia. Upon insult, cells begin to die and the integrity of the plasma membrane is compromised. Eventually, the membrane is degraded enough to allow intracellular LDH to escape into the extracellular media in a manner that is directly proportional to the number of necrotic or apoptotic cells (Lobner, 2000).

By sampling the extracellular media we can determine the amount of LDH released by kinetic assay in which LDH catalyzes the production of lactic acid from pyruvate using NADH. Because NADH absorbs light at ~340 nm, we can measure the rate of its loss (i.e. use in this reaction). We design our experiments with appropriate controls that include an untreated group and a group that has been exposed to an insult known to kill 100% of the neurons (500 micromolar NMDA) or 100% of the neurons and glia (20 micromolar A23187, a calcium ionophore). Once we have the data from the control groups and our experimental groups, we subtract the LDH value observed in the untreated group from each of the others (i.e. experimental and 100% kill) and then normalize the experimental values to the amount of LDH in the 100% kill group. This technique is briefly illustrated in FIGURE 1.4.

Since LDH can be released from both neurons and glia, we often pair these results with another assay in order to confirm which cell type(s) has/have died. For this we use the trypan blue staining based on the same membrane-integrity principle as the LDH assay. Only cells that have compromised membrane integrity allow trypan blue into the cytosol. After a short incubation with the dye, we wash the excess from the media and visually confirm which cells (i.e. cell types: neurons or glia) are stained. For the experiments in this thesis, all of the

neurotoxicants were confirmed to kill only neurons under the conditions presented. This is not surprising given that neurons have long been known to be more sensitive to many insults and injuries and glia are often preserved.
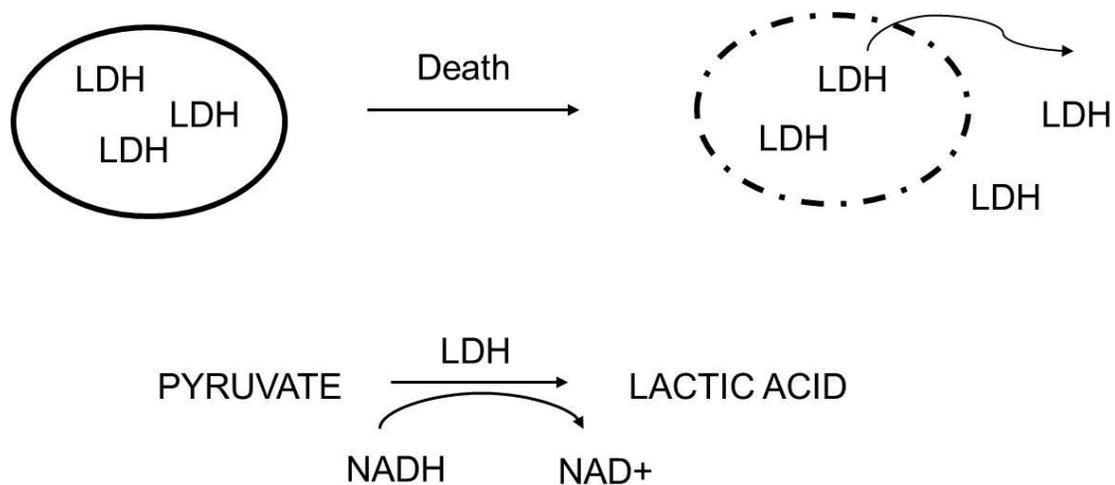
FIGURE 1.4. LDH assay.

Upon cell death, the cytosolic enzyme, lactate dehydrogenase (LDH), is able to escape to the extracellular media because the plasma membrane is compromised. Sampling the media allows quantification of the amount of enzyme released by exploiting the lower equation and measuring the loss of NADH absorbance over time. Since the amount of LDH released is directly proportional to the number of dead cells, this allows the inference of how toxic an insult is.

CHAPTER II

MECHANISMS OF CHLORPYRIFOS AND DIAZINON INDUCED NEUROTOXICITY IN CORTICAL CULTURE

Abstract

The main action of organophosphorous insecticides is generally believed to be the inhibition of acetylcholinesterase (AChE). However, these compounds also inhibit many other enzymes, any of which may play a role in their toxicity. We tested the neurotoxic mechanism of two organophosphorous insecticides, chlorpyrifos and diazinon, in primary cortical cultures. Exposure to the insecticides caused a concentration-dependent toxicity that could not be directly attributed to the oxon forms of the compounds, which caused little toxicity but strongly inhibited AChE. Addition of 1 mM acetylcholine or carbachol actually attenuated the toxicity of chlorpyrifos and diazinon. The muscarinic receptor antagonist, atropine, and the nicotinic receptor antagonist, mecamylamine, did not attenuate the toxicity of either insecticide. These results strongly suggest that the organophosphorous toxicity observed in this culture system is not mediated by buildup of extracellular acetylcholine resulting from inhibition of AChE. The toxicity of chlorpyrifos was attenuated by antagonists of either the NMDA or AMPA/kainate-type glutamate receptors, but the cell death was potentiated by the caspase inhibitor ZVAD. Diazinon toxicity was not affected by glutamate receptor antagonists, but was attenuated by ZVAD. Chlorpyrifos induced diffuse nuclear staining characteristic of necrosis, while diazinon induced chromatin condensation characteristic of apoptosis. Also, chlorpyrifos exposure increased the levels of extracellular glutamate, while diazinon did not. The results suggest two different mechanisms of neurotoxicity of the insecticides, neither one of which involved acetylcholine. Chlorpyrifos induced a glutamate-mediated excitotoxicity, while diazinon induced apoptotic neuronal death.

Introduction

The organophosphorous insecticides chlorpyrifos and diazinon are potent inhibitors of acetylcholinesterase (AChE) activity. However, organophosphates inhibit many other serine hydrolases (Casida and Quistad, 2005).   Exposure to these compounds can induce both acute toxicity and long-term neurological deficits (Dahlgren et al., 2004; Lotti and Moretto, 2005)lottie(Roy et al., 2005; Slotkin et al., 2008). While they have been banned from residential use in the United States, they continue to be widely used throughout the commercial agricultural industry. This widespread use leaves many people at risk for both acute and chronic exposure to toxic levels of organophosphorous insecticides.   The focus of research on these compounds has been on their ability to inhibit AChE, and how this leads to hyperstimulation of cholinergic systems. This action is clearly important for many of the acute effects of organophosphates, such as constriction of the pupils, bronchial constriction, increased sweating, urinary and fecal incontinence, convulsions, seizures, inhibition of respiratory drive, and muscle fasciculations. The ability of organophosphates to inhibit AChE has led to the hypothesis that they exert their neurotoxic effects by increasing acetylcholine concentrations, leading to overstimulation of cholinergic receptors and thereby inducing seizure activity and excitotoxic neuronal death (Lallement et al., 1998).   However, chlorpyrifos and diazinon can have deleterious effects on the nervous system through a variety of mechanisms (Howard et al., 2005; Giordano et al., 2007). Chlorpyrifos can increase the expression of NMDA receptors (Gultekin et al., 2007), while both chlorpyrifos and diazinon have been shown to modify expression of neurotrophic factors (Slotkin and Seidler, 2007; Slotkin et al., 2007), induce oxidative stress (Kovacic, 2003; Saulsbury et al., 2009), and impair spatial memory learning in rats (Terry et al., 2003; Timofeeva et al., 2008). Interestingly, many of the effects of chlorpyrifos and diazinon have been shown to occur at concentrations where they have little effect on AChE (Slotkin and Seidler, 2007; Slotkin et al., 2007; Timofeeva et al., 2008).   These findings have led to recent interest in the neurotoxic mechanisms of chlorpyrifos and diazinon, independent of AChE inhibition. Our study utilized

primary murine cortical cultures to further define the mechanism of chlorpyrifos- and diazinon-induced neuronal death.

Materials and Methods

Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE). Fetal bovine serum and horse serum were obtained from Atlanta Biologicals (Lawrenceville, GA). Laminin and glutamine were from Invitrogen (Carlsbad, CA). Chlorpyrifos-oxon (97.5% pure) was purchased from ChemService (West Chester, PA). Diazinon-oxon (92.7%) was purchased from Accustandard (New Haven, CT). Chlorpyrifos (99.2% pure), diazinon (99.0% pure) and all other chemicals were obtained from Sigma (St. Louis, MO).

Cortical cell cultures

Mixed cortical cell cultures were prepared from fetal (15-16 day gestation) mice as previously described (Lobner, 2000). Briefly, dissociated cortical cells were plated on 24 well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Cultures were maintained in humidified 5% CO_2 incubators at 370C. Nearly-pure glia cultures were prepared as described above except from postnatal day 1-2 mice in which only glia survive (McCarthy and de Vellis, 1980; Choi et al., 1987). Nearly-pure neuronal cultures were prepared as described for mixed cultures with cytosine arabinoside (10 μM) added to the cultures 48 hours after plating to inhibit glial replication. Less than 1% of cells in these cultures stain for glial fibrillary acidic protein (GFAP) (Dugan et al., 1995).

Induction of neuronal death

Toxicity experiments were performed on cultures 13-15 days in vitro (DIV). Neuronal death was induced by 24-hour exposure to chlorpyrifos, chlorpyrifos-oxon, diazinon or diazinon-oxon in media the same as in which cells were plated except lacking serum.

Assay of cell death (LDH Release)

Cell death was assessed in mixed cultures by the measurement of lactate dehydrogenase (LDH) released from damaged or destroyed cells to the extracellular fluid 24 hours after the beginning of the insult. Control LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 500 μM NMDA. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Lobner, 2000).

Propidium iodide staining

Cultures were exposed to the non-vital dye propidium iodide (10 μM) for 30 minutes, following 24-hour exposure to the insecticides. Digital images were taken with a Zeiss LSM 5 PASCAL confocal microscope at a magnification of 630X.

Analysis of extracellular glutamate

Extracellular glutamate was measured following 6-hour exposure to chlorpyrifos or diazinon. Samples of the bathing media from the cell cultures are assayed for glutamate by using phenylisothiocyanate (PITC) derivatization, HPLC (Agilent 1100) separation using a Hypersil-ODS reverse phase column, and ultraviolet detection at a wavelength of 254 nm (Cohen et al., 1986; Lobner and Choi, 1996). Bathing media (200 μL) was derivatized with 100 μL of PITC, methanol, triethylamine (TEA) and dried under vacuum. These samples are then reconstituted in

solvent consisting of 0.14 M sodium acetate, 0.05% TEA, 6% acetonitrile, brought to pH 6.4 with glacial acetic acid. The above solvent is used as the mobile phase, with the column being washed in 60% acetonitrile, 40% water between each sample run. Media glutamate concentrations are calculated by normalizing to glutamate standards. Glutamate measurements are found to be linear over the range 0.1 - 10 μM.

Measurement of acetylcholinesterase activity in vitro.

Acetylcholinesterase activity in mixed cultures was measured by a modified Ellman assay (Ellman et al., 1961). Briefly, cells were washed into phosphate-buffered saline (PBS) with 0-100 μM concentrations of chlorpyrifos, chlorpyrifos-oxon, diazinon or diazinon-oxon. 300 μM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) was added to all wells at the start of the assay. Control and experimental wells were also supplied with 0.42 μM acetylthiocholine iodide (ASChI). Following 1-hour incubation, 50 μL samples of the extracellular media were taken and absorbency at 405 nm determined on a plate reader. AChE activity was normalized to protein levels and calculated as percent control after subtraction of background absorbance measured in those cultures supplied with only PBS and DTNB.

Statistical analysis.

Differences between test groups were examined for statistical significance by means of one-way ANOVA followed by the Bonferroni t-test, with $p < 0.05$ being considered significant.

Results

Concentration dependent toxicity of chlorpyrifos and diazinon

We first tested chlorpyrifos and diazinon for toxicity in mixed neuronal and glial cortical cultures. Both compounds were toxic, with diazinon causing significant toxicity at a lower concentration (30 μM) than chlorpyrifos (100 μM) (FIGURE 2.1 A and C). However, we found

that chlorpyrifos-oxon induced only low-level toxicity (FIGURE 2.1 B), even at a concentration of 100 µM, while diazinon-oxon caused no neuronal death (FIGURE 2.1 D). Therefore, the majority of the neuronal death induced by 100 µM chlorpyrifos and 30 µM diazinon cannot be caused by the oxon forms of the compounds. In the experiments that follow, 30 µM diazinon and 100 µM chlorpyrifos were used because these were concentrations that induce intermediate levels of neuronal death. The toxicity at these concentrations was primarily due to neuronal cell death, as they induced minimal cell death when tested on pure glial cultures (30 µM diazinon induced 3+1 % glial cell death; 100 µM chlorpyrifos induced 2+1% glial cell death; n = 16).

Acetylcholinesterase inhibition by chlorpyrifos, diazinon and their respective oxons

We next measured the ability of chlorpyrifos, diazinon and their respective oxons to inhibit AChE activity. Chlorpyrifos and diazinon were capable of substantial AChE inhibition at high concentrations (FIGURE 2.2 A and C), while the oxon forms of these compounds proved to be more potent and efficacious AChE inhibitors than their precursors (FIGURE 2.2 B and D).
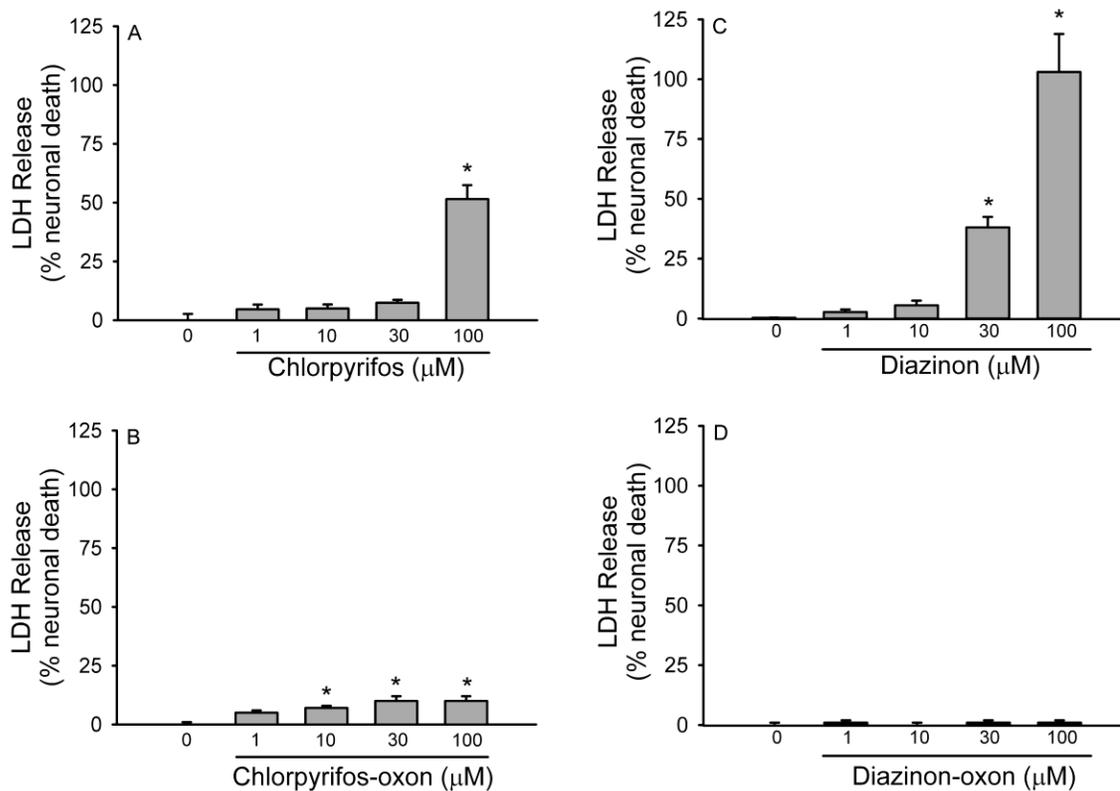
FIGURE 2.1. Organophosphorous pesticides induce toxicity in cortical culture.

Chlorpyrifos (A), chlorpyrifos-oxon (B), diazinon (C), and diazinon-oxon (D) induce toxicity in

primary cortical cultures. Bars show % neuronal cell death (mean + SEM, n=8) quantified by

measuring release of LDH 24 hrs after the beginning of the insult. * indicates significantly

different from control.
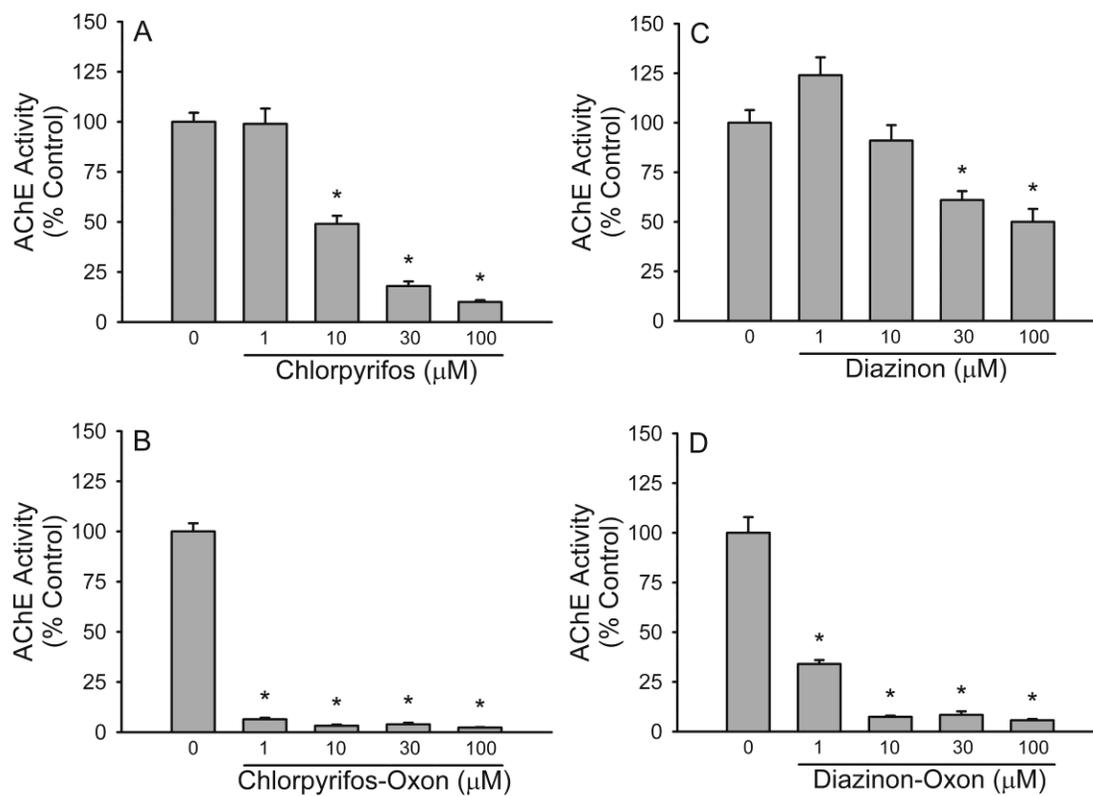
FIGURE 2.2. Organophosphorous pesticides inhibit acetylcholinesterase activity in cortical culture.

Chlorpyrifos (A), chlorpyrifos-oxon (B), diazinon (C) and diazinon-oxon (D) inhibit AChE activity in cortical culture. Bars show % acetylcholinesterase activity (mean + SEM, n=8) relative to control cultures after normalization to protein following 1-hour incubation with ASChI and DTNB. * indicates significant difference from control.

Toxicity of chlorpyrifos and diazinon is not mediated by increased extracellular acetylcholine

The next set of experiments was to determine whether the toxicity of these agents was due to inhibition of AChE. We first tested the effects of acetylcholine and its non-hydrolyzable analog, carbachol. Neither of these compounds was toxic, even at a concentration of 1 mM (FIGURE 2.3). If chlorpyrifos or diazinon was toxic by inhibiting AChE, causing acetylcholine buildup, the addition of acetylcholine or carbachol would be expected to potentiate their toxicity. Instead, acetylcholine and carbachol were protective against chlorpyrifos and diazinon toxicity (FIGURE 2.3). We also tested if acetylcholine receptor antagonists were protective against chlorpyrifos and diazinon toxicity. Neither the muscarinic acetylcholine receptor antagonist, atropine, nor the nicotinic acetylcholine receptor antagonist, mecamylamine, were protective against chlorpyrifos or diazinon toxicity (FIGURE 2.4).
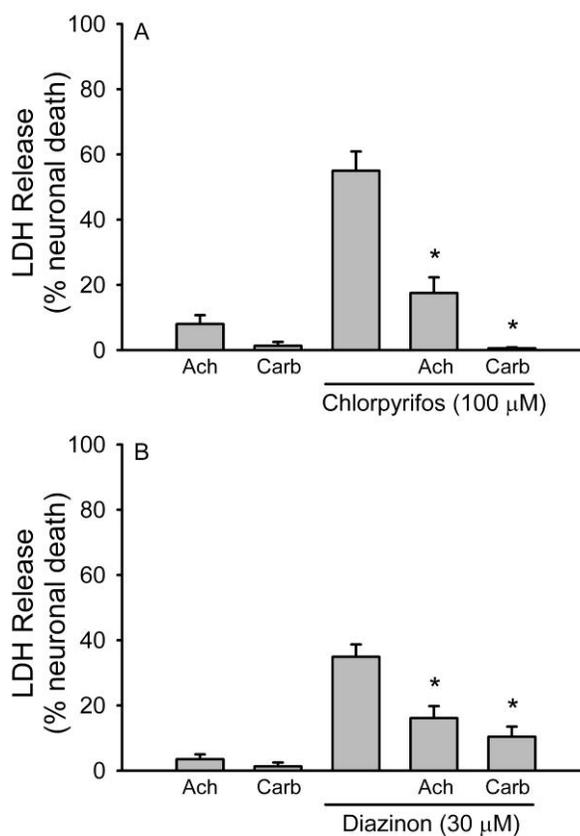
FIGURE 2.3. Cholinergic agonists are not toxic and partially protect against chlorpyrifos and diazinon.

Acetylcholine and carbachol are not toxic and partially protect against chlorpyrifos (A) and diazinon (B) toxicity. ACh: 1 mM acetylcholine; Carb: 1 mM carbachol. Bars show % neuronal cell death (mean + SEM, n =12-20) quantified by measuring release of LDH, 24 hrs after the beginning of the insult. * indicates significantly different from chlorpyrifos or diazinon alone.
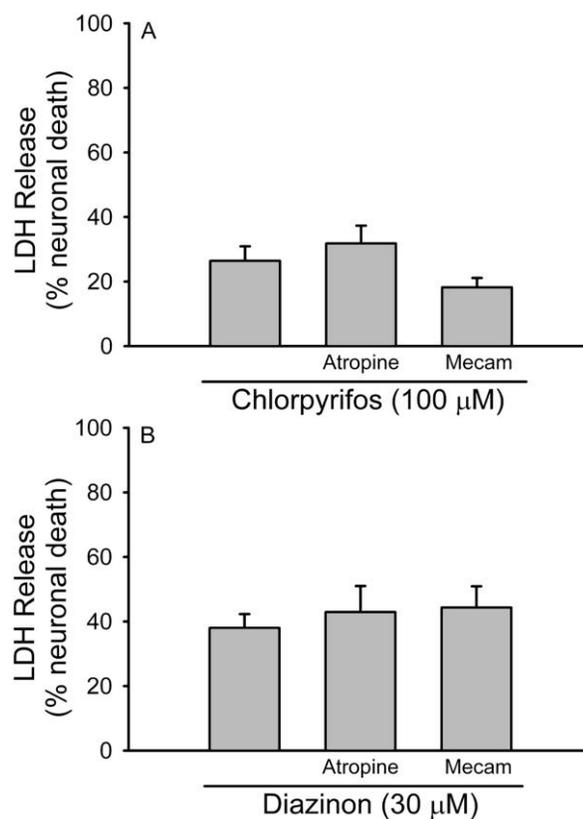
FIGURE 2.4. Cholinergic antagonists are not protective against chlorpyrifos and diazinon.

The muscarinic receptor antagonist atropine and the nicotinic receptor antagonist mecamylamine

are not protective against chlorpyrifos (A) or diazinon (B) toxicity. Atropine: 1 mM atropine;

Mecam: 10 μM mecamylamine. Bars show % neuronal cell death (mean + SEM, n = 8-16)

quantified by measuring release of LDH, 24 hrs after the beginning of the insult.

Chlorpyrifos induces excitotoxicity, diazinon induces apoptosis

To determine the mechanism of toxicity of chlorpyrifos and diazinon, we first tested the protective effects of various pharmacological agents. Chlorpyrifos toxicity was attenuated by NMDA-type glutamate receptor antagonists, the non-competitive antagonist dizocilpine (MK-801), and the competitive antagonist D-2-amino-5-phosphovaleric acid (APV). The competitive AMPA/kainate-type glutamate receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (NBQX), was also protective. However, the caspase inhibitor, Z-Val-Ala-Asp-fluoromethylketone (ZVAD), actually enhanced chlorpyrifos toxicity (FIGURE 2.5 A). In contrast, diazinon toxicity was not affected by glutamate receptor antagonists, but was attenuated by the caspase inhibitor ZVAD (FIGURE 2.5 B). These results suggest that chlorpyrifos induces an excitotoxic neuronal death, while diazinon induces apoptosis. To test this further, we observed the cells following propidium iodide staining. Propidium iodide binds to DNA of dying cells in which the cell membrane has been disrupted. Following chlorpyrifos exposure there was diffuse nuclear staining indicating generalized, random, DNA breakdown, indicative of necrosis (Lobner et al., 2003), while following diazinon exposure many of cells showed nuclear condensation and fragmentation into discrete spherical or irregular shapes, characteristic of apoptosis (Lobner et al., 2003)(FIGURE 2.6).

The pharmacological studies indicate that chlorpyrifos induces excitotoxic neuronal death. This may be caused either by increased extracellular glutamate or by the cells being more sensitive to glutamate toxicity. We found that chlorpyrifos did increase extracellular glutamate, while diazinon did not have this effect (FIGURE 2.7 A). Extracellular glutamate was assayed after a 6 hour insecticide exposure because at that time there was not cell death assessed by LDH release. To determine the source of glutamate, we tested the effects of chlorpyrifos on both pure neuronal and pure glial cultures. Chlorpyrifos increased extracellular glutamate in each type of culture (FIGURE 2.7 B & C).
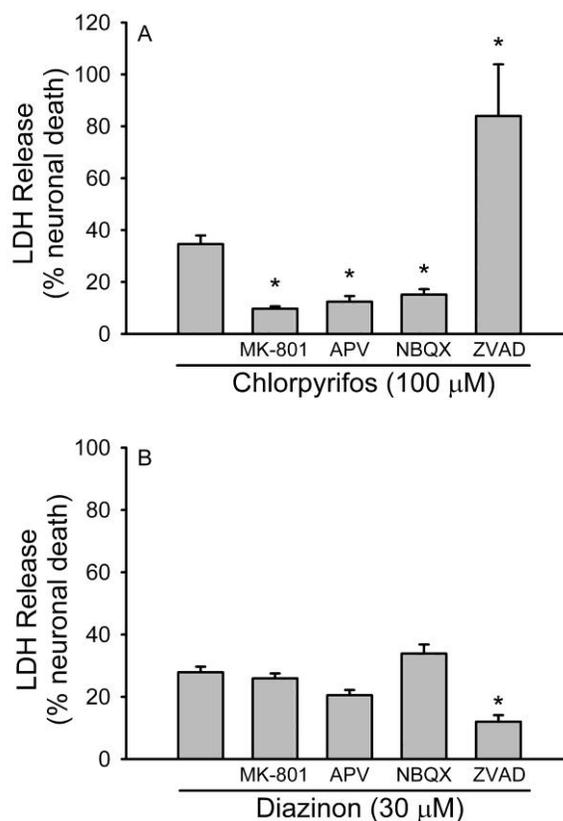
FIGURE 2.5. Glutamate receptor antagonists block chlorpyrifos toxicity; an apoptosis inhibitor blocks diazinon toxicity.

The glutamate receptor antagonists MK-801, APV, and NBQX protect against chlorpyrifos (A) but not diazinon (B) toxicity, while the caspase inhibitor ZVAD protects against diazinon but not chlorpyrifos toxicity. MK-801: 10 μM MK-801; APV: 200 μM APV; NBQX: 20 μM NBQX; ZVAD: 100 μM ZVAD. Bars show % neuronal cell death (mean + SEM, n=8-16) quantified by measuring release of LDH, 24 hrs after the beginning of the insult. * indicates significantly different from chlorpyrifos or diazinon alone.
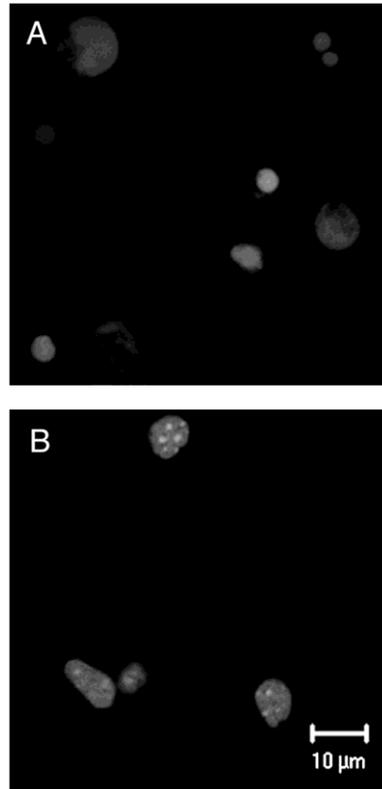
FIGURE 2.6. Chlorpyrifos causes necrosis; diazinon induces apoptosis.

Chlorpyrifos induces DNA breakdown characteristic of necrosis (A), while diazinon induces

DNA fragmentation characteristic of apoptosis (B) as detected by propidium iodide staining.

Propidium iodide (10 μM) was added for 30 minutes, 24 hours after the beginning of the insult.
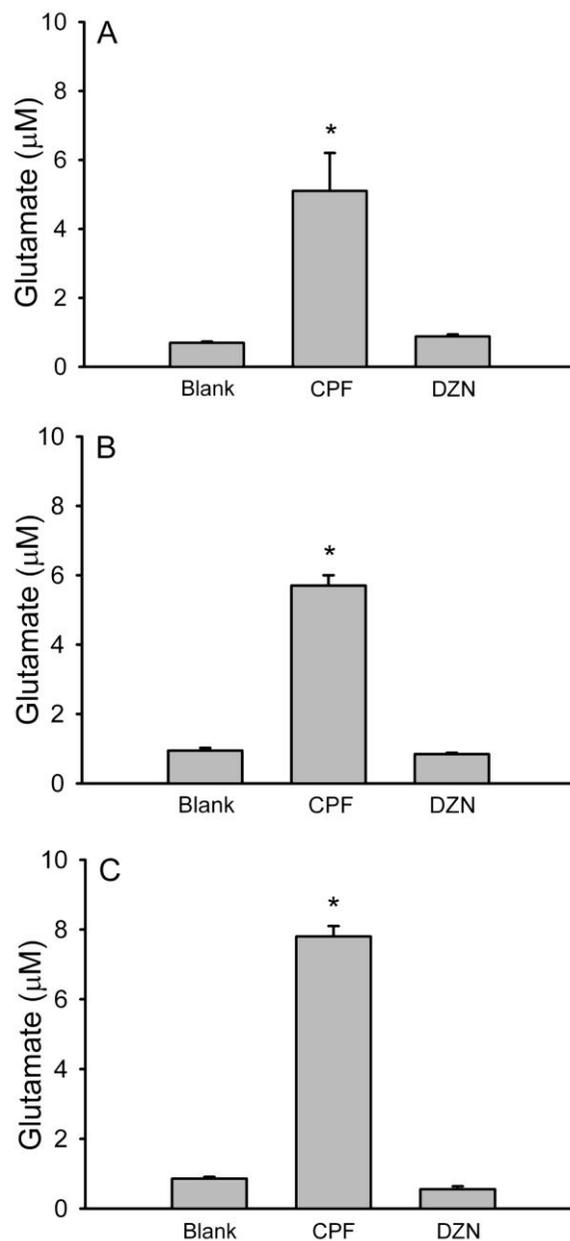
FIGURE 2.7. Extracellular glutamate levels following chlorpyrifos and diazinon exposures. Chlorpyrifos, but not diazinon, induces increased extracellular glutamate in mixed neuronal and glial cultures (A), pure neuronal cultures (B), and pure glial cultures (C). CPF: 100 μM chlorpyrifos; DZN: 30 μM diazinon. Bars indicate glutamate concentrations (μM) in media (mean + SEM, n=8-16) quantified by HPLC determination. * indicates significantly different from control.

Discussion

Chlorpyrifos and diazinon both induced neuronal death, but through very different mechanisms. Chlorpyrifos induced a clear excitotoxic neuronal death. The fact that both the NMDA receptor antagonists MK-801 and APV, and the AMPA/kainate antagonist NBQX were protective suggests that both types of receptors were involved in the neuronal death. This was not unexpected. While the NMDA receptor/channel is most often implicated in excitotoxic neuronal death because of its high calcium permeability (Choi, 1992), the cell membrane must be depolarized to remove the magnesium block of the NMDA receptor/channel before the calcium influx can occur. Opening of the sodium permeable AMPA/kainate receptor channels can provide this required depolarization. Two different NMDA antagonists were used because non-competitive antagonists, including MK-801, have been found to directly inhibit AChE, while also preventing its inactivation by diisopropylfluorophosphate (Galli and Mori, 1996). The competitive antagonist, APV, is not known to have these effects. Chlorpyrifos has also been shown to induce excitotoxicity in vivo. However, this action has been attributed to its inhibition of AChE leading to increased extracellular acetylcholine, which has been proposed to induce seizure activity (Lallement et al., 1998). This is clearly not the mechanism involved in our studies. The current studies indicate an alternative, or complementary, mechanism for chlorpyrifos toxicity. That is, chlorpyrifos increases extracellular glutamate through a non-acetylcholine mechanism. The mechanism by which chlorpyrifos increased extracellular glutamate was not determined. It may involve either increased glutamate release, decreased glutamate uptake, or both.

A recent study found that chlorpyrifos induced death of oligodendrocyte progenitor cells in culture (Saulsbury et al., 2009). The result is consistent with our study in that atropine and mecamylamine were also found to not be protective. The mechanism of toxicity was determined to be induction of oxidative stress. The difference is that in our culture system we studied

neuronal cell death where excitotoxicity is more likely to play a prominent role, although it is quite possible that oxidative stress also is involved.

The toxic effects of diazinon are very different than those of chlorpyrifos, although its effects are also not mediated by inhibition of AChE. Diazinon-induced neuronal death did not involve excitotoxicity, but had characteristics of apoptosis. The toxicity was blocked by the non-selective caspase inhibitor ZVAD, and the propidium iodide staining indicated condensed and fragmented DNA. In contrast, chlorpyrifos induced an excitotoxic and necrotic cell death. This was confirmed by the propidium iodide staining, which showed diffuse nuclear staining consistent with necrosis. While excitotoxicity can induce apoptosis in some systems, in the culture system used for these studies it has been shown that excitotoxicity causes necrosis (Gwag et al., 1997). Chlorpyrifos has been shown to induce apoptosis in human T cells (Li et al., 2009) and placental cells (Saulsbury et al., 2008), but these cells are not electrically excitable and would not be vulnerable to excitotoxicity. Interestingly, chlorpyrifos has also been shown to induce apoptosis in rat cortical cultures (Caughlan et al., 2004). The difference between that study, and our current study, is likely that in the previous study chlorpyrifos exposure was initiated in cultures DIV 5-7, while in our studies exposure was begun on cultures DIV 13-15. Sensitivity to glutamate-induced excitotoxicity is known to increase with the length of time in culture prior to glutamate insult (Choi et al., 1987).

A recent study tested concentrations of chlorpyrifos and diazinon that caused minimal AChE inhibition, too low to cause cholinergic hyperstimulation, for their ability to induce changes in gene transcription in rats (Slotkin and Seidler, 2007). Using microarray technology they observed large numbers of changes. There were a number of results of interest for the current studies. First, both chlorpyrifos and diazinon increased expression of caspases, suggesting possible induction of apoptosis. Interestingly, the authors indicate "DZN had more widespread effects on bax, bmf, casp1 and casp4, implying that DZN may produce greater apoptosis than CPF." Second, chlorpyrifos caused significantly greater increases in expression of

NMDA receptor subunits, particularly of the gene encoding for the NR2B subunit, than diazinon. The NR2B subunit is believed to play a particularly prominent role in excitotoxicity due to the increased calcium permeability of channels containing the NR2B subunit (Liu et al., 2007). This finding is consistent with our results that chlorpyrifos induces excitotoxicity. We found evidence for actions of chlorpyrifos on extracellular glutamate, not on postsynaptic receptors. However, this does not exclude a concomitant effect at the level of glutamate receptors. Unfortunately, expression of genes involved in glutamate release and uptake were not studied in the microarray paper.

The finding that high concentrations of acetylcholine or carbachol were protective against both chlorpyrifos and diazinon toxicity was somewhat surprising in that the mechanisms by which the insecticides induce cell death are different. However, acetylcholine receptor activation, of either the nicotinic or muscarinic receptor, has been shown previously to be protective against both excitotoxicity (Felipo et al., 1998; Dajas-Bailador et al., 2000) and apoptosis (Yan et al., 1995; Fucile et al., 2004). In addition to examining the effects of cholinergic agonists and antagonists, we also measured the capabilities of each compound to inhibit AChE in cortical culture. Chlorpyrifos and diazinon can be converted to their oxon forms which are more effective at inhibiting AChE (Capodicasa et al., 1991; Nigg and Knaak, 2000) and these oxons may therefore be responsible for their toxicity. We included these oxon forms in the studies in order to analyze the correlation between neurotoxicity and AChE inhibition. Interestingly, and in accordance with the lack of toxicity of acetylcholine and carbachol, the ability of each compound did not correlate well with the toxicity results. That is, chlorpyrifos-oxon and diazinon-oxon were the least toxic despite being the most potent and efficacious inhibitors of AChE. Taken together, the data strongly suggests that the toxicity of chlorpyrifos and diazinon, in this system, is not mediated by inhibition of AChE and increased extracellular acetylcholine.

CHAPTER III

EFFECTS OF CHELATORS ON MERCURY, IRON, AND LEAD NEUROTOXICITY IN
CORTICAL CULTURE

Abstract

Chelation therapy for the treatment of acute, high dose exposure to heavy metals is accepted medical practice. However, a much wider use of metal chelators is by alternative health practitioners for so called "chelation therapy". Given this widespread and largely unregulated use of metal chelators it is important to understand the actions of these compounds. We tested the effects of four commonly used metal chelators, calcium disodium ethylenediaminetetraacetate ($CaNa_2EDTA$), D-penicillamine (DPA), 2,3 dimercaptopropane-1-sulfonate (DMPS), and dimercaptosuccinic acid (DMSA) for their effects on heavy metal neurotoxicity in primary cortical cultures. We studied the toxicity of three forms of mercury, inorganic mercury ($HgCl_2$), methyl mercury (MeHg), and ethyl mercury (thimerosal), as well as lead ($PbCl_2$) and iron (Fe-citrate). DPA had the worst profile of effects, providing no protection while potentiating $HgCl_2$, thimerosal, and Fe-citrate toxicity. DMPS and DMSA both attenuated $HgCl_2$ toxicity and potentiated thimerosal and Fe toxicity, while DMPS also potentiated $PbCl_2$ toxicity. $CaNa_2EDTA$ attenuated $HgCl_2$ toxicity, but caused a severe potentiation of Fe-citrate toxicity. The ability of these chelators to attenuate the toxicity of various metals is quite restricted, and potentiation of toxicity is a serious concern. Specifically, protection is provided only against inorganic mercury, while it is lacking against the common form of mercury found in food, MeHg, and the form found in vaccines, thimerosal. The potentiation of Fe-citrate toxicity is of concern because of iron's role in oxidative stress in the body. Potentiation of iron toxicity could have serious health consequences when using chelation therapy.

Introduction

Environmental and occupational exposure to heavy metals such as mercury and lead are of significant concern world-wide. Such metals are known to target the mammalian central nervous system and have been implicated in the development of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Praline et al., 2007). Childhood exposure to mercury or lead is of specific concern given the deleterious effects that each of these metals impose on the developing nervous system (Ellis and Kane, 2000).

Mercury is found in multiple forms in the environment. Elemental mercury can be released from dental amalgam restorations (Patterson et al., 1985) and the mercury vapor can be readily absorbed (Hursh et al., 1976). The elemental mercury can then be converted into inorganic mercury in the body which can accumulate in the brain (Bjorkman et al., 2007). Methyl mercury accumulates in fish inhabiting mercury-contaminated lakes and oceans and is of specific concern because it is readily absorbed from the GI tract and is actively transported across the blood-brain barrier (Kerper et al., 1992; Kostial et al., 2005). While MeHg is thought to be cleared from the brain, it is possible that over multiple exposures significant amounts of MeHg can become demethylated to inorganic mercury and accumulate in brain tissue. Monkeys that have undergone MeHg exposure were found to have little organic mercury in their brains 6 months later, but had higher than normal inorganic mercury (Burbacher et al., 2005). Mercury has been shown to have a substantially longer half-life in the brain than in the blood stream (Rice, 1989). Thimerosal, an antiseptic containing ethyl mercury, continues to be used as a preservative of vaccines distributed and administered worldwide with its use in the US only recently decreasing (Geier et al., 2007). Direct intramuscular injection of this compound provides rapid access to the blood stream and thereby privileged access to its target organs, possibly including nervous tissue.

Lead exposure is also widespread. Commercial use of lead is quite broad, with various lead compounds serving as components in many common products such as lead-based paints found in older homes, as well as being used in the manufacture of batteries (Shukla and Singhal, 1984; Jarup, 2003).

The concerns with iron are somewhat different. While environmental exposure to iron does occur, for example, from drinking water, iron pipes, and cookware, the main concern is the inappropriate release of iron in the body. Iron can be released from the breakdown of hemoglobin following aneurysm or blood disease. The free iron is dangerous because of its ability to generate oxygen free radicals by catalyzing the Fenton reaction (Yamazaki and Piette, 1990).

A controversial use of metal chelators has been by alternative health practitioners for the treatment of chronic health conditions. The basic idea is that chronic high levels of heavy metals are responsible for health problems such as heart disease, Alzheimer's disease, and autism, and that these diseases can be treated by chelation therapy to remove the heavy metals from the body. Statistics on how often such procedures are performed, what they are used to treat, and whether they are effective, or harmful, are limited. A published study (Barnes et al., 2008) estimated that in the year 2002, 66,000 people in the United States underwent chelation therapy. The number is likely to be increasing. Small scale clinical trials have been performed using chelation therapy with EDTA for the treatment of atherosclerotic vascular disease, none of which has showed any benefit (Guldager et al., 1993; van Rij et al., 1994; Knudtson et al., 2002). The National Institutes of Health have recently initiated a major clinical trial testing EDTA in people with coronary artery disease, the results from this trial should be available in 2012 (website: http://www.nccam.nih.gov/chelation).

At present, the primary clinical treatment of acute heavy metal poisoning is by administration of metal chelators. Chelators such as calcium disodium ethylenediaminetetraacetate (CaNa2EDTA), D-penicillamine (DPA), 2,3 dimercaptopropane-1-

sulfonate (DMPS), and dimercaptosuccinic acid (DMSA) have been designed to effectively mobilize and remove toxic metal molecules while limiting the disruption of homeostatic levels of essential metals such as zinc. Chelators have been shown to have varying efficacies depending on the metal to which the patient was exposed (Andersen, 2004). The purpose of the present study is to characterize the effects of each chelator on the cytotoxic effects of $HgCl_2$, MeHg, Thimerosal, $PbCl_2$, and Fe-citrate. To study these interactions, we exposed murine primary cortical cultures to toxic concentrations of each metal of interest both with and without the chelators present.

Materials and Methods

Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE). Serum was from Atlanta Biologicals (Atlanta, GA). All chemicals were obtained from Sigma (St. Louis, MO).

Cortical cell cultures

Mixed cortical cell cultures containing both neuronal and glial cells were prepared from fetal (15-16 day gestation) mice as previously described (Rose and Ransom, 1997; Lobner, 2000). Timed pregnant mice were anesthetized with isoflurane and euthanized by cervical dislocation. Dissociated cortical cells were plated on 24 well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2mM glutamine and glucose (total 21 mM). Cultures were plated at approximately 8 hemispheres per plate. Glial cultures were prepared identical to mixed cultures except cortical cells were obtained from postnatal day 1-3 mice (McCarthy and deVellis, 1980; Choi et al., 1987). The postnatal mice were anesthetized with isoflurane and euthanized by decapitation. Glial cultures were plated at approximately 3

hemispheres per plate. Cultures were maintained in humidified 5% CO2 incubators at 370C. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Induction of neuronal death

All experiments were performed on cultures 13-15 days in vitro (DIV), at this time there are approximately 150,000 neurons/well (145,900 + 5800; n = 4; data from 4 plates from 4 different dissections). Metals and/or chelators were added to the cultures in media identical to plating media except lacking serum for 24 hours, at the end of which time a sample of the media was taken to perform the LDH release assay to determine the level of cell death.

Assay of cell death (LDH Release)

Cell death was assessed in mixed or pure glial cultures by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 hours after the beginning of the insult. Blank LDH levels were subtracted from insult LDH values, and results normalized to 100% neuronal death caused by 500 µM NMDA in mixed cultures and 100% glial death caused by 20 µM A23187 in glial cultures. Approximately 50% (51 + 2%, n= 12) of the LDH present in mixed cultures is present in the neurons. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Gwag et al., 1995; Lobner 2000).

Statistical analysis

Differences between test groups were examined for statistical significance by means of one-way ANOVA followed by the Bonferroni t-test, with $p < 0.05$ being considered significant.

Results

Concentration-dependent toxicity of HgCl2, MeHg, thimerosal, PbCl2, and Fe-citrate.

Figure 3.1 A-E demonstrates the concentration-dependent cytotoxicity with 24-hour exposure to increasing concentrations of each of HgCl2, MeHg, thimerosal, PbCl2, and Fe-citrate. Cell death was assayed in each case through measuring release of the cytosolic enzyme lactate dehydrogenase. HgCl2 had the most notable effects, especially at 5 μM concentrations, while causing approximately 40% cell death at a concentration of 1μM. MeHg and Thimerosal produced similar toxicity profiles, both causing approximately 40% neuronal death at 5μM. Fe-citrate required approximately 30 μM to produce 40% neuronal death. We chose these concentrations of each compound for the following experiments to produce an intermediate level of cell death. This allowed for the observation of both increased and decreased toxicity caused by the chelators tested. PbCl2 produced only 25% cell death at a concentration of 100 μM. We decided that this lower toxicity at 100μM would be sufficient to reveal any protection or potentiation brought on by presence of a chelator and did not use a higher concentration in order to maintain physiological relevance.
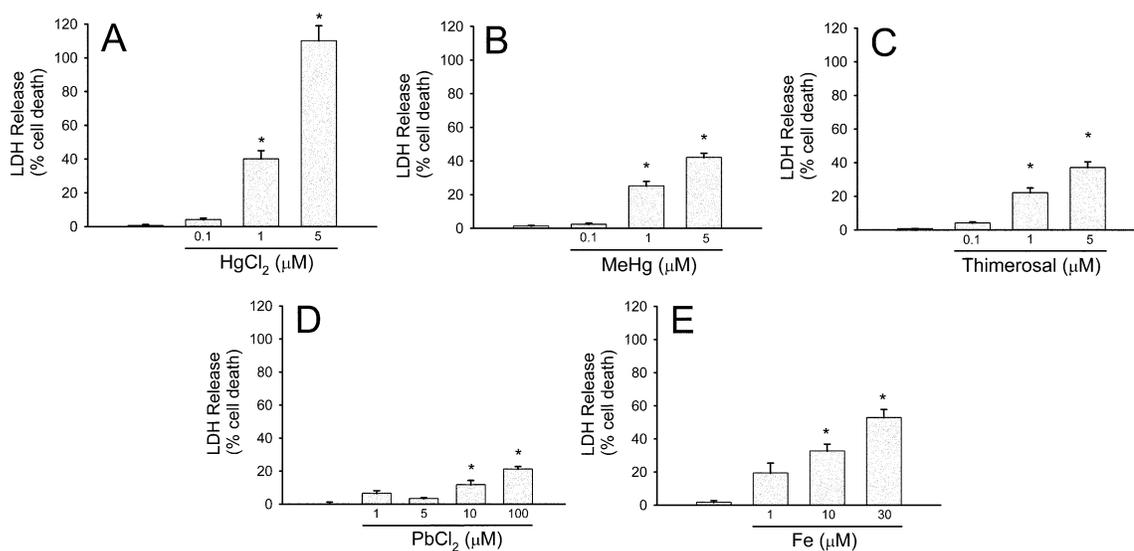
FIGURE 3.1. Toxicity of heavy metals in cortical culture.

Toxicity of (A) inorganic mercury (HgCl2), (B) methyl mercury (MeHg), (C) ethyl mercury

(thimerosal), (D) lead (PbCl2), and (E) iron (Fe-citrate) in mixed cortical cultures. Bars show %

cell death (mean + SEM, n = 16-24) quantified by measuring release of LDH, 24 hrs after the

beginning of the insult. * indicates significant difference from control.

Metal-induced cell death is neuron-specific.

The experiments in Figure 1 were performed using mixed neuronal and glial cultures. To

confirm which cells each metal was targeting, we exposed glial cultures to HgCl2 (1 µM), MeHg

(5 µM), thimerosal (5 µM), PbCl2 (100 µM) or Fe-citrate (30 µM). Each of the compounds

induced less than 5% glial death (HgCl2, 0.4 + .2; MeHg, 3.5 + .7; thimerosal, 4.0 + .4; PbCl2,

1.2 + .3; Fe-citrate, 3.5 + 1.7; n = 8). Therefore, the death observed in mixed cultures is likely

primarily neuronal death.

CaNa2EDTA, DPA, DMPS and DMSA alone are not neurotoxic.

The inherent toxicity of each chelator was tested. CaNa2EDTA, DPA, DMPS or DMSA alone, at a concentration of 100 μM, caused less than 5% cell death in mixed cortical cultures (EDTA, 2.6 + 1.1; DPA, 2.4 + 2.3; DMPS, 4.8 + 2.1; DMSA, 3.7 + 1.3; n = 8).

Effects of chelators on metal toxicity.

Co-application of 100μM CaNa2EDTA attenuated HgCl2 toxicity, but not MeHg, thimerosal or lead toxicity. The CaNa2EDTA also caused a dramatic potentiation of Fe-citrate toxicity. The death caused by the combination of CaNa2EDTA and Fe-citrate is indicated to be greater than 100%. This number is an artifact of the calculation, in our studies 100% neuronal death is defined as complete neuronal death caused by a high concentration of NMDA. The fact that in this case there is more LDH release than that occurring during NMDA exposure indicates that the combination of CaNa2EDTA and Fe-citrate causes not only death of 100% of the neurons, but also death of some of the glial cells present in these cultures (FIGURE 3.2 A). If the calculation were based on total LDH release possible (neurons and glia) the values presented for all of the other studies in which the death was purely neuronal would be misleading. Addition of 100μM DPA failed to provide protection against toxicity induced by any of the metals. Furthermore, it enhanced the toxicity of HgCl2, thimerosal, and Fe-citrate (FIGURE 3.2 B). Addition of 100μM DMPS significantly attenuated HgCl2-induced neuronal death, but it potentiated thimerosal, Fe-citrate, and PbCl2 toxicity (FIGURE 3.2 C). Similar to DMPS, DMSA also attenuated HgCl2 toxicity, while potentiating thimerosal and Fe-citrate toxicity, but it had no effect on MeHg or PbCl2 toxicity (FIGURE 3.2 D).
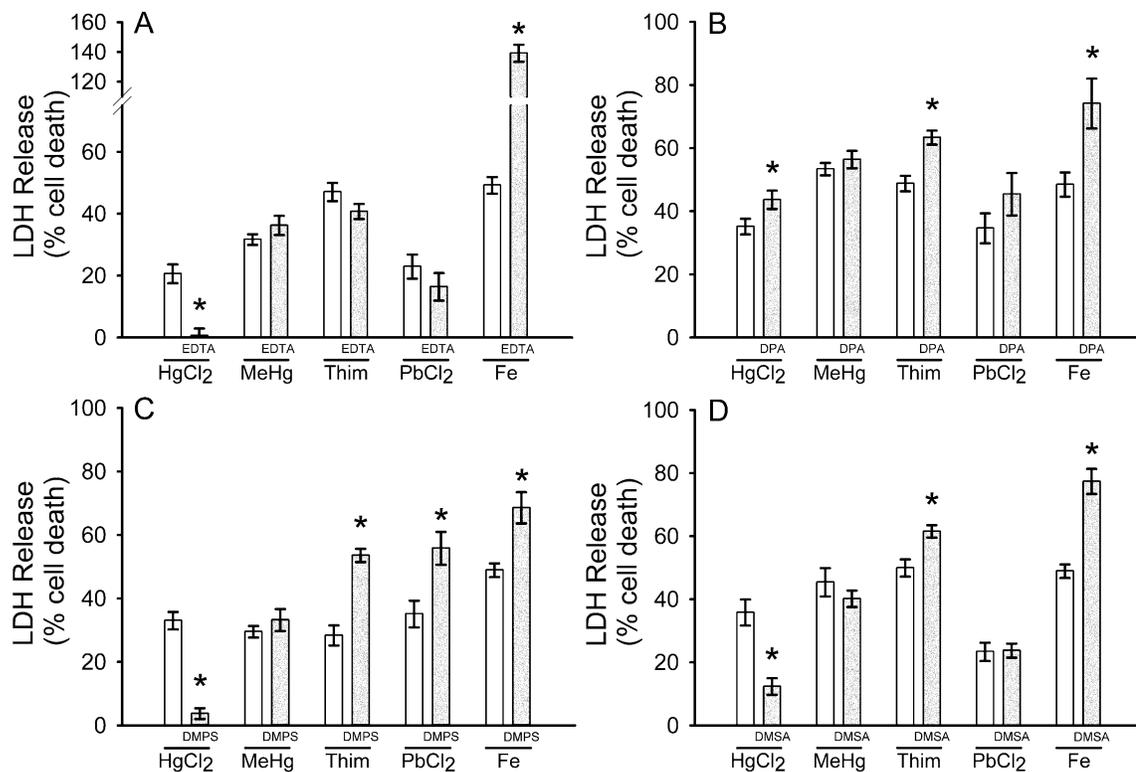
FIGURE 3.2. Effects of chelators on heavy metal toxicity.

Effects of (A) CaNa2EDTA, (B) DPA, (C) DMPS, and (D) DMSA on metal toxicity in mixed cortical cultures. HgCl2 (1 µM), MeHg (5 µM), Thimerosal (5 µM), PbCl2 (100 µM), Fe-citrate (30 µM), CaNa2EDTA (100 µM), DPA (100 µM), DMPS (100 µM), DMSA (100 µM). All compounds were present for 24 hours. Bars show % cell death (mean + SEM, n = 8-16) quantified by measuring release of LDH, 24 hrs after the beginning of the insult. Open bar represents the metal without chelator. * indicates significant difference from control injury (metal without chelator present).

Discussion

An important question concerning the use of metal chelators is whether they increase or decrease levels of heavy metals in the brain. British Anti-Lewisite (BAL), the first chelator used clinically for mercury intoxication, was far from ideal as it was found to mobilize and relocate mercury and lead to the brain increasing their neurotoxic effects (Andersen, 2004). Derivatives of BAL, DMPS and DMSA, have come into use because they are significantly less toxic than BAL. Their effects on brain metal levels are more complex. Aposhian et al. 1996 exposed rats to inorganic lead or inorganic mercury for 86 or 41 days, respectively, prior to treatment with DMPS (Aposhian et al., 1996). In their study, DMPS reduced kidney levels of mercury while causing no change in brain levels of mercury or lead. DMSA, which is approved in the US for the treatment of lead-poisoned children has been shown to decrease brain lead (Cory-Slechta, 1988) and methyl mercury (Aaseth and Frieheim, 1978). However, DMSA, as well as DMPS, have been shown to increase the uptake of inorganic mercury into motor neurons (Ewan and Pamphlett, 1996) and both DMPS and DMSA have been found to be ineffective at eliminating mercury from the body (Eyer et al., 2006). Whether chelator metal complexes enter the brain is not clear. We have found that the ability of the chelators to attenuate neurotoxicity of metals is very limited. While $CaNa_2EDTA$, DMPS, and DMSA each attenuated the toxicity of HgCl2, they did not attenuate the toxicity of the organic forms of mercury, and potentiated the toxicity of at least one metal.

Of the forms of mercury tested, HgCl2 was the most toxic, particularly at a concentration of 5 μM. This may be surprising in that MeHg is generally believed to the most toxic form of mercury. However, the high toxicity of MeHg is due to its ability to be readily absorbed in the gastrointestinal tract and to cross the blood brain barrier (Sasser et al., 1978; Burbacher et al., 2005). Neither of these events is modeled in the cell culture system used in these studies.

The most commonly used metal chelator for chronic disease is EDTA, either simply as EDTA, or as $CaNa_2EDTA$. The problem with using EDTA, not chelated with calcium, is that it can lead to hypocalcemia and potentially death (Brown et al., 2006). However, treatment with $CaNa_2EDTA$ has been shown to cause a temporary increase in brain lead or mercury levels following its administration (Berlin et al., 1965; Cory-Slechta et al., 1987).

Chelators, while thought to directly limit metal toxicity through binding (and thereby blocking harmful reactivity), have been shown to have varying effects when accompanied by their target metals. Binding of iron by EDTA actually increases the ability of iron to induce free radicals because it makes iron more soluble in physiological solutions and increases the ability of iron to promote the formation of hydroxyl radicals via the Fenton reaction (Smith et al., 1990). Therefore, binding of metals to chelators may actually increase their ability to induce cell death. This is, in fact, what we observed with each of the chelators tested.

Of the chelators tested, DMSA appears to have the best profile of effects. It attenuates HgCl2 toxicity, while causing only moderate enhancement of thimerosal and Fe-citrate toxicity. DPA had the worst profile of effects, providing no protection, and enhancing HgCl2, thimerosal, and Fe-citrate toxicity. The other chelators that attenuated HgCl2 toxicity, DMPS and CaNa2EDTA, each have other injury enhancing actions. DMPS enhances PbCl2 toxicity, and CaNa2EDTA causes a severe potentiation of Fe-citrate toxicity. The fact that all of the chelators cause potentiation of some form of metal toxicity is of concern regarding their long-term, and widespread, use for chelation therapy. It has been shown previously that EDTA can increase iron induced free radical formation, but the fact that each of the chelators tested was found to increase iron toxicity is worrying. Considering the variety of results on chelator effects on brain levels of heavy metals, it is important to gain further understanding of how chelator-binding alters the toxicity of these metals.

CHAPTER IV

GLUTATHIONE-MEDIATED NEUROPROTECTION AGAINST METHYLMERCURY IN
CORTICAL CULTURE IS DEPENDENT ON MRP1

Abstract

Methylmercury (MeHg) exposure poses significant neurotoxic threat to humans worldwide. The present study investigated the mechanisms of glutathione-mediated attenuation of MeHg neurotoxicity in primary cortical culture. MeHg caused depletion of mono- and disulfide glutathione in neuronal, glial and mixed cultures. Supplementation with exogenous glutathione, specifically glutathione monoethyl ester (GSHME) protected against the MeHg induced neuronal death. MeHg caused increased reactive oxygen species (ROS) formation measured by dichlorodihydrofluorescein (DCF) fluorescence with an early increase at 30 minutes and a late increase at 6 hours. This oxidative stress was prevented by the presence of either GSHME or the free radical scavenger, trolox. While trolox was capable of quenching the ROS, it showed no neuroprotection. Exposure to MeHg at subtoxic concentrations caused an increase in system x_c^- mediated 14C-cystine uptake that was blocked by the protein synthesis inhibitor, cycloheximide (CHX). Interestingly, blockade of the early ROS burst prevented the functional upregulation of system x_c^-. Inhibition of multidrug resistance protein-1 (MRP1) potentiated MeHg neurotoxicity and increased cellular MeHg. Taken together, these data suggest glutathione offers neuroprotection against MeHg toxicity in a manner dependent on MRP1-mediated efflux.

Introduction

Methylmercury (MeHg) is an ubiquitous and potent neurotoxicant posing significant threat to humans primarily through dietary exposure. Exposure to MeHg has been implicated as a factor in the development of neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, and Amyotrophic Lateral Sclerosis (ALS)(Hock et al., 1998; Monnet-Tschudi et al., 2006; Praline et al., 2007). Humans are primarily exposed to MeHg through consumption of contaminated fish and shellfish. MeHg is formed by bacterial methylation of inorganic mercury in aquatic sediments (Clarkson, 1997).   MeHg then travels up the food chain and accumulates in fish and shellfish, and is subsequently consumed by humans (Mahaffey et al.,

2004). There is clear evidence for neurological deficits following exposure to high levels of mercury. For example, a large release of MeHg into Minamata Bay in Japan led to toxic levels of exposure and severe injury to the local population, including neurological dysfunctions (Harada, 1995; Tsuda et al., 2009). In a separate incident, methylmercury-contaminated seed grain was sent to a population in Iraq with its consumption leading to severe neurological injuries (Myers et al., 2000). Acute exposure to high levels of mercury is known to target cerebellar granular neurons, however, cortical damage from environmental exposure to mercury is likelier to underly its implicated role in the etiology of the aforementioned neurodegenerative diseases. While humans are exposed to other forms of mercury, such as elemental mercury found in dental amalgam restorations, or ethylmercury found in the commercial preservative, thimerosal, MeHg is of specific concern because it is easily absorbed in the gastrointestinal tract, and readily traverses the blood-brain barrier (BBB)(Bridges and Zalups, 2005, 2010).

Proposed mechanisms for MeHg neurotoxicity are primarily focused on three possibilities: disruption of intracellular calcium and zinc ion homeostasis, likely involving mitochondrial deficits (Atchison and Hare, 1994; Kawanai et al., 2009), redox imbalance by increased production of reactive oxygen species and/or by decreasing endogenous cellular antioxidant defenses (Ali et al., 1992; Aschner, 2000), and direct interactions with free protein sulfhydryl groups (Rooney, 2007). The present study is aimed at determining the effects of MeHg on the glutathione (GSH) cycling system and oxidative stress.

Astrocytes are thought to be responsible for de novo synthesis of glutathione from glutamate, glycine and the rate limiting substrate, cysteine, which is brought into the astrocytes primarily in its oxidized form, cystine. Glutathione can be utilized by the cell to reduce reactive oxygen species, such as superoxide, produced as a byproduct of mitochondrial energy production; this superoxide rapidly reacts to form hydrogen peroxide which is then reduced by glutathione to form glutathione-disulfide (GSSG) and water in a reaction catalyzed by glutathione peroxidase.

Glutathione may also be utilized as a xenobiotic detoxicant as has been well characterized involving chemotherapeutics in cancer treatment. That is, glutathione can be directly conjugated to exogenous substrates via a disulfide bond with the free sulfhydryl groups; these reactions are directed by a class of enzymes known as glutathione-S-transferases (GSTs)(Dringen, 2000; Dringen and Hirrlinger, 2003). GSH, GSSG and the glutathione-conjugates are then exported from the cell in a glutathione-dependent manner via multi-drug resistance proteins (MRP), specifically MRP1 in the CNS (Hirrlinger and Dringen, 2005; Minich et al., 2006). These glutathione molecules can then be broken down in the extracellular space by glutathione reductase, amino-peptidase N, or gamma-glutamyl transpeptidase. This metabolism produces the substrate cysteine, which can be taken up and utilized by neurons to produce their own glutathione. In this way, neurons are dependent on astrocytes to supply glutathione (Dringen et al., 1999). Since glutathione serves a dual role as both an antioxidant and detoxicant, and both of these roles may offer independent protective mechanisms against MeHg, the present study examines the impact of MeHg on these aspects of glutathione action.

Materials and Methods

Materials. Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE, USA). Serum was from Atlanta Biologicals (Atlanta, GA, USA). NADPH was from Applichem (Darmstadt, Germany). Radiolabeled ^{14}C-Cystine was purchased from PerkinElmer (Boston, MA, USA). S-(4)-carboxyphenylglycine (CPG) and MK571 were obtained from Tocris Bioscience (Ellisville, MO, USA). DCF was from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma (St. Louis, MO, USA)

Cortical Cell Cultures. Mixed cortical cell cultures containing glial and neuronal cells were prepared from fetal (15-16 day gestation) mice as previously described (Lobner, 2000).

Dissociated cortical cells were plated on 24-well plates (2.0 cm^2 surface area per well) coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum, 2 mM glutamine and D-glucose (total 21 mM). Neuronal cultures were prepared exactly as above with the addition of 10 μM cytosine aribinocide 48 hours after plating to inhibit glial replication (Dugan et al., 1995; Rush et al., 2010). Cultures were plated at approximately 8 hemispheres per plate in a volume of 0.5 mL per well. Glial cultures were prepared as described for mixed cultures from cortical tissue taken from post-natal day 1-3 mice and plated at approximately 3 hemispheres per plate (Choi et al., 1987; Schwartz and Wilson, 1992; Rush et al., 2009). Cultures were maintained in humidified 5% CO_2 incubators at 37^{0}C. Mice were handled in accordance with a protocol approved by our institutional animal care committee and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All experiments were performed in media identical to growth media except lacking serum (MS) at a volume of 0.4 mL per well. All experiments were performed on mixed cortical cultures, except where noted.

Assay of neuronal death. Cell death was assessed in cultures by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 hours after the beginning of the insult. Insults were performed by washing cultures into MS or MS containing the indicated chemicals and incubating overnight. Control LDH levels were subtracted from insult LDH values, and results normalized to 100% neuronal death caused by 500 μM NMDA. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Lobner 2000). Trypan blue staining indicated that the cell death observed was neuron selective. Cell-free experiments were performed to exclude the possibility of direct inhibition of LDH by the pharmacological agents used.

Glutathione Assay. Total cellular glutathione and media glutathione were assayed using a modified enzymatic method (Baker et al., 1990; Lobner et al., 2003). Cultures were washed into MS with or without MeHg (5 μM) and incubated for 6 hours. Media samples of 25 μl were taken after the drug application period and assayed as aliquots of supernatant below. Cultures were washed with cold (4 °C) HEPES buffered saline solution, dissolved in 200 μl of 1% sulfosalicylic acid, and centrifuged. A 25 μl aliquot of the supernatant was combined with 150 μl of 0.1 M phosphate/5 mM EDTA buffer, 10 μl of 20 mM dithiobis-2-nitrobenzoic acid, 100 μl of 5 mM NADPH, and 0.2 U of glutathione reductase. Total glutathione was determined by kinetic analysis of absorbance changes at 402 nm for 1.5 min, with concentrations determined by comparison to a standard curve. GSSG was measured as above except samples were treated with 2-vinylpyridine and triethanolamine for 1 hour prior to beginning the reaction. Cell-free experiments were performed to exclude the possibility of assay inhibition by the experimental reagents used. Due to inter-plate culture variance in absolute glutathione measures data in Table 1 are reported as percent respective intra-plate control glutathione measures. Untreated control values across all culture types were typically in the following ranges (represented as mean nmol/well): 1.99-3.33 cellular GSH, 0.24-0.50 media GSH, 0.14-0.74 cellular GSSG, 0.060-0.16 media GSSG.

Assay for oxidative stress. Oxidative stress was measured with 5-(and -6)-2'7'-dichlorodihydrofluorescein diacetate (DCF) using a fluorescent plate reader following a modification of a previous method (Wang and Joseph, 1999; Lobner et al., 2007). Cultures were washed into MS and subsequently exposed to treatments by adding drug for the exposure times indicated.   10 μM DCF is added to the cultures 30 minutes prior to data acquisition. Fluorescence is read using a Fluoroskan Ascent plate reader (Thermo LabSystems) with excitation and emission filters set to 485 nm and 538 nm,respectively. Background fluorescence (no DCF added) was subtracted and the results normalized to control conditions.

[14]C-Cystine Uptake. Radiolabeled cystine uptake was performed as previously described with modifications. Cultures were exposed to MS containing the indicated drug treatments for 24 hours, or for 1 hour followed by 23 hours in drug-free MS. 24 hours after the start of the experiment, cultures were washed into HEPES buffered saline solution and immediately exposed to ^{14}C-cystine (0.025 μCi/mL, 200 nM total cystine) for 20 minutes. Following ^{14}C-cystine exposure, cultures were washed with ice cold HEPES buffered saline solution and dissolved in 250 μl warm sodium dodecyl sulfate (0.1%). An aliquot (200 μl) was removed and added to scintillation fluid for counting. Values were normalized to ^{14}C-cystine uptake in untreated controls of the same experimental plate.

Determination of MeHg content in cells by ICP-MS. Following 6 hour exposure to MS with or without the indicated treatments, cultures were washed 3x with cold MS and then dissolved in 100 μl 1% sulfosalicylic acid. Samples were combined with 900 μl 5% HNO_3 containing 500 ppb Au and digested at 70°C for 2 hours. Digestions were then centrifuged at 6000 x g for 5 minutes and the supernatants were diluted with an additional 1 mL of 5% HNO_3. Mercury content was determined using a Micromass Platform ICP-MS controlled by MassLynx software (Waters Corporation, Milford, MA). Isotopes [198]Hg, [199]Hg, [200]Hg, [201]Hg, [202]Hg, and [204]Hg were recorded and total mercury was quantified by comparing sample responses to those produced by commercial standards treated identically to the samples.

Statistical Analysis. Differences between test groups were examined for statistical significance by means of one-way ANOVA followed by the Student-Neuman Keuls post-hoc analysis, with $p < 0.05$ being considered significant.

Results

Methylmercury exposure induced concentration-dependent neurotoxicity in mixed neuronal and glial cortical cultures 24 hours after onset of insult (FIGURE 4.1). Cell death was

assayed by release of the cytosolic enzyme lactate dehydrogenase (LDH). Further, trypan blue

staining of these cultures demonstrated that the MeHg-induced toxicity at the concentrations

tested was purely neuronal (data not shown). From these data we chose 5 μM MeHg as a

moderate insult for subsequent experiments as it allows bidirectional alterations to MeHg-induced

neurotoxicity (i.e. neuroprotection or potentiation). For other experiments we used 3 μM MeHg

as a low-level insult as no significant neurotoxicity was observed at this concentration (FIGURE

4.1).

MeHg toxicity has been shown to cause glutathione depletion in several cell types

(Kitahara et al., 1993; Kaur et al., 2006; Amonpatumrat et al., 2008). We found that exposure of

mixed neural and glial, pure neuronal and pure glial cultures to 5 μM MeHg significantly

decreased total GSH and disulfide glutathione (GSSG) levels in the cells as well as in the

extracellular media (Table 1). This glutathione depletion occurred following a 6 hour MeHg

exposure, a time that precedes cell death as no LDH activity was detectable at this time (data not

shown). We next tested whether glutathione supplementation or addition of the free radical

scavenger, trolox could protect the neurons. 24 hr co-treatment of MeHg with glutathione

monoethyl ester (GSHME) but not trolox, was neuroprotective (FIGURE 4.2). Since glutathione

supplementation was protective, and this effect could be due to its nature as an antioxidant or a

xenobiotic detoxicant, we next assessed the abilities of GSHME and Trolox to block MeHg-

induced oxidative stress.

Mixed neuronal and glial cortical cultures exhibited a biphasic pattern of oxidative stress

induced by exposure to 5 μM MeHg for 0, 30, 60, 180 or 360 minutes (FIGURE 4.3) as measured

by fluorescence of DCF. DCF fluorescence peaked at 30 minutes, returned to control levels at 3

hours and increased once again by 6 hours following onset of MeHg insult. Both 100 μM

GSHME and 100 μM trolox were capable of preventing the MeHg-induced rise in DCF

fluorescence indicative of their ability to block MeHg-induced oxidative stress. However, trolox

was much more effective than GSHME actually decreasing the DCF signal well below control

levels.
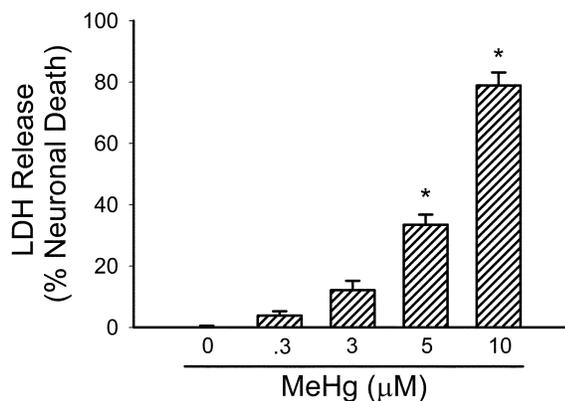
FIGURE 4.1. Concentration response curve of MeHg in cortical culture.

24-hour exposure to MeHg induces LDH release in mixed cortical cultures. Results are expressed as mean+SEM (n=8-16). * indicates significant difference from untreated control.
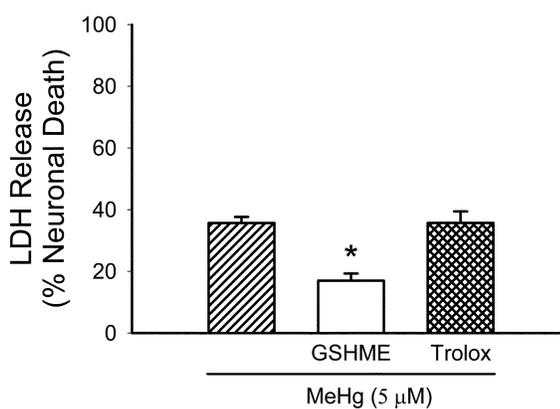
FIGURE 4.2. MeHg-induced neuronal death is attenuated by glutathione monoethyl ester (GSHME, 100 µM) but not trolox (100 µM).

Results are expressed as mean+SEM (n=16-20). * indicates significantly different from MeHg-alone.

Table 4.1

Effect of MeHg (5 µM) on glutathione levels in glial, neuronal and mixed cortical
cultures after 6 hour exposure.

Culture Type	GSH			GSSG	
	Cellular	Media		Cellular	Media
Mixed	83.4±4.7	22.8±1.0		60.7±5.7	12.6±2.4
Glia	44.8±5.2	62.3±9.8		18.4±2.9	29.3±3.7
Neurons	59.6±6.5	36.9±1.8		20.6±6.2	N/D

Results are expressed as % untreated control (mean±SEM, n=8-16). All values are
significantly different from control. N/D = not detectable.
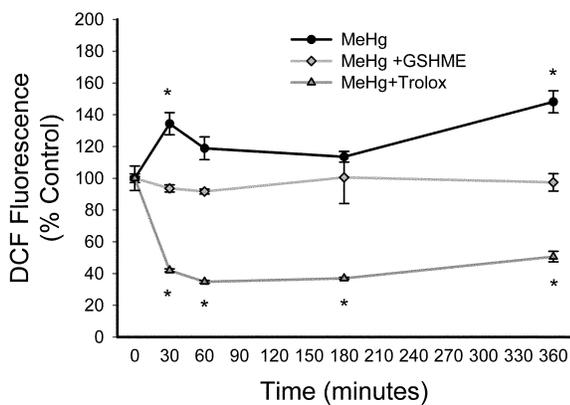
FIGURE 4.3. Timecourse of MeHg (5 µM) induced ROS formation as detected by DCF

fluorescence.

Results are expressed as mean+SEM (n=8-16) after normalizing to average fluorescence of

untreated controls (i.e. DCF only). * indicates significantly different from untreated controls.

To further examine MeHg induced alterations to glutathione-related systems, we next tested whether low-level MeHg exposure had an effect on cellular uptake of cystine, the rate limiting substrate for GSH synthesis. Acute exposure to MeHg had no effect on ^{14}C-Cystine uptake (3 μM MeHg, 20 minute exposure, data not shown); however, 24-hour exposure to low-level MeHg (3 μM) caused a robust increase in subsequent ^{14}C-cystine uptake that was blocked by co-treatment with the protein synthesis inhibitor, cycloheximide (CHX, 500 ng/mL) (FIGURE 4.4 A). Further, this MeHg-induced increase in cystine uptake was sensitive to blockade by CPG (300 μM, present only during the 20 minute uptake period), an inhibitor of cystine-glutamate exchange (system x_c^-), suggesting that the increase was entirely mediated by functional upregulation of system x_c-. In a similar experiment aimed at determining the effect of trolox on the MeHg-induced increase, overnight exposure to trolox alone induced a large increase in subsequent cystine uptake (data not shown). Thus, we used an altered protocol shortening the exposure time to 1 hour and followed by an overnight incubation in identical exposure media except lacking the experimental agents. 60 minute exposure to MeHg produced a similar increase in xCT-mediated cystine uptake 23 hours later (FIGURE 4.4 B). Co-treatment with trolox blocked this increase while trolox alone had no effect on uptake.

It is possible that glutathione conjugates to MeHg intracellularly and that this conjugate is exported via MRP1. To test whether the export of glutathione is necessary for its protective capabilities, we co-treated cultures with MeHg and an inhibitor of MRP1, MK571. MK571 alone caused no significant cell toxicity; however, by inhibiting MRP1-mediated glutathione export MeHg neurotoxicity was severely potentiated (FIGURE 4.5 A). Supporting the idea that MeHg conjugates to GSH, we found that inhibiting MRP1-mediated glutathione export with MK571 led to a doubling of MeHg accumulation within the cellular compartment (FIGURE 4.5 B). Interestingly, GSH supplementation with GSHME had no effect on MeHg accumulation in the cells.
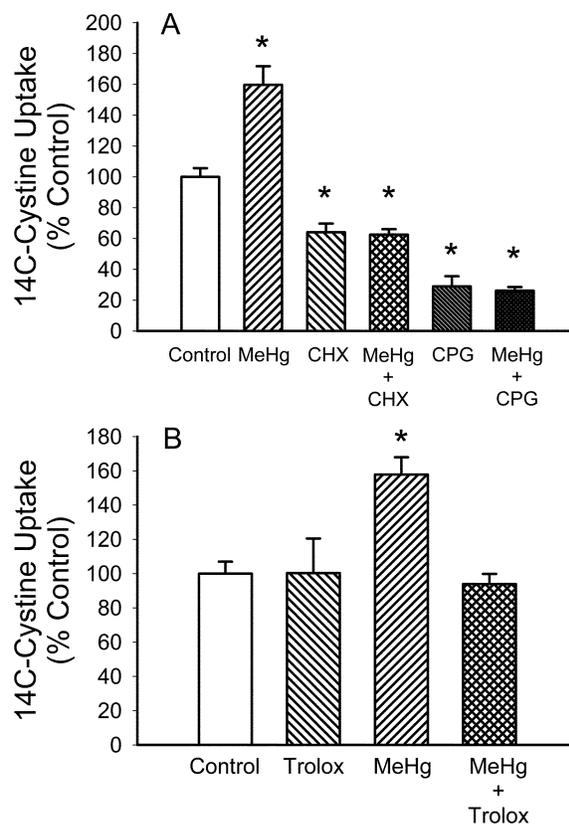
FIGURE 4.4. Subtoxic MeHg exposure causes a robust increase in system x_c^- mediated ^{14}C-cystine uptake.

A- Cultures were exposed to MeHg (3 µM), cycloheximide (CHX, 500 ng/mL), or in combination for 24 hours prior to measuring uptake. Carboxyphenylglycine (CPG, 300 µM) was present only during the 20 minute uptake period. B- Cultures were exposed to MeHg (3 µM), trolox (100 µM) or in combination for 60 minutes, washed into drug-free media and incubated overnight (23 hours) prior to assay for cystine uptake. Results are expressed as mean+SEM (n=8-16) after normalizing to untreated control uptake. * indicates significantly different from control
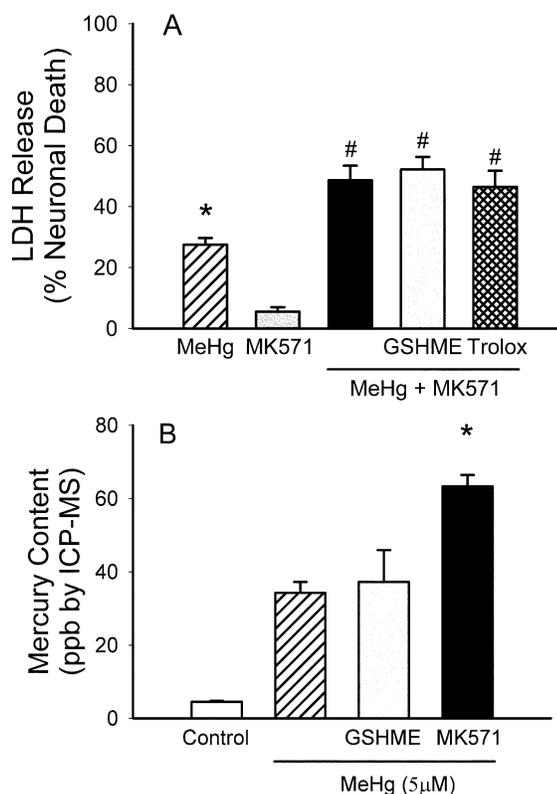
FIGURE 4.5. Blockade of glutathione efflux augments MeHg toxicity and cellular accumulation of mercury.

A-Cultures were exposed to MeHg (5 µM), MK571 (10 µM), or both in combination with or without GSHME (100 µM) for 24 hours at which time samples were taken and neuronal death quantified by LDH release. Results are expressed as mean+SEM (n=8-12). * indicates significantly different from untreated control, # indicates significantly different from MeHg-only treated. B-Cultures were exposed to MeHg (5 µM) in the presence of GSHME (100 µM) or MK571 (50 µM) for 6 hours. Samples were then taken and subsequently analyzed for mercury content by ICP-MS. Results are expressed as mean+SEM (n=3-6).

Discussion

The present study offers several insights into the contributions of alterations in glutathione cycling and oxidative stress to methylmercury-induced neurotoxicity. We observed significant toxicity with overnight exposure of mixed cortical cultures to 5 μM MeHg. This concentration is similar to those used in other studies demonstrating cytotoxicity, oxidative stress and mitochondrial deficits in cortical and cerebellar granular neuron (CGN) cultures (Gasso et al., 2001; Morken et al., 2005; Kaur et al., 2006; Yin et al., 2007). However, it is important to note that CGNs have been shown to be more sensitive to MeHg toxicity (Sarafian and Verity, 1991; Marty and Atchison, 1997; Sakaue et al., 2005). Though not the only factor recognized, the increased sensitivity of CGNs has been posited as attributable to the relatively low glutathione content of these cells (Yee and Choi, 1996; Shafer et al., 2002; Kaur et al., 2007; Wang et al., 2009).

Previous studies have shown that MeHg causes a depletion of cellular glutathione (Yee and Choi, 1996; Franco et al., 2007a; Amonpatumrat et al., 2008; Wang et al., 2009). Here, we also report that MeHg depletes both total and disulfide glutathione in cortical culture; total GSH and disulfide GSSG were decreased in cellular and media samples from glia-enriched, neuron-enriched and mixed cultures. This is noteworthy given the protective roles of GSH in the cell. GSH can reduce free radicals or eliminate exogenous molecules from the cell. The MeHg-induced loss of glutathione may be due to the formation of ROS and subsequent use of GSH by the cells to reduce the oxidative damage. However, since reduction of oxidative stress by GSH results in the formation of GSSG, the observed depletion of both GSH and GSSG suggests that the loss of glutathione was not due to oxidative stress. This is consistent with the hypothesis that glutathione is acting to detoxify MeHg by direct conjugation rendering the utilized GSH molecules undetectable by our assay, and perhaps unrecoverable by the cell. It has also been shown that modulation of cellular thiols (including GSH) alters sensitivity to MeHg in culture

(Kaur et al., 2006) and that exogenous supply of glutathione precursors such as N-acetyl-cysteine

(NAC) or cystine is neuroprotective (Fujiyama et al., 1994; Kaur et al., 2006, 2007). Specifically,

NAC is able to boost cellular GSH availability and leads to decreased MeHg-induced cytotoxicity

and ROS formation. Inhibition of glutathione synthesis with buthionine sulfoxamine (BSO)

increases sensitivity to MeHg and increases subsequent ROS formation (Toyama et al., 2011).

These studies also demonstrate that modulation of glutathione availability alters cellular ROS

formation in response to MeHg insult. Further, this ROS formation is sensitive to antioxidants

(Gasso et al., 2001; Shanker and Aschner, 2003; Kaur et al., 2010). Importantly, however, in our

hands trolox does not offer the neuroprotective capacity that glutathione supplementation with

GSHME provides. This suggests that oxidative stress alone is not primary to the toxicity of

MeHg under these conditions. Others have shown that trolox does offer neuroprotection against

early MeHg-induced mitochondrial deficits detected by MTT metabolism after short, 1 hour

exposure (Kaur et al., 2010). Notably, however, these studies did not investigate later timepoints

as seen in the present data.

We also examined the function of the cystine-glutamate antiporter in response to MeHg

exposure by radiolabeled ^{14}C-cystine uptake. As discussed above, cystine uptake is an important

aspect of glutathione synthesis and subsequent cycling. Low-level MeHg insult induced a robust

increase in cystine uptake. This functional upregulation of xCT was blocked by CHX suggesting

that the upregulation requires translation of new protein. As well, CPG blocked this increased

radioligand uptake to CPG-treated control levels suggesting the entire MeHg-induced increase

was mediated by system x_c-. Trolox also blocked the MeHg-increased cystine uptake.

Considering that upregulation of xCT is likely an endogenous mechanism of cytoprotection in

response to MeHg-insult, the fact that trolox blocks upregulation of xCT may account for the

inability of trolox to protect against the neuronal injury. The most likely mechanism for

increased system x_c- activity is upregulation of xCT by activation of the nrf2-ARE pathway

(Mysona et al., 2009; Wang et al., 2009; Qin et al., 2010). Consistent with this explanation is a recent report demonstrating nrf2-dependent methylmercury detoxification in hepatocytes (Toyama et al., 2011) and an earlier report in astrocytes (Wang et al., 2009).

The current data indicate that blockade of oxidative stress alone is insufficient to prevent MeHg-induced neuronal death, and that elimination of MeHg from the cell by conjugation to GSH is necessary. Consistent with this, inhibition of glutathione export via the MRP1 inhibitor, MK571, not only potentiated MeHg-induced neurotoxicity, but this potentiation was associated with increased accumulation of MeHg in the cell. Similar results have been reported in primary mouse hepatocytes where it was found that upregulation of MRP1 by isothiocyanates decreased MeHg accumulation and toxicity (Toyama et al., 2011). Interestingly, and consistent with a previous report using NAC (Kaur et al., 2006), glutathione supplementation with GSHME did not affect MeHg accumulation in the cell. Trolox has previously been reported to have no effect on MeHg accumulation nor on glutathione availability (Kaur et al., 2010); these and the present data, support the assertion that oxidative stress is not primary to MeHg-induced neurotoxicity. Rather, conjugation with MeHg and subsequent export of glutathione from the cytosol is crucial to alleviate or prevent MeHg-induced neurotoxicity and that solely blocking oxidative stress is not likely to offer significant neuroprotection.

CHAPTER V

SYNERGISTIC TOXICITY OF THE ENVIRONMENTAL NEUROTOXINS
METHYLMERCURY AND BMAA

Abstract

Determining the environmental factors involved in neurodegenerative diseases has been elusive. Methylmercury and β-N-methylamino-L-alanine (BMAA) have both been implicated in this role. However, studying these factors in isolation probably does not accurately mimic the human condition. Neurodegenerative diseases likely involve a complex interaction between genetic predisposition and multiple environmental factors. In the current study we tested the interaction of the environmental neurotoxins methylmercury and BMAA. Exposure of primary cortical cultures to methylmercury or BMAA independently induced concentration dependent neurotoxicity. Importantly, concentrations of BMAA that caused no toxicity by themselves potentiated methyl mercury toxicity. Since both BMAA and methylmercury toxicity have been associated with depletion of glutathione we examined the role of glutathione in the combined toxicity. BMAA plus methylmercury, at concentrations that had no effect by themselves, induced depletion of cellular glutathione. The combined toxicity of methylmercury and BMAA was attenuated by the cell permeant form of glutathione, glutathione monoethyl ester, and the free radical scavenger, trolox, but not by the NMDA receptor antagonist, MK-801. The results indicate a synergistic toxic effect of the environmental neurotoxins BMAA and methyl mercury and that the interaction is at the level of glutathione depletion. Since glutathione depletion is known to occur in neurodegenerative diseases these results provide a potential mechanism for the involvement of methylmercury and BMAA in neurodegenerative diseases.

Introduction

The cause of most neurodegenerative diseases is unclear. Huntington's disease is a purely genetic disease, but Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) involve varying degrees of genetic and environmental factors. The exact role of environmental factors, and what those factors are, has been difficult to determine. Two of the environmental factors that have been implicated in the etiology of neurodegenerative diseases are β-N-methylamino-L-alanine (BMAA) and methylmercury.

BMAA is a non-protein amino acid neurotoxin that was first implicated in the neurodegenerative disease ALS/ Parkinson's Dementia Complex (ALS/PDC) on Guam (Cox and Sacks, 2002; Papapetropoulos, 2007).   Since these initial reports, a number of studies have suggested that BMAA may be involved in neurodegenerative diseases, not only on Guam, but also in the rest of the world. First, cyanobacteria present throughout the world have been shown to produce BMAA (Cox et al., 2005; Banack et al., 2007; Esterhuizen and Downing, 2008; Metcalf et al., 2008). Second, BMAA is biomagnified in systems not only on Guam (Cox et al., 2003), but also in the Baltic Sea (Jonasson et al., 2010) and the Gulf of Mexico (Brand et al., 2010). Third, BMAA can become protein-associated which allows for it to build up in tissue and provides a mechanism for slow release (Murch et al., 2004a). Forth, BMAA was found not only in brain samples of ALS/PDC patients from Guam, but also in the brains of Alzheimer's disease and ALS patients from North America (Murch et al., 2004b; Pablo et al., 2009).   These results suggest that BMAA may be of concern not only for people on select pacific islands, but is a general health concern for the world population.

Mercury exposure to humans occurs primarily in the forms of elemental mercury, ethylmercury, or methylmercury. Elemental mercury exposure can come from dental amalgam. Mercury vapor is released from amalgam restorations (Clarkson, 1997) and a strong correlation between the level of mercury exposure and poor performance on motor and cognitive tests was

found in dentists (Ngim et al., 1992). However, with modern dental materials, and proper handling of amalgam by dental professionals, this problem can be minimized and there is no evidence that the number of amalgam fillings is correlated with cognitive performance (Saxe et al., 1995). Ethylmercury, often in the form of the compound thimerosal, is present as a preservative in a number of products, including vaccines. It has been reported that thimerosal containing vaccines play a causal role in the development of autism (Mutter et al., 2004), but there is no clear evidence for such a connection (Aschner and Ceccatelli, 2010). Methylmercury exposure to humans is widespread. Methylmercury is formed through the methylation of inorganic mercury by bacteria in aquatic sediment (Clarkson, 1997). It is found at high levels in many fish and shellfish (Mahaffey et al., 2004). It is clear that exposure to high levels of methylmercury can induce neurological deficits. For example, a large industrial release of methylmercury into Minamota Bay in Japan led to high levels of methylmercury exposure and severe injury to the population, including neurological deficits (Harada, 1995). There is some evidence that methylmercury at lower concentrations can cause neurological problems. The best evidence comes from studies showing that exposure to even low levels of methylmercury prenatally can cause neurological deficits (Grandjean et al., 1998). However, fairly low exposure of methylmercury to adults has also been shown to induce neurological deficits (Yokoo et al., 2003). It is important to note that methylmercury is more easily absorbed into the body than other forms of mercury (Bains and Shaw, 1997). Furthermore, methylmercury crosses the blood brain barrier as a complex with cysteine (Kerper et al., 1992) and the main form of methylmercury found in fish is as a complex with cysteine (Harris et al., 2003).

One problem with most studies involving environmental neurotoxins is that they have typically been studied in isolation, while the actual situation clearly involves interaction between multiple toxins and genetic factors. As a first step to assess how such factors may interact we studied the interaction of BMAA and methyl mercury.

Materials and Methods

Materials.

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE, USA). Serum was from Atlanta Biologicals (Atlanta, GA). Trolox and glutathione monoethyl ester were from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cortical cell cultures.

Mixed cortical cell cultures containing neuronal and glial cells were prepared from fetal (15-16 day gestation) mice as previously described (Lobner, 2000). Dissociated cortical cells were plated on 24 well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2mM glutamine and glucose (total 21 mM). Cultures were maintained in a humidified 5% CO_2 incubator at 37°C. Mice were handled in accordance with a protocol approved by our institutional animal care committee.

Induction of neuronal death.

All experiments were performed on mixed cultures 13-15 days in vitro (DIV). Toxicity was induced by exposure to the toxic agents for 24 hours in media as described for plating except without serum. All exposure media contained 26 mM $NaHCO_3$, as it has been shown previously that HCO_3^- is required for expression of NMDA receptor mediated BMAA toxicity (Weiss and Choi, 1988).

Assay of neuronal death (LDH Release).

Cell death was assessed in mixed cultures by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 hours after the beginning of the insult. Blank LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 500 mM NMDA. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Lobner, 2000). Glial cell death (assessed by trypan blue staining) was not observed in any of the current studies. Therefore results are presented as percent neuronal death.

Glutathione assay.

Total glutathione was assayed using a modification of a previous method (Baker et al., 1990; Lobner et al., 2003). Briefly, following exposure to BMAA and/or methylmercury for 6 hours, cells were washed with a HEPES buffered saline solution, dissolved in 200 µl of 1% sulfosalicylic acid, and centrifuged. A 25 µl aliquot of the supernatant was combined with 150 µl of 0.1 M phosphate/5 mM EDTA buffer, 10 µl of 20 mM dithiobis-2-nitrobenzoic acid, 100 µl of 5 mM NADPH, and 0.2 U of glutathione reductase. Total glutathione was determined by kinetic analysis of absorbance changes at 402 nm for 1.5 minutes, with concentrations determined by comparison to a standard curve.

Statistical analysis.

Differences between test groups were examined for statistical significance by means of one-way ANOVA followed by the Bonferroni correction post-hoc test, with $p < 0.05$ being considered significant.

Results

Methylmercury and BMAA both induced concentration dependent neurotoxicity in mixed neuronal and glial cortical cell cultures (FIGURE 5.1 A and B). Cell death was assayed by release of the cytosolic enzyme lactate dehydrogenase (LDH). The toxicity at these concentrations was purely neuronal as assessed by trypan blue staining (data not shown). We next tested for a synergistic interaction between BMAA and methylmercury by testing the effects of sublethal concentrations of BMAA on low level toxicity induced by 3 µM methylmercury. BMAA at concentrations from 10-100 µM causes potentiation of the methylmercury toxicity (FIGURE 5.2).
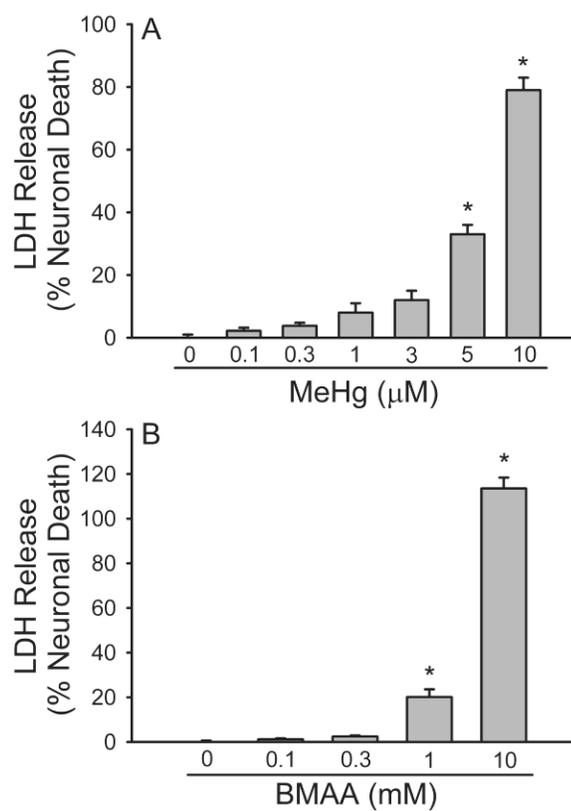
FIGURE 5.1. Concentration dependent toxicity of methylmercury and BMAA in cortical

cultures.

Bars show % neuronal cell death (mean + s.e.m., n=16-20) quantified by measuring release of

LDH, 24 hrs after the beginning of the insult. * indicates significant toxicity.
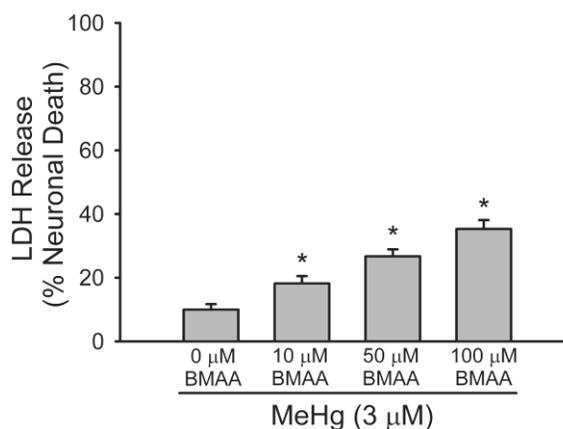
FIGURE 5.2. Synergistic toxicity of BMAA and methylmercury.

BMAA at concentrations that do not cause any toxicity by themselves (10-100 µM) significantly potentiate methylmercury (3 µM) toxicity.   Bars show % neuronal cell death (mean + s.e.m., n=16-20) quantified by measuring release of LDH, 24 hrs after the beginning of the insult.   * indicates significant difference from methylmercury alone.

Both BMAA and methylmercury toxicity have been associated with disruption of the glutathione system (Kaur et al., 2006; Liu et al., 2009). We found that, at relatively low concentrations, 100 µM BMAA and 3 µM methylmercury, they do not cause significant depletion of cellular glutathione. However, the two toxicants administered together did cause significant glutathione depletion (FIGURE 5.3). The glutathione assays were performed at the end of 6 hour exposure to the toxins, a time before significant cell death occurred (less than 10% measured by LDH release).

Toxicity caused by a high concentration of BMAA is mediated primarily by activation of NMDA receptors, with oxidative stress apparent when NMDA receptors are blocked (Lobner et al., 2007).   Toxicity of higher concentration methylmercury in this culture system is attenuated by the cell permeant form of glutathione, glutathione monoethyl ester, but not by the free radical

scavenger, trolox (Rush et al., 2010). The combined toxicity of low level methylmercury and

BMAA was not attenuated by the NMDA antagonist MK-801, but was attenuated by trolox or

glutathione monoethyl ester (FIGURE 5.4).
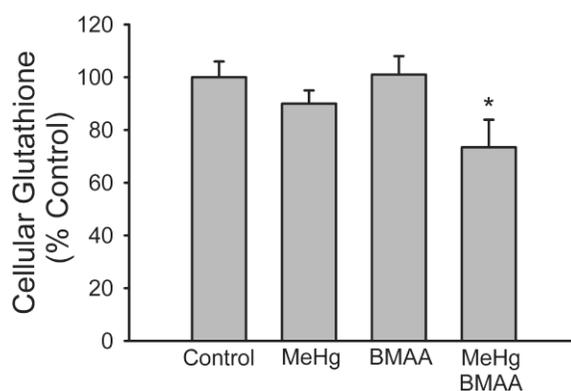
FIGURE 5.3. Methylmercury plus BMAA, but not either compound alone, significantly

decreases cellular glutathione.

Methylmercury (3 µM), BMAA (100 µM). Bars show glutathione levels following a 6 hour

exposure presented as % Control (mean + s.e.m., n = 16). * indicates significant difference from

control.
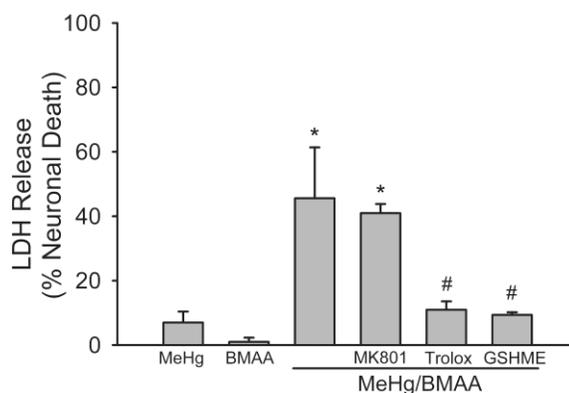
FIGURE 5.4. Synergistic toxicity of BMAA and MeHg is dependent on oxidative stress. Synergistic toxicity of BMAA and methylmercury is not attenuated by the NMDA antagonist MK-801, but is attenuated by the free radical scavenger trolox, and the cell permeant form of glutathione, glutathione monoethylester. Methylmercury (3 µM), BMAA (100 µM), MK-801 (10 µM), Trolox (100 µM), GSHME (100 µM). Bars show % neuronal cell death (mean + s.e.m., n=16-20) quantified by measuring release of LDH, 24 hrs after the beginning of the insult.   * indicates significant toxicity. # indicates significant difference from BMAA plus methylmercury without drug treatment.

Discussion

There are two main findings of the current study. First, there is a synergistic toxicity between methylmercury and BMAA. Second, the mechanism of the synergistic toxicity appears to be at the level of depletion of cellular glutathione. Depletion of glutathione as the mechanism of combined methylmercury and BMAA toxicity is consistent with the known actions of each of the compounds. We have shown previously that high concentration BMAA inhibits cystine/glutamate exchange-mediated cystine uptake leading to decreased cellular glutathione (Lobner et al., 2007). The prominent role of cystine/glutamate exchange in the regulation of cellular glutathione and how its inhibition can lead to neuronal death has been well characterized (Murphy and Baraban, 1990). The process of glutathione production in neurons involves a complex series of steps in which cystine is taken up primarily into astrocytes (Sagara et al., 1993). The glutathione produced by astrocytes is released and converted extracellularly into cysteine, which is taken up by neurons and used by them to produce glutathione (Wang and Cynader, 2000).

BMAA acts to specifically block the initial step of uptake of cystine into astrocytes (Lobner, 2009; Liu et al., 2010). Methylmercury can act at a number of levels on the glutathione system. It has been shown to inhibit glutathione peroxidase (Franco et al., 2009) and can directly conjugate to glutathione (Fujiyama et al., 1994). The combination of the effects on the glutathione system by BMAA and methylmercury could potentially cause neurodegeneration. There is evidence that glutathione depletion plays a role in Alzheimer's disease, Parkinson's disease, and ALS (Bains and Shaw, 1997; Liu et al., 2004; Zeevalk et al., 2008). The type of low level, long-term, depletion of glutathione that may be caused by exposure to methylmercury and BMAA could cause the long-term oxidative stress that may underlie aspects of neurodegenerative diseases.

The toxicity of the combination of methylmercury and BMAA is somewhat different from the toxicity of either compound by itself at higher concentrations. Toxicity of a high concentration of BMAA is mediated primarily by NMDA receptor activation, with significant protection provided by the NMDA receptor antagonist MK-801, and protection by trolox only occurring when NMDA receptors are blocked (Lobner et al., 2007). Neurotoxicity of methylmercury is sensitive to manipulations that increase intracellular glutathione (Kaur et al., 2006; de Melo Reis et al., 2007). The effects of trolox are less clear. The oxidative stress induced by methylmercury is decreased by trolox (Shanker and Aschner, 2003), but trolox does not prevent the toxicity (Kaur et al., 2010), and specifically does not prevent the toxicity in our culture system (Rush et al., 2010). The combined toxicity of low level BMAA and methylmercury is not sensitive to MK-801, but is attenuated by either glutathione ethyl ester or trolox. This result indicates the prominence of oxidative stress in the toxicity of combined treatment.

Is exposure to both BMAA and methylmercury likely to occur in the same person? The simple answer is that since both are ubiquitous in the environment it is likely that exposure to both will occur in many people. The ability to answer the more specific question of whether exposure to both will occur through the consumption of certain fish or shellfish from specific locations is limited by the available information. BMAA levels in certain type of fish and shellfish species have only been measured in two location: the Baltic Sea (Jonasson et al., 2010) and southern Florida (Brand et al., 2010). Certain trends appear in this data. The highest levels of BMAA appear to be found in filter feeders such as shellfish. This is different than the methylmercury which accumulates more higher up the food chain. However, there is some overlap. For example, in the Caloosahatchee river in Florida, fish such as largemouth bass, and bowfin have high levels of BMAA (Brand et al., 2010), and these same fish have also been shown to accumulate mercury (Katner et al., 2010). The Caloosahatchee River specifically has been shown to have high levels of mercury in its sediment and the one fish type tested, catfish, had

high mercury levels (Kannan et al., 1998). Interestingly, high levels of both BMAA and methylmercury have been found in the brains of deceased Alzheimer's disease patients (Hock et al., 1998; Pablo et al., 2009). Of course exposure to BMAA and methylmercury does not have to come from the same source and more studies of levels of BMAA in various systems must be performed.

CHAPTER VI

GENERAL DISCUSSION

General Discussion

The data presented in this thesis not only provide valuable insights into the etiology of several environmental toxicants but also lay an important framework for studying potential interactions of toxicants likely to be coexposed and have overlapping targets leading to augmented (neuro)toxicity. This final section presents a discussion of the major findings from the presented studies as well as that of the broad implications thereof. Included is a discussion of the impact of future research derived from these studies.

Organophosphorous Pesticides

One of the most significant findings of this study was the observation that both chlorpyrifos and diazinon can cause neurotoxicity by distinct mechanisms and each independent of acetylcholinesterase inhibition. Whereas CPF was shown to elevate extracellular glutamate and cause an excitotoxic necrosis, DZN produced an apoptotic neurodegeneration by a yet to be determined mechanism. These findings contrast with the major theory that OP agent-induced neurotoxicity results from AChE inhibition and subsequent cholinergic hyperstimulation. However, recent interest in OP chemicals has shifted to studies such as mine.

One such in vitro study demonstrated CPF as toxic to oligodendrocyte precursors by a mechanism that was insensitive to antagonists of cholinergic receptors (Garcia et al., 2001; Garcia et al., 2002). While this study did not suggest a prominent role for glutamatergic excitotoxicity as seen in my study, but rather an oxidative stress-induced cell death, it is not in direct conflict with my data because of the difference in cultured cell types utilized. My studies were performed using cortical neurons cultured with glia (mostly astrocytes). Quantified cell death was composed entirely of neurons and suggested to be a result of AMPA and NMDA receptor stimulation by the elevated glutamate levels in the extracellular media. It is likely that

neurons, by their electrically excitable nature, are sensitive to this mechanism of CPF action. The oligodendrocyte progenitors from the Garcia study, on the other hand, are far less excitable and thus, less likely to be susceptible to excitotoxic stimulation. Interestingly, there have been reports of CPF-induced apoptosis in other non-excitable cell types such as human T lymphocytes (Li et al., 2007, 2009). In vitro and in vivo evidence suggests that damage to these immune cells leads to immunological abnormalities including an association with increased allergies in animals and humans (Thrasher et al., 1993; Blakley et al., 1999; Thrasher et al., 2002).

Further support for CPF having glutamatergic effects comes from genetic microarray data. CPF and DZN at concentrations unlikely to cause significant inhibition of AChE were demonstrated to cause transcriptional alterations in rats (Slotkin and Seidler, 2007, 2010). Specifically, CPF increased transcription of glutamate receptor proteins (of specific note, the calcium-permeable NMDA receptor subunit, NR2B) and later shown to alter glutamate transporter expression. This may suggest a bimodal mechanism that could account for the CPF-induced glutamatergic hyperstimulation I observed. That is, by CPF increasing ionic glutamate receptors the cultured neurons would become more sensitive to the elevated glutamate concentrations resulting from decreased glutamate transporter expression. My results are consistent with both microarray findings for CPF. Also consistent with my data were the DZN findings from the microarray study. In particular, DZN was reported to increase apoptosis related genes (i.e. Caspases) to a greater degree than CPF. DZN did not alter expression of glutamate receptors or transporters (Slotkin et al., 2010). Of course, further experiments are necessary to confirm these suggestions as relevant to CPF and DZN-induced neurotoxicity in vitro and in vivo.

Importantly, these results highlight the potential for OP agents to act beyond their anticholinesterase actions. The likelihood that other OP compounds exert non-cholinergic actions is great and this is underlined by the finding that CPF and DZN both can cause neurotoxicity by distinct, non-cholinergic mechanisms. There are other recent reports suggesting that other non-cholinergic mechanisms exist for OP nerve agents sarin, VX and tabun, as well (Chebabo et al.,

1999; Rocha et al., 1999). While cholinergic hyperstimulation is obviously key to the acute lethality of these compounds, non-cholinergic mechanisms are likely to contribute, at least in part, to the ability of OP agents to increase the risk of developing neurodegenerative disease. Further study along this avenue is imperative to understand, and if possible address, the risks associated with OP exposure.

Heavy Metals

Chelation

One potentially worrying finding with regard to my studies is that every chelator examined exacerbated the damage caused by at least one heavy metal, and several of the chelators tested potentiated more than one metal. DPA had the most detrimental effects in our hands, in that it potentiated the damage of inorganic mercury, thimerosal and iron while failing to provide protection from any of the metals tested. That said, EDTA, DMPS and DMSA each provided near complete protection against inorganic mercury. These data suggest that it is potentially very important to consider which metals may be present in a patient when selecting a chelator in order to at least avoid further damage. There are certain key limitations to this study, such that clinical applications of these chelators may produce less obvious effects. Specifically, the renal system would, in theory, remove the chelated metal complex perhaps before any further damage could be done. Our data suggests that without targeting any specific heavy metal, but rather, targeting multiple metal species with a single chelator is likely to potentiate the insult caused by at least one other metal likely to be present. While my study did not investigate the exact nature of the potentiating effects of these metal chelates, others have provided similar evidence in the past. For instance, EDTA has been shown to improve the ability of iron to catalyze the Fenton reaction producing excessive ROS.

Mercury toxicity

Perhaps the most important contribution I made with regard to the neurotoxicity of mercurials is the clarification that oxidative stress is not the primary mechanism underlying the toxicity of these compounds. In fact, the data suggest that at least the early increase in ROS production following onset of mercurial insult is important to activation of an endogenous cytoprotective mechanism involving system x_c^- and glutathione. The evidence for this is the lack of protection by the antioxidants trolox and PBN against MeHg insult; and the potentiation of inorganic mercury- or thimerosal-induced toxicity by these antioxidants.

My experiments exploring the role of glutathione in the detoxification of mercurials also provided evidence to support a secondary role for oxidative stress. While many other studies have reported a depletion of glutathione in response to inorganic mercury, methylmercury or thimerosal, my data provides a more complete picture. Specifically, I have demonstrated that each of these mercurials depletes total glutathione as well as disulfide glutathione in cellular and extracellular compartments across all three culture types: mixed cortical neurons and glia, glia-enriched and neuron-enriched. The most likely explanation is that the mercurials, with their high affinity for sulfhydryl interactions, conjugate directly to any available glutathione thereby removing glutathione from the cycling pool and rendering it undetectable by our assay. Supporting this hypothesis is evidence from several mass-spectrometry studies demonstrating that mercurials directly conjugates with glutathione and its cysteinyl precursors.

Another important finding was the obligate role of the glutathione exporter, MRP1, in the detoxification of mercurials. Inhibiting MRP1-mediated export with MK571 produced a severe potentiation of mercurial-induced neurotoxicity. While glutathione supplementation is well-known to protect against mercury insult, inhibition of MRP1 eliminated the protective effects of glutathione supplementation. Further analysis demonstrated that in the face of methylmercury insult, preventing the export of glutathione nearly doubled the amount of cell-associated mercury

measured by inductively-coupled plasma mass spectrometry. While others have reported similar findings with regard to a detoxifying role for glutathione (Toyama et al., 2007; Wang et al., 2009), this study is the first to clearly demonstrate an obligate role for MRP1-mediated export in this detoxification. Only one recent study has produced similar findings, and have actually provided a more complete story (Toyama et al., 2011). In this study, researchers targeted upregulation of genes under the control of the transcription factor NRF2 as a means of increasing cellular defense against methylmercury insult in mouse hepatocytes and a neuronal cell line. By exposing mice to isothiocyanate compounds, NRF2-regulated genes which include system x_c^-, MRP1, and glutathione sythesizing enzymes were upregulated. This upregulation decreased subsequent mercury burden in an MK571-dependent manner in hepatocytes cultured from these mice and SH-SY5Y cells treated similarly.

I propose here that cytosolic glutathione conjugates with mercury that has gained access to the intracellular compartment and is subsequently exported by MRP1 preempting the metal's damaging effects. When the cell first detects mercury, likely through a ROS-sensing NRF2-dependent mechanism, or perhaps through a loss of glutathione, a signaling mechanism shown recently in a similar culture system, the cell responds by upregulating NRF2-responsive genes in order to maintain glutathione levels (Seib et al., 2011). Of course, if the mercury remains at high levels for prolonged periods, such as in our culture conditions, eventually this system is overwhelmed and the neurons die. With an exogenous supply of glutathione or its precursors, the cell can better maintain intracellular glutathione available for the detoxification of mercury and neurodegeneration is prevented (Zeevalk et al., 2007). However, an alternate explanation for protection with glutathione supplementation is that the glutathione symply provides an abundance of sulfhydryl groups with which the mercurials can interact without causing any cellular damage. While this is possible, data from my current study argue against it. First, glutathione supplementation does not, by itself, alter the amount of mercury associated with the cell, despite its ability to protect against mercurial insult. This by itself may actually speak to the hypothesis

that abundant sulfhydryl groups account for glutathione-mediated protection. However, the

second piece of data demonstrates that the same glutathione supplementation fails to protect

against mercury toxicity when MRP1-mediated glutathione export is inhibited. This finding

suggests that availability of the sulfhydryl groups of glutathione has little to do with its protection

against methylmercury toxicity. An important measure that may clarify this point is one that has

not been made. It would be useful to know whether cell-associated mercury content remains

doubled in the presence of MK571 when glutathione is supplied exogenously. FIGURE 6.1

depicts the working model of MeHg neurotoxicity as it relates to glutathione cycling.
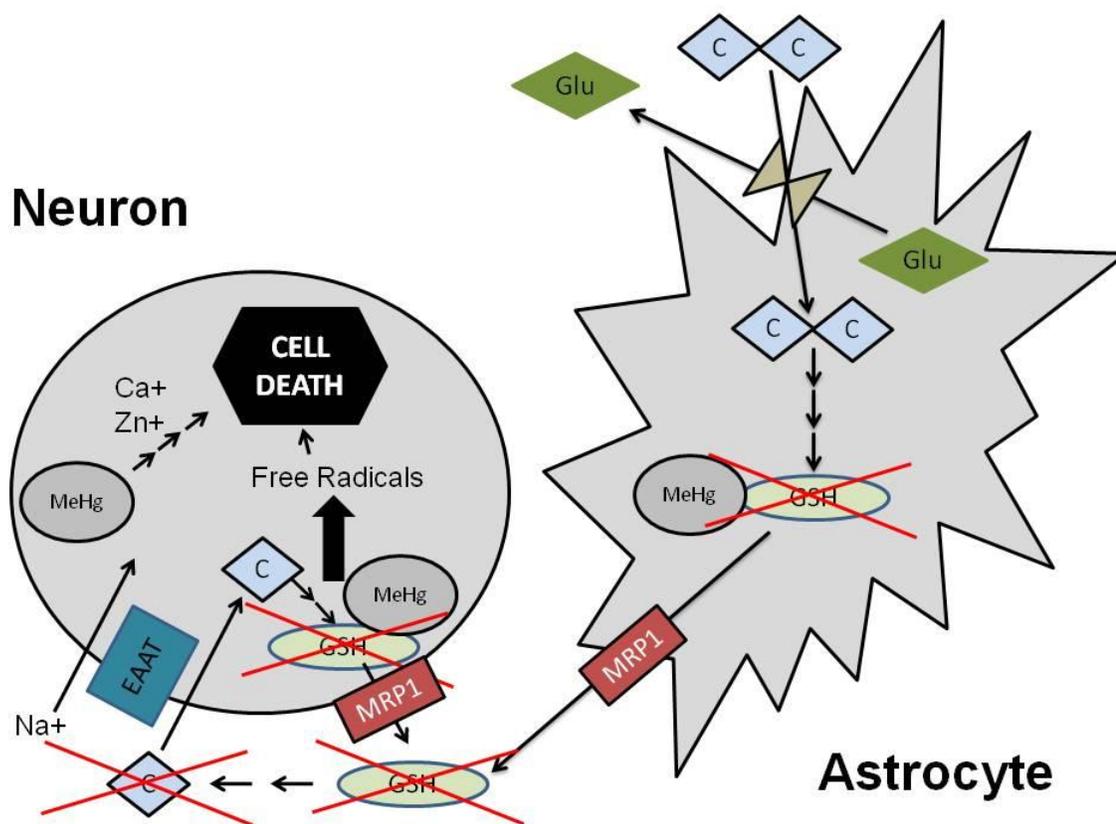
FIGURE 6.1. MeHg effects on GSH cycling.

Upon accessing the cytosolic compartment, MeHg is able to conjugate to free mono-sulfide

glutathione (GSH) via suflydryl bond. Glutathione conjugation to MeHg leads to a depletion of

GSH available for homeostatic use as an antioxidant. Once conjugated, the MeHg-GSH complex

is exported from the cell via MRP-1, thereby limiting the damaging effects of MeHg. However,

depletion of GSH may lead to accumulation of free-radicals produced endogenously or by some

mechanism induced by MeHg in/directly. MeHg has other well-defined damaging effects,

namely disruption of calcium and zinc ion homeostasis.

Combinatorial Neurotoxicity

The major finding from my studies of MeHg and BMAA was the identification of their convergence on the glutathione system. While these agents are neurotoxic by themselves at relevant concentrations, of greater concern is that these toxicants can act synergistically to cause significant cell death at concentrations that are not toxic in isolation. Specifically, the data suggest that MeHg, at a concentration that does not cause neuronal death, becomes neurotoxic when paired with a concentration of BMAA that is also not toxic by itself. Given that previous research in the Lobner lab has demonstrated that both of these compounds can adversely affect the glutathione system, the hypothesis is that MeHg and BMAA act synergistically by attacking separate arms of the glutathione cycle. Indeed, when MeHg and BMAA insults were combined, cellular glutathione levels were depleted, an effect not seen with either compound alone at these relatively low concentrations. The combined exposure was necessary to induce neurotoxicity, and the toxicity was blocked by application of the antioxidant trolox or by glutathione supplementation. This suggests that oxidative stress and glutathione depletion play key roles in the toxicity induced by these two toxicants. A model of combined exposure can be seen in FIGURE 6.2.

Since each of these agents has been implicated in the etiology of neurodegenerative disease (i.e. AD, PD and ALS/ALS-PDC) and exposure to more than one of these compounds is increasingly likely due to their ubiquity and overlapping routes of exposure, it is important that their effects are understood in combination. This study is a platform for future work with these compounds. Of course, MeHg and BMAA are not the only toxic compounds present in the environment. MeHg and BMAA are also not the only environmental neurotoxicants that disrupt the glutathione system or cellular redox state. Genetic factors, many of which are implicated in

sporadic neurodegenerative diseases, are also known to negatively impact these measures. Thus, many factors (i.e. multiple environmental toxicants and genetic factors) may converge in an immensely complicated manner to produce a cell-type specific disease. While studies must eventually be designed to demonstrate these complicated interactions, the data within this thesis contribute important first steps in identifying cellular targets common to environmental toxicants and demonstrating a proof of principle synergistic action by exploiting these targets.

Others have previously investigated the mechanism(s) of other neurotoxicants likely to be co-exposed. Polychlorinated biphenyls (PCBs) are also found in fish. Low-dose exposures to PCBs and MeHg during periods of development are now recognized to cause long-term neurological deficits (Vitalone et al., 2010). Iron and paraquat act synergistically to cause death of dopaminergic neurons of the substantia nigra, perhaps leading to increased likelihood of developing PD in individuals co-exposed to these toxicants (Peng et al., 2010). Recent studies have demonstrated that arsenic and fluoride are often both present in water supplies, and have begun investigating neurotoxic mechanisms of these compounds in combination (Chouhan and Flora, 2010). While many of these studies are quite recent, the scientific community has long recognized the impact of exposure to multiple toxins. Early studies examined combined exposure of high manganese levels and ethanol (Shukla et al., 1978), or multiple pesticides in factory workers producing these products (Peters et al., 1986). Another important new prospect in this direction is that of combining environmental factors with genetic susceptibilities suspected as risk factors for disease. An important first study in this area found that chronic, low-dose exposure to dietary MeHg hastened the onset and progression of ALS-like symptoms in the SOD1-G93A rat model of ALS (Johnson et al., 2011). It is expected that more studies will appear in the near future providing enormous insight into the etiology of these debilitating disease.

The next logical steps will involve research designed to extend our understanding of the role of environmental and genetic factors that can lead to neurodegenerative disease. These studies should include combinations of two and more environmental toxicants on wild-type *in*

vitro and *in vivo* models. *In vivo* studies of combined environmental toxicants in wild-type and genetically predisposed animal models (such as SOD1-G93A rodents) should also be conducted. Epidemiological studies to identify genetically susceptible populations also exposed to environmental toxicant(s) would be immensely helpful in understanding these relations. The data presented in this thesis provide evidence suggesting environmental exposures to toxicants in isolation or in combination, may compromise neuronal health and potentially lead to development of neurodegenerative disease.
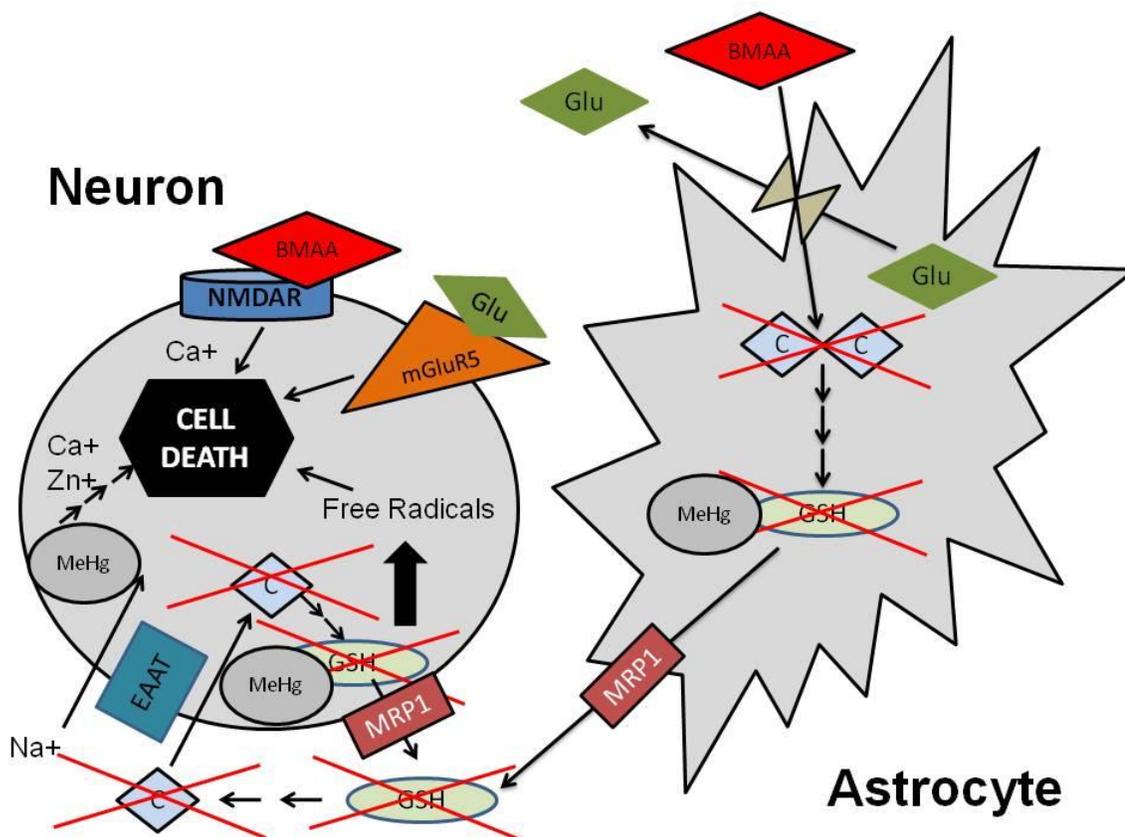
FIGURE 6.2. Combined effects of MeHg and BMAA on GSH cycling.

MeHg and BMAA both impact glutathione cycling leading to synergistic enabling of individual

toxic effects. By competing for cystine import via system x_c^-, BMAA decreases glutathione

production thereby rendering neurons and astrocytes more susceptible to MeHg. Additionally,

simultaneous stimulation of NMDA receptors by BMAA directly and stimulation of metabotropic

glutamate receptors (mGluR 5) by glutamate released via system x_c^- in exchange for BMAA and

cystine exacerbates the calcium and zinc dishomeostasis imposed by MeHg.

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TABLE OF ABBREVIATIONS

Table of Abbreviations

AC	adenylyl cyclase
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
ALS-PDC	Amyotrophic Lateral Sclerosis-Parkinson's-like Dimentia Complex
AO	antioxidant
APV	D-2-amino-5-phosphovaleric acid
ASChI	acetylthiocholine iodide
BBB	blood-brain barrier
BMAA	beta-N-methylamino-L-alanine
CaNa2EDTA	calcium disodium ethylenediaminetetraacetate
CGN	cerebellar granular neuron
CHX	cycloheximide
CNS	central nervous system
CPF	chlorpyrifos
CPG	S-(4)-carboxyphenylglycine
CPO	chlorpyrifos-oxon
CysGly	cysteinylglycine
DCF	dichlorodihydrofluorescein
DIV	day *in vitro*
DMPS	2,3-dimercaptopropane-1-sulfonate
DMSA	dimercaptosuccinic acid
DPA	D-penicillamine
DZN	diazinon
DZO	diazinon-oxon
EAAT	excitatory amino acid transporter
GFAP	glial fibrillary acidic protein
GSH	glutathione
GSHME	glutathione monoethyl ester
GSSG	glutathione (disulfide)
GST	glutathione-S-transferase
GSx	glutathione conjugates
LDH	lactate dehydrogenase
MeHg	methylmercury
MRP-1	multidrug resistance protein-1
NAC	N-acetylcysteine
NBQX	6,7-dinitroquinoxaline-2,3-dione
NRF2-ARE	nuclear factor (erythroid derived-2)-like 2-antioxidant response element

OP	organophosphorous pesticides
OPIDP	organophosphate-induced delayed polyneuropathy
PBN	N-phenyl-2-naphthylamine
PCBs	polychlorinated biphenyls
PD	Parkinson's Disease
PITC	phenylisothiocyanate
ROS	reactive oxygen species
TCP	3,5,6-trichloropyridinol
TEA	triethylamine
ZVAD	Z-Val-Ala-Asp-fluoromethylketone