Characterization Of Methylmercury Demethylation In The Central Nervous System

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Abstract

The toxicity of mercury is dependent on its chemical composition at its point of entry and site of toxicity. Differences in toxicity indicate that inorganic mercury (iHg) and methylmercury (MeHg) mediate adverse reactions via different mechanisms. Based on the available toxicity data, demethylation of MeHg to iHg has proven detrimental as it increases the severity of toxic insult. Accordingly, an in vitro system was established in order to characterize the demethylation reaction using primary astrocytes from neonatal rat cerebellum. Incubation of MeHg with a pro-oxidant increased the rate of demethylation (control vs. rotenone = -1.86±5.57% vs. 16.27±2.68%, $p<0.05$) and accumulation (control vs. rotenone = 86.53±4.14ng/mg vs. 123.6±3.80ng/mg, $p<0.001$) relative to control. These findings suggest that demethylation is not only harmful as a result of increased iHg levels, but also because total mercury is increased. In light of rising atmospheric mercury levels, it is important that this pathway be fully characterized.

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Glossary

Anti-oxidant: A molecule capable of neutralizing a free radical.

Asphyxiation: A condition in which the amount of oxygen decreases and carbon dioxide increases. This leads to a loss of consciousness and death in more severe cases. Neonatal animals contain special oxygen carriers which are more resistant to low oxygen levels.

Astrocytes: Support cells in the brain. Astrocytes are responsible for maintaining the blood-brain-barrier and carry out metabolic and immunological support for the neurons.

Blood-brain-barrier (BBB): A specialized pairing of endothelial cells (blood vessels cells) and astrocytes which provide a strong barrier to prevent contents present in blood from entering the brain.

Cell: The basic unit of life. Cells are comprised of organelles which are essential for proper function.

Central Nervous System (CNS): The part of the nervous system which encompasses the brain and spinal cord.

Demethylation: The conversion from organic mercury to inorganic mercury

Electron transport chain: A series of enzymes which function to produce energy as ATP (see mitochondrion) in the mitochondrion through movement of protons and electrons.

Endogenous: Something present in an organism that was produced by that organism.

Exogenous: Something present in an organism that was not produced by that organism.

FACS: Flow assisted cell sorting. An analytical device used to sort and quantify particles based on size, granularity, and presence of specific fluorescent markers.

Free Radical: A highly reactive atom or molecule with a free electron.

Glia: The "housekeeping" cells in the brain. Glial cells (astrocytes, microglia, and oligodendrocytes) are responsible for maintaining and optimizing the microenvironment of the nervous system.

Glutathione (GSH): A molecule consisting the amino acids glycine, cysteine, and glutamate. GSH is involved in many metabolic reactions and also acts as an anti-oxidant.

***In vitro*:** Latin for "in glass". In biological terms, this refers to growing biological cells and tissues in specialized dishes.

***In vivo*:** Latin for "in life". This refers to subjecting a whole animal to an experiment while still living.

Minamata disease: Mercury toxicity resulting from chronic exposure to mercury contaminated fish from Minamata Bay, Japan.

Mitochondrion: An organelle within the cell which is responsible for the cell's energy production in the form of adenosine triphosphate (ATP). The number of mitochondria within a cell is proportionate to the cell's energy requirements.

Neurons: The cells of the nervous which transmit electrical impulses for the purpose of thought, emotion, and movement.

Oxidative stress: An overwhelming excess of free radicals which surpass an organism's antioxidant capabilities.

Postnatal: After-birth

Pro-oxidant: A molecule which itself releases or increases production of free radicals. Anti-oxidants in high concentrations have the potential to become pro-oxidants.

Reactive Oxygen Species (ROS): A free radical which contains oxygen.

Superoxide anion: A highly reactive free radical which is formed when an extra electron binds to an oxygen molecule.

Synchrotron: A high powered particle accelerator used for a variety of scientific applications spanning several disciplines.

Toxicant: Any chemical, naturally-occurring or anthropogenic in origin, that causes an adverse effect to an organism

Toxicokinetics: The study of the effects of a biological system on a chemical. This includes the absorption, distribution, metabolism, and elimination.

Toxicodynamics: The study of the effects of a chemical on a biological system. For example, increased neurotransmitter levels in the brain following exposure to mercury.

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1 Introduction

1.1 Mechanisms of mercury toxicity

The primary mechanism by which mercury, both inorganic (iHg) and organic (MeHg), exerts its toxic effects is through the binding of sulfhydryl groups on protein residues which may lead to altered protein function (2005b; Cabanero et al., 2005; Costa et al., 2004; Fitsanakis & Aschner, 2005; Gailer et al., 2000; Szasz et al., 2002). The manifestation of Hg toxicity is likely due to secondary effects including alterations of calcium homeostasis (Limke et al., 2004), and generation of reactive oxygen species (ROS) (Dreiem et al., 2005; Chapman & Chan, 1999; Allen et al., 2002); which ultimately lead to cell death and other toxic responses.

1.1.1 Sulfhydryl Binding

The binding of mercury to proteins containing sulphur groups has been noted in the literature in both iHg and MeHg (ATSDR, 1999). This mechanism results in a broad range of toxicities consequential of the modification of protein structure and function (Costa et al., 2004) although there appears to be some degree of specificity (Limke et al., 2004) as noted by the distribution of MeHg in the adult brain (Eto, 2000). This may therefore translate to various pathologies: Allen *et al.* (2001) noted an inhibition of the enzyme glutamine synthetase, in part, by thiol binding. Moreover, Hg has been shown to inhibit sarcoplasmic ATPases resulting in alteration of calcium homeostasis (Abramson et al., 1983).

1.1.2 Alterations to calcium homeostasis

Hg-mediated toxicity, as described above, often leads to secondary effects which are the true basis for the observed toxicity. One such effect is the alteration of calcium

homeostasis. The mechanisms in which this occurs are numerous. Basu *et al.* (2005b) noted inhibition of muscarinic ACh receptors following incubation with mercury *in vitro* which has been suggested to cause calcium release from intracellular stores (Limke et al., 2004). Moreover, Hg-induced damage to organelles which store calcium, such as the smooth endoplasmic reticulum and the mitochondrion, has been noted to lead to increased intra-cellular calcium levels (Somlyo et al., 1985). The mechanism in which calcium results in its toxicity has been well characterised. An early study showed that removal of extracellular calcium increased survival of neurons *in vitro* (Choi, 1985). Elevated calcium levels can lead to cell death through either apoptosis or necrosis pathways. Calcium has been found to activate the protease calpain which, if overexpressed, can lead to cleavage of vital proteins resulting in cytoskeleton disruption and subsequent death (Wang, 2000). Additionally, activation of calcium-dependent caspases and endonucleases leads to apoptosis and may lead to necrosis in the latter. Finally, calcium-induced activation of phospholipase A_2 has been found to disrupt membrane stability leading to necrosis (Boelsterli, 2003).

1.1.3 Oxidative Stress

Mitochondria have been implicated as targets of MeHg – likely due to their lipophilicity (Aramaki et al., 2004) - leading to the formation of reactive oxygen species (ROS) (Dreiem et al., 2005). The presence of ROS in Hg toxicity cases has been reinforced by studies showing that antioxidants, such as vitamin E and selenium, increases survival in murine *in vivo* models (Beyrouty & Chan, 2006). Using primary rat neuron cultures, Dringen *et al.* (1999) showed that the major deoxidizing pathway in neurons involves catalase suggesting a rationale for greater susceptibility to toxicity in neurons than glial cells. Furthermore, Ali *et al.* (1992) found increased ROS production in rat synaptosomes, using

the fluorescent dye 2',7'-dichlorofluorescein-diacetate, especially in the cerebellum. It has been suggested that ROS production from mercury arises from homolytic cleavage of the carbon-mercury bond resulting in an alkyl radical (Ganther, 1978). However, if this were the source of ROS, the production of free radicals would only be equivalent to the rate of demethylation. As discussed in section 2, demethylation is a very slow process. Accordingly, this is not a significant source of ROS if it is indeed correct. A mechanistic study investigating the source of ROS in the brain found that mercury inhibited the ubiquinol: cytochrome c oxidoreductase region (complex III) of the electron transport chain (Yee & Choi, 1996).

Oxidative stress may induce cytotoxicity by damaging DNA, proteins, or lipids. Damage to DNA in the form of base or sugar oxidation may lead to strand breaks, crosslinkages between two molecules (DNA-DNA or DNA-protein), and errors in DNA replication – all leading to cell death or tumour formation (Boelsterli, 2003). Similarly, oxidative protein damage can result in protein-protein cross-linking, fragmentation, and alternate folding if side chains are affected (Josephy, 1996). Lipid oxidation is slightly different in that the interaction of a lipid with a ROS results in the creation of a reactive lipid. Directly, lipid oxidation can lead to membrane damage and indirectly to any number of other forms of oxidative damage (Boelsterli, 2003).

1.1.4 Glutathione

As an addendum to oxidative stress, it is prudent to make mention the role of glutathione (GSH). GSH is a three amino acid complex consisting of cysteine, glutamate, and glycine. It functions to metabolize compounds by binding through its sulphur group and acts as an antioxidant and biomarker by quenching radicals through the formation of a

dithiol linkage with another GSH molecule (Josephy, 1996). Dringen *et al.* (1999) noted that neurons are less capable of handling oxidative stress than astrocytes, suggesting that GSH deficiencies play a key role in Hg-mediated toxicity in neurons.

As mentioned above, Hg has the potential to cause toxicity mediated through the production of ROS. Astrocytes have been shown to be more resistant to cytotoxicity than neurons using *in vitro* models (Allen et al., 2002; Dringen et al., 1999; Morken et al., 2005). It should be noted, however, that a study conducted in monkeys showed a decrease in astrocytes and no change in numbers of neurons in chronic MeHg exposure studies (Charleston et al., 1996). This is in contrast to human cases in which chronic exposure showed loss of neurons in adults - particularly granule cells within the cerebellum and neuronal swelling in the cerebral cortex (Eto, 2000). Acute studies found loss of neurons and reactive proliferation of astrocytes (Eto et al., 1999). A rationale for the results seen through the *in vitro* models deals with the role of cysteine transport from astrocytes to neurons through a Na^+ dependent cysteine transporter – a transporter which is absent in neurons and is controlled by glutamate and aspartate (Allen et al., 2002). Morken *et al.* (2005) showed that treating neuroglia co-cultures with 5μM MeHg increased neuronal GSH levels which improved survival rates suggesting a protective role of astrocytes on neuronal endurance.

1.1.5 Glutamate Excitotoxicity

Glutamate, the most common excitatory neurotransmitter in the brain, has been shown to exhibit toxicity when in excess in the extracellular matrix of the brain (Fitsanakis & Aschner, 2005). As previously mentioned, the cerebellum is a major target of Hg toxicity (Costa et al., 2004). This may be related to glutamate toxicity as the cerebellum is one of the most abundant areas of glutaminergic receptors in the brain (Matyja & Albrecht, 1993).

Following exposure to Hg, analyses have shown that there is a decrease in glutamate levels in neurons and an increase in astrocytes (Morken et al., 2005). This may be explained by the findings by Allen *et al.* (2001) that glutamine synthetase – the enzyme responsible for the conversion of glutamate to glutamine in astrocytes – is inhibited by $HgCl_2$ in vitro. Further studies have shown impairment of the excitatory amino acid transporters (EAAT) resulting in a build-up of glutamate in astrocytes and the extracellular matrix and a decrease inside neurons (Fitsanakis & Aschner, 2005). Excess glutamate in the extracellular space is thought to overstimulate the NMDA receptor (Allen et al., 2001; Allen et al., 2002; Fitsanakis & Aschner, 2005; Mariussen & Fonnum, 2001; Matyja & Albrecht, 1993) resulting in alterations to calcium homeostasis (Choi, 1985). Influxes of calcium result in a change in membrane potential and initiation of apoptosis (Limke et al., 2004).

Boveris and Chance (1973) noted that the addition of glutamate increased ROS levels in mitochondria from pigeon hearts. A surplus of glutamate can cause impairments of Na^+-dependent cysteine transporters (Allen et al., 2002) leading to deficiencies in glutathione in neurons. This is likely why Dringen *et al.* (1999) found that neurons are less capable of detoxifying ROS by the GSH pathway than astrocytes.

1.1.6 Selenium

The role of selenium has been extensively studied as a possible treatment for Hg toxicity because, when co-administered, it appears to reduce the toxicity of iHg (Gailer et al., 2000) and, when administered in combination with vitamin E, decreases MeHg toxicity (Beyrouty & Chan, 2006). Kasuya (1976) noted a four-fold increase in effectiveness in selenite when compared to the selenate species stating that a dose of $2x10^{-6}$M sodium selenite was sufficient to protect against 10^{-5}M MeHg when co-administered with primary

cerebellar neurons. *In vivo* studies have made similar findings. In Hybro-G female broiler chickens, addition of 0.2 mg/kg feed of both iHg and selenium reduced total Hg concentrations by as much as 60-100% while chickens exposed to MeHg prior to selenium administration did not show significant antagonistic effects from selenium (Cabanero et al., 2005). In pike (*Esox lucius*), dietary intake of selenium reduced the amount of mercury absorbed from contaminated perch consumption by 5-11% but had no effect on MeHg absorption from water (Cuvin-Aralar & Furness, 1991) suggesting that the protective effect of selenium occurs prior to absorption. Separately, Se and Hg contribute to oxidative stress in mallard ducks; however, when co-administered, they decrease toxicity (Hoffman & Heinz, 1998). It has been reported that the addition of selenium before Hg results in the methylation of selenium to dimethylselenide which is said to be toxic (Gailer et al., 2000). Interestingly, Yamane *et al.* (1977) found that ^{14}C-labelled MeHg acted as a methyl donor to dimethylselenium, which was immediately exhaled (and is therefore non-toxic), resulting in the formation of iHg.

1.2. Rationale for experiment

MeHg and iHg have been compared in a number of studies (Monnet-Tschudi et al., 1996; Basu et al., 2005b; Chapman & Chan, 1999). It has been found that although MeHg can readily cross the blood-brain barrier, iHg is more toxic and persistent once inside the brain (Dringen et al., 1999; Yasutake et al., 1998). Oxidative stress plays a key role in the demethylation pathway. The ability of antioxidants such as selenium and vitamin E to moderate toxicity through moderating demethylation is of key interest in decreasing the potential for toxicity.

1.3. General thesis objective

There is overwhelming support for the theory of demethylation of MeHg in mammalian systems (Allen et al., 2001; Charleston et al., 1996; Hansen & Danscher, 1995; Monnet-Tschudi et al., 1996; Vahter et al., 1994; Vahter et al., 1995; Watanabe, 2002; Yasutake & Hirayama, 2001). The different rates of elimination of the two mercury species (Dringen et al., 1999; Yasutake et al., 1998) suggest that demethylation is a detrimental consequence. Although there have been mechanisms proposed to explain this reaction in the liver (Suda & Hirayama, 1992; Yasutake & Hirayama, 2001), no functional studies on this reaction have been evaluated in the brain to date.

This thesis will examine the mechanism of demethylation of MeHg to iHg *in vitro* using primary astrocytes from rat cerebellum. First, an attempt will be made to establish a culture system in which doses low enough to be sublethal but sufficient for detection are utilized. Secondly, I will determine the effect of ROS on mercury concentration. Finally, I will attempt to identify the role of ROS in MeHg demethylation. The findings of this research may aid in decreasing mercury toxicity in exposed individuals.

1.4. General thesis hypothesis

Oxidative stress is responsible for driving the demethylation of MeHg into iHg. I believe that it can be altered such that the relative toxicity will decrease as elimination increases.

2 Publication I: Demethylation of methylmercury in the brain and its effects on neurotoxicity

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Abstract

Mercury is a naturally occurring environmental contaminant and a known neurotoxicant. A dynamic equilibrium exists between the elemental, inorganic, and organic forms, which affects the toxicokinetics. Methylmercury (MeHg) has been shown to accumulate in tissues, making it more toxicologically relevant than the non-cumulative inorganic form. However, *in vitro* data has shown that inorganic mercury (iHg) has greater potential for neurotoxicity through astrocyte dysfunction. This paper reviews the accumulation and relative toxicities of MeHg and iHg as they relate to the process of demethylation. Accumulation studies indicate that MeHg can access the brain but reaches a threshold. iHg continues to rise despite lowering mercury exposure over time. In neuronal primary cells and cell lines, MeHg is more potent a toxicant than iHg (MeHg vs. iHg 1.93±0.27 vs. 39.88±23.32, n=3). However, glia and the brain as a whole are more sensitive to iHg than MeHg. *In vivo* results were difficult to compare due to the number of possible endpoints and a lack of reported internal doses. Demethylation has been shown *in vitro* under physiological conditions within 3 weeks and *in vivo* within a matter of months. Given that MeHg is more bioavailable from oral ingestion but iHg elicits greater toxicity in the brain, demethylation may exacerbate mercury-induced toxicity. It is therefore prudent that we further investigate the mechanism of demethylation in the brain to decrease mercury neurotoxicity.

Keywords: Demethylation, Methylmercury, Inorganic Mercury, Brain

Introduction

Mercury is an omnipresent environmental contaminant. Its accumulation in the environment is the consequence of both natural and anthropogenic factors: the former results from erosion of mercury-containing earth by wind and water and from volcanic activity. The latter is, in essence, an acceleration of the natural process through industrial practices such as mining, smelting, and incineration (ATSDR 1999). Three types of mercury are present in the environment. Elemental mercury (Hg^0), a silver liquid commonly found in thermometers, certain fungicides, and electrical switches, has been noted to volatilize causing a significant exposure from inhalation in occupational settings (Tang & Li, 2006). Inorganic mercury (iHg), which includes the Hg^+ and Hg^{2+} ions, is water soluble and is often bound to negatively charged elements forming mercury salts (U.S.EPA., 1997a). Organic mercury compounds, of which methylmercury (MeHg) is the most abundant, are highly lipophilic substances created by methylation of iHg through both abiotic and biotic pathways. Abiotic factors include UV radiation from the sun (Siciliano et al., 2005) and the complexing of methylated compounds to iHg in aquatic ecosystems (Celo et al., 2006). Biotic methylation occurs mainly in anoxic conditions where sulphates are limiting by sulphate reducing bacteria in aquatic sediments (Compeau & Bartha, 1985).

Absorption of MeHg and iHg by oral exposure is 85-100% and 7-15%, respectively, making MeHg the more toxicologically relevant form of mercury (National Research Council, 2000). The primary route of exposure to MeHg in humans is through the consumption of fish and marine mammals.

Hg speciation – the conversion between species of mercury - is an important factor for determining the internal dose in the target organ (i.e. the CNS). Toxicokinetics including absorption, distribution, and elimination are all governed by the species of mercury.

Yasutake and Hachiya (2006) found accumulation of iHg in rat hair following exposure to 20μg $HgCl_2$/mL drinking water which was about 1/6 that of MeHg accumulation. Intraperitoneal exposure of MeHg in pregnant Sprague-Dawley rats showed liver and kidney concentrations of total mercury to be approximately double that of the brain in 1 and 7 day postpartum progeny (Smith et al., 1983). Human autopsy cases from Japan following MeHg exposure have reported accumulation rates in hundreds of ng/g for the liver and kidneys and only a tenth of the concentration in the cerebellum and cerebrum (Matsuo et al., 1989).

Differences in the kinetics of accumulation also exist between iHg and MeHg. A study in rats looking at mercury vapour as a source of iHg determined a biological half-life of over 30 weeks following 12 hour exposure to 25 $\mu g/m^3$ of mercury vapour in air (Pamphlett & Coote, 1998). Another study in rats revealed a biological half-life of approximately 7 days following a single intravenous injection of 0.5mg/kg MeHg (Sundberg et al., 1998). MeHg feeding trials in monkeys investigated the toxicokinetics in the thalamus. Levels of MeHg reached a plateau at about 12 months of dosing while iHg continued to rise (Charleston et al., 1996) suggesting a half-life of MeHg in the order of months and an indeterminate clearance rate for iHg. Voluntary ingestion of ^{203}Hg-methylmercury revealed a whole body half-life of 70-80 days while blood half-lives ranged from 48-53 days (Miettinen et al., 1971), which is consistent with previous findings (Aberg et al., 1969). In contrast, biological half-lives for iHg are 60 days in whole body which is consequent of retention in the kidneys following either inhalation of mercury vapour and oral consumption of iHg (Agency for Toxic Substances and Disease Registry, 1999). Using oral doses of iHg in human volunteers, Rahola *et al.* (1973) found a half-life of 20 days in the brain.

The above data suggests that MeHg has greater potential to accumulate in the body than iHg with the exception of in the kidneys. However, demethylation has been seen

observed over time in both animal models (Charleston et al., 1996; Pamphlett & Coote, 1998; Vahter et al., 1995) and human populations (Eto, 2000; Amin-Zaki et al., 1974). The determining factors for the process of demethylation, particularly in the CNS, are not fully understood.

The purpose of this paper is to review the difference between the toxicities of iHg and MeHg in the CNS. We also want to investigate the potential factors affecting demethylation - the conversion from MeHg to iHg - in different brain regions.

Accumulation in the CNS from Oral Exposure

Chronic exposure data from Minamata, Japan has shown that MeHg has the ability to accumulate in the brain (Takeuchi & Eto, 1999). Although exposure data from human volunteers exists, the number of volunteers per study and doses do not provide adequate data for accumulation studies. Accordingly, this section will address quantitative data collected in rats and monkeys following oral ingestion of MeHg as summarized in Table 1. The values expressed in the column "*% of total ingested*" were calculated according to the formula presented in Figure 1.

$$Percent\ accumulated = \frac{Brain\ Hg(\mu g / g)\ x\ brain\ mass(g)}{Oral\ Dose(mg / kg)\ x\ Body\ Mass(kg)\ \mathrm{x}\ 1000\mu g/\mathrm{mg}} \cdot 100\%$$

Figure 1: Calculation of percent mercury accumulation in the brain following oral exposure. Values are given in Table 1. Reported brain and body masses were used where applicable. Reported averages were used when brain and body masses were not given.

Table 1: Accumulation in the CNS from oral consumption

Rat							
Tissue type	Ingested quantity of MeHg	Number of doses	External Dose (mg/kg)	Internal Dose (μg/g)	Clearance Time (days)	% of total ingested accumulated in the brain	Reference
Whole brain PD1	1 mg/kg/day	10	10	1.58	0	0.84	(Sakamoto & Nakano, 1995)
Whole brain PD1	10 mg/kg/day	10	100	20.3	0	1.08	(Sakamoto et al., 1993)
Whole brain PD14	1 mg/kg/day	10	10	2.34	0	0.56	(Sakamoto & Nakano, 1995)
Whole brain PD14	10 mg/kg/day	10	100	30.4	0	0.73	(Sakamoto et al., 1993)
Whole brain PD35	1 mg/kg/day	10	10	1.42	0	0.15	(Sakamoto & Nakano, 1995)
Whole brain PD35	10 mg/kg/day	10	100	24.3	0	0.26	(Sakamoto et al., 1993)
Cerebellum Adult Male	5mg/kg/day	12	60	14.0	13	0.01	(Yamashita et al., 1997)
Whole Brain Adult Male	10mg/kg	1	10	3.78	5	0.19	(Clausing et al., 1984)
Whole Brain Adult Female	0.84mg/kg 5 times/week	4	3.36	1.00	0	0.22	(Magos & Butler, 1976)
Whole Brain Adult Female	0.84mg/kg 5 times/week	9	7.56	2.20	0	0.22	(Magos & Butler, 1976)
Whole Brain Adult Female	0.84mg/kg 5 times/week	14	11.76	2.90	0	0.19	(Magos & Butler, 1976)
Whole Brain Adult Female	3.36mg/kg 5 times/week	4	13.44	4.20	0	0.24	(Magos & Butler, 1976)
Whole Brain Adult Female	3.36mg/kg 5 times/week	9	30.24	9.80	0	0.24	(Magos & Butler, 1976)
Whole Brain Adult Female	3.36mg/kg 5 times/week	14	47.04	16.3	0	0.26	(Magos & Butler, 1976)

Cerebrum PD1	5mg/kg/day	30	150	18.9	0	0.21	(Sakamoto et al., 1998)
Cerebellum PD1	5mg/kg/day	30	150	18.5	0	0.07	(Sakamoto et al., 1998)
Whole Brain Male	1mg/kg/day	1	1	0.22	7	0.11	(Suda et al., 1989)
Whole Brain Male	5mg/kg/day	1	5	1.25	7	0.13	(Suda et al., 1989)
Whole Brain Male	5mg/kg/day	4	20	5.30	7	0.13	(Suda et al., 1989)
Whole Brain Male	5mg/kg/day	8	40	12.5	7	0.16	(Suda et al., 1989)
Whole Brain Male	5mg/kg/day	12	60	23.6	7	0.20	(Suda et al., 1989)
Monkey							
Tissue type	Ingested quantity of MeHg	Number of doses	External Dose (mg/kg)	Internal Dose (µg/g)	Clearance Time (days)	% of total ingested accumulated in the brain	Reference
Whole Brain Infant	0.02mg/kg	4	0.08	0.1	0	12.798	(Burbacher et al., 2005)
Thalamus Female	0.05mg/kg/day	180	9	1.7	0	0.037	(Vahter et al., 1994)
Occipital Lobe female	0.05mg/kg/day	180	9	0.6	0	0.006	(Vahter et al., 1994)
Thalamus Female	0.05mg/kg/day	360	18	1.8	0	0.018	(Vahter et al., 1994)
Occipital Lobe female	0.05mg/kg/day	360	18	0.3	0	0.001	(Vahter et al., 1994)
Thalamus Female	0.05mg/kg/day	480	24	1.6	0	0.012	(Vahter et al., 1994)
Occipital Lobe female	0.05mg/kg/day	480	24	0.3	0	0.001	(Vahter et al., 1994)
Thalamus Female	0.05mg/kg/day	360	18	0.1	180	0.001	(Vahter et al., 1994)
Occipital Lobe female	0.05mg/kg/day	360	18	0.0	180	0.000	(Vahter et al., 1994)
Thalamus Female	0.05mg/kg/day	180	9	3.517	0	0.077	(Charleston et al., 1996)
Thalamus Female	0.05mg/kg/day	360	18	5.133	0	0.057	(Charleston et al., 1996)
Thalamus Female	0.05mg/kg/day	480	24	5.162	0	0.043	(Charleston et al., 1996)
Thalamus Female	0.05mg/kg/day	360	18	0.775	180	0.009	(Charleston et al., 1996)

Using the chemical abstracts database for MeHg from SciFinder Scholar and the keywords accumulation and brain, we found 21 examples of MeHg dosing ranging from 0.84mg/kg/day to 5mg/kg/day, in rats, for a period ranging from 1 to 30 days. External doses ranged from.1 to 150 mg/kg and internal doses ranged from 0.22 to 30.4 μg/g. Percent of accumulation from oral ingestion ranged from 0.01 to 1.08%. Discrepancies between external and internal doses are resultant of different tissues and reported brain regions.

Monkey studies are rare, although thorough. Two studies were found in which female monkeys were exposed chronically (3 to 18 months). Additionally, one sub-chronic study examined whole brain in infants. The infant study reported a substantially higher percent ingested relative to brain and body mass when compared to the adult study (12.798% vs. $0.0218 \pm 7.4 \times 10^{-3}$%). Dosing for the adult monkeys was 0.05mg/kg/day while the infant monkeys in the Burbacher *et al.* (2005) study received 0.02mg/kg/day. Accumulation in the adult studies ranged from 0 to 0.077% of the ingested quantity relative to brain and body mass. Interestingly, the internal doses reported by Charleston *et al.* (1996) were about 2-3 fold higher than those reported by Vahter *et al.* (1994).

It is difficult to draw conclusions involving the monkey data given the relatively few studies. Moreover, rat data is very short term (1-30 days) when compared to that of monkeys (3-18 months). This is likely due to the cost and labour intensiveness of working with primates when compared to rodents.

Charleston *et al.* (1996) was the only report that we found to show speciation of mercury in the brain and report both internal and external doses. Following 6 months of MeHg exposure, 11% of the total mercury in the brain was iHg. This increased in the 12 and 18 month exposure groups to 12% and 33%, respectively. In a fourth treatment group, in which monkeys were exposed for 12 months (as previous) then a 6 month clearance period,

79% of the total mercury (0.775 μg/g wet weight) in the brain was iHg. In this experiment, iHg was slightly higher than the normal 12 month exposed group (0.608 vs. 0.616 μg/g wet weight) while MeHg was substantially lower in the clearance group (4.526 vs. 0.159 μg/g). This shows that the rate of demethylation exceeds the rate of elimination of iHg from the brain (Charleston et al., 1996).

Experimental findings from rat and primate models have revealed that minute but significant amounts of mercury accumulate in the brain following oral exposure. Variability may be resultant of the use of average brain masses when actual masses were not reported. Chronic MeHg exposure has been shown to decrease brain weights and is related to age of exposure (Sakamoto & Nakano, 1995) and brain region of interest (Pan et al., 2005). Therefore, calculated accumulation rates may be slightly lower and more inconsistent than actual values. However, the general trends – such as females accumulating more than males – should remain consistent. Conflicting results make it impossible to differentiate between groups at the neonatal stage. What is clear, however, is that infants accumulate more mercury in their brains following oral exposure than adults.

Relative Potencies of Organic and Inorganic Mercury on Neuronal Endpoints

The literature surrounding the relative toxicities of organic and inorganic mercury is somewhat divided. Discrepancies in the data may be attributed to the specific endpoint tested. This section summarizes the literature comparing both organic and inorganic mercury on various endpoints. *In vitro* data are tabulated in Table 2. *In vivo* studies are summarized in Table 3.

In Vitro

The cases described in Table 2 show conflicting results with respect to which mercury species is the more potent toxicant. Interestingly, MeHg was the more potent

species as measured by LD_{50} in all cases where purified neuronal cultures and cell lines (MeHg vs. iHg 1.93±0.27 vs. 39.88±23.32, n=3).In contrast, whole brain studies and purified astrocyte cultures showed greater toxicity when treated with iHg with the exception of the 25 day old culture conducted by Monnet-Tschudi *et al.* (1996). This suggests that there may be a different mechanism for toxicity in neurons and glia. This is in agreement with *in vitro* tests which show different rates of accumulation and metabolism of neurons and astrocytes (Syversen et al., 2006; Aschner et al., 2007).

In an immature differentiated brain cell culture assay, iHg was reported to induce far greater toxicity than MeHg in astrocytes as assessed by glutamine synthetase activity (Monnet-Tschudi et al., 1996) and is consistent with results involving the use of purified astrocyte cultures (Allen et al., 2001). However, in more differentiated cultures, MeHg induced slightly greater toxicity than iHg in astrocytes and oligodendrocytes and drastically greater toxicity in neurons (Monnet-Tschudi et al., 1996). It has been suggested that the discrepancy is related to a holistic response caused by neuron dysfunction (Monnet-Tschudi et al., 1996) likely resulting from the establishment of cell-cell interactions such as gap junctions.

Table 2: Summary of the available toxicity data comparing MeHg and iHg toxicities *in vitro*

Cell line					
Tissue type	MeHg Quantity	iHg Quantity	Endpoint	Remarks	Reference
HEK 293 (human kidney cells)	13.0μM	0.63μM	IC_{50} Neuronal T-type Ca^{2+} channel inhibition	iHg is a more potent inhibitor of T-type Ca^{2+} channels than MeHg	(Tarabova et al., 2006)
NSC-34 (neuron spinal cord hybrid)	1.74μM	7.95μM	LD_{50}	MeHg is a more potent inducer of cell death	(Chapman & Chan, 1999)
N18TG-2 (neuroblastoma)	1.59μM	85.3μM	LD_{50}	MeHg is a more potent inducer of cell death	(Chapman & Chan, 1999)
Rat					

Tissue type	MeHg Quantity	iHg Quantity	Endpoint	Remarks	Reference
neonatal Cortical astrocytes	10 μM	5 μM	Glutamine Synthetase from Astrocytes	iHg significantly lowered glutamine synthetase activity by 45% ($p<.001$), MeHg inhibition was not significant	(Allen et al., 2001)
Fetal telencephalon (5-15 day old culture)	1 μM	1 μM	Astrocyte, oligodendrocyte, and neuron specific enzymes	iHg toxicity was greater for astrocytes than MeHg (90% reduction of Glutamine Synthetase versus 40% in MeHg)	(Monnet-Tschudi et al., 1996)
Fetal telencephalon (25-35 day old culture)	1 μM	1 μM	Astrocyte, oligodendrocyte, and neuron specific enzymes	MeHg toxicity was greater for all cell types than iHg. Neuron specific enzymes choline acetyltransferase and glutamic acid decarboxylase were the most drastically affected.	(Monnet-Tschudi et al., 1996)
Neurons from granule layer Cerebellum (from PD7)	2.47 μM	26.40 μM	LD_{50}	MeHg was approx. 10-fold more cytotoxic	(Gasso et al., 2001)
Cerebral cortex	IC_{50}: M1:3.4μM M2:149μM	IC_{50}: M1:2.2μM M2:5μM	50% binding inhibition	For M2 binding inhibition, iHg was 30 times as potent as MeHg. Little difference in potency for M1 binding inhibition.	(Castoldi et al., 1996)
Dorsal root ganglia neurons	100 μM	1-10 μM	GABA-induced electrical current	MeHg: suppression of electrical current iHg: enhancement of electrical current	(Arakawa et al., 1991)
Other					
Otter, mink, mouse, human Adult Female Cortex	3.32 μM	1.67 μM	IC_{50} for muscarinic receptor binding	inhibited by iHg with greater potency than MeHg	(Basu et al., 2005b)
Otter, mink, mouse, human Adult Female Cerebellum	1.97 μM	1.7 μM	IC_{50} for muscarinic receptor binding	inhibited by iHg with greater potency than MeHg	(Basu et al., 2005b)
Otter, mink, mouse, human Cerebral	2.58-17.9 μM	1.41-8.13 μM	Inhibition of muscarinic receptor binding	Otter is most sensitive, Human is least sensitive. Cerebellum is the	(Basu et al., 2005b)

cortex				most sensitive brain region	
Otter, mink, mouse, human Cerebellum	1.59-13 μM	1.16-9.05 μM	Inhibition of muscarinic receptor binding	Otter is most sensitive, Human is least sensitive. Cerebellum is the most sensitive brain region	(Basu et al., 2005b)

The degree of toxicity is also related to the specific endpoint. For instance, iHg was 30-fold more potent an inhibitor of M2 muscarinic receptor binding than MeHg hydroxide, although there were no significant differences between the mercury species on the M1 receptor (Castoldi et al., 1996). Similarly, the neurotransmitter GABA has been shown to be affected by mercury. GABA-induced electrical current was enhanced by concentrations up to 10μM of iHg and suppressed by 100μM MeHg (Arakawa et al., 1991). These results further substantiate the theory that the mechanism of toxicity between the two species is different.

Table 3: Summary of the available toxicity data comparing MeHg and iHg toxicities in different brain regions of various species in vivo. Estimated internal doses are given in italics based on most conservative values in table 1.

Species	MeHg Quantity	iHg Quantity	Internal Dose (μg Hg/g wet tissue weight)	Remarks	Reference
Rat Sprague-Dawley adult female	0.5, 2 mg/kg/day for 16 days, oral	---	*1.83, 7.30*	Both doses significantly increased receptor density in the cerebellum. Only 2 mg/kg/day increased hippocampal receptor density.	(Coccini et al., 2000)
Rat Wistar PD2	---	4 mg/kg/day for 58 days	---	Noradrenalin elevated in olfactory bulb, visual cortex, brain stem	(Lakshmana et al., 1993)
Rat Wistar PD2	---	4 mg/kg/day for 58 days	---	Decreased acetylcholinesterase activity in hippocampus	(Lakshmana et al., 1993)
Rat Sprague-Dawley adult male	5 mg/kg/day for 7 days gavage (LOEL)	---	*5.37*	Significant decrease in MAO activity in cortex, striatum, hypothalamus, hippocampus, brain stem, cerebellum	(Chakrabarti et al., 1998)
Rat Sprague-Dawley adult male	7.5 mg/kg/day for 10 days gavage (maximum)	---	*8.05*	Significant decrease in MAO activity in cortex, striatum, hypothalamus, hippocampus, brain stem, cerebellum	(Chakrabarti et al., 1998)
Rat Sprague-Dawley adult female	0.5 mg/kg/day for 2 months, 0.1 mg/kg/day for 3 months	---	*6.85* – 2 months *2.05* – 3 months	Significantly greater release of dopamine in the brain independent of exposure route	(Faro et al., 1997)
Rat Sprague-Dawley adult female	40, 400, 4000 μM constant perfusion for 4 hours	---	---	Dopamine levels increased by 900-9000% of basal levels	(Faro et al., 2000)
Rat Sprague-Dawley adult female	40, 400, 4000 μM constant perfusion for 4 hours	40, 400, 4000 μM constant perfusion for 4 hours	---	MeHg and iHg increased dopamine levels at different times. MeHg caused far greater dopamine release. Suggests different mechanisms	(Faro et al., 2007)
Rat Sprague-Dawley adult female	---	10, 100, 1000 μM constant perfusion for 3 hours	---	Dopamine levels significantly increased in 100, 1000 μM groups	(Vidal et al., 2007)
Mouse ICR Hippocampus	2, 4, 10 mg/kg i.p.	---	---	Glutamine synthetase activity was significantly	(Kwon & Park, 2003)

				inhibited in 4, 10 mg/kg groups	
Rat Sprague-Dawley adult male	---	0, 1, 2.0 or 4.0mg/kg/day for 7 days	---	SOD activity decreased in the cerebellum but not in cortex or brain stem. GPx activity decreased (dose dependent) in the cerebellum (only significant at 4mg/kg)	(Hussain et al., 1997)
Male mice (1mg/kg), rats (5mg/kg)	1, 5 mg/kg i.p.	---	---	Significant increase in ROS production in the cerebellum from 5mg/kg MeHg. No other regions affected	(Ali et al., 1992)
Swiss albino mouse postnatal	---	0.5 mg/kg/day i.p. in mother	---	Weaning rats experienced increased Glutathione Reductase activity, impairment in motor performance in the rotarod task and decreased locomotor activity	(Franco et al., 2007)
Swiss albino mouse postnatal	---	1.5 mg/kg/day i.p. in mother	---	Weaning rats experienced increased TBARS activity	(Franco et al., 2007)
Rat Wistar	---	5 mg/kg/day for 5 days sc in PD1-5,8-12 or 17-21	---	PD1-5 group is most susceptible to toxicity as measured by behavioural deficits. No observed toxicity in PD17-21 group.	(Peixoto et al., 2007)
Rat Wistar PD1	5 mg/kg/day for 30 days	---	Cerebrum: 18.9 Cerebellum: 18.5 (*21*)	Severe hindlimb paralysis, loss of equilibrium, severe neurodegeneration	(Sakamoto et al., 1998)
Rat Wistar PD 14 & PD35	10 mg/kg/day for 10 days	---	PD14: 30.4 (*64.5*) PD35: 24.3 (*20.5*)	Weight gain was significantly lower when compared to control	(Sakamoto et al., 1993)
Rat Wistar Adult male	5 mg/kg/day for 12 days	---	14.0 (*9.2*)	Short-term increase in glutamine, nitrates, and nitrites in cerebrospinal fluid, increase in nitric oxide synthase in cerebellum	(Yamashita et al., 1997)
Monkey Adult female Contralateral Occipital Lobe	0.05 mg/kg/day for 6 months oral	---	iHg: 0.388 MeHg: 3.129 Total: 3.517 (*18*)	Majority of iHg was found in astrocytes. Astrocyte numbers declined in 6 month dosing group.	(Charleston et al., 1996)
Monkey Adult female Contralateral Occipital Lobe	---	0.2 mg/kg/day i.v.	iHg: 0.616 MeHg: 0.159 Total: 0.775	Microglia numbers increased significantly in the presence of low level iHg	(Charleston et al., 1996)

There is increasing literature investigating the use of neurochemical endpoints as biomarkers for toxicity due to their limited invasiveness and time utilization (Manzo et al., 2001). Basu *et* al. (2005a) studied the inhibitory effects of Hg on muscarinic acetylcholine (ACh) and dopamine (D2) receptors in wild river otter. A negative correlation was found between concentrations of mercury and ACh and D2 receptor levels in the cerebral cortex and cerebellum. Similar correlations existed between iHg and MeHg on receptor binding (-0.466 and -0.452, respectively) in the cerebral cortex although iHg was more potent than MeHg (Basu et al., 2005a). Further studies by Basu *et al.* (2005b) have shown that iHg is a more potent inhibitor of muscarinic receptor binding than MeHg and that the cerebellum is the most sensitive to mercury induced inhibition.

It has been shown that neurons are more susceptible to MeHg toxicity than iHg while glia, of which astrocytes are the most abundant, are more vulnerable to iHg. This discrepancy implores the question of which species of mercury is more toxic to the brain as a whole. When considering that astrocytes comprise approximately 50% of the brain by volume and that astrocytes take up much of the mercury that reaches the brain, it may be inferred that neurotoxicity is a consequence of astrocyte dysfunction (Aschner et al., 2007; Brookes, 1992; Allen et al., 2001; Dave et al., 1994). It is therefore reasonable to conclude that iHg should be the species of greatest concern to neurological health.

In Vivo

The library of neurotoxicity studies involving animals exposed to MeHg is extensive. The purpose of this section is to provide a number of key examples for the purpose of comparing data to the *in vitro* reports and further comparing iHg and MeHg toxicity. Internal doses were only reported from oral MeHg exposure based on the percent accumulation values obtained in Table 1. Internal doses, where reported, were compared to estimates to

assess accuracy. The mean estimate was 119±33% of reported internal doses. Values ranged from 65.71% to 212.17%. There is considerable variability between the estimates and reported values likely due to the number of extrinsic factors (such as diet, age, weight, and handling).Coccini *et al.* (2000) evaluated the effects of *in vivo* exposure of MeHg on muscarinic receptors in adult female rats. Interestingly, MeHg dosing was found to increase muscarinic receptor density in the hippocampus and cerebellum at 2 mg/kg/day with no change in ligand binding affinity immediately after sacrificing the animals (Coccini et al., 2000). However, binding affinity increased in an experimental group which was given a two week recovery period following MeHg treatment (Coccini et al., 2000). This suggests that receptor affinity and density may change over time. This may account for the differences observed *ex vivo* in otter, mink, mouse, and human tissues by Basu *et al.* (2005b). Lakshmana *et al.* (1993) noted a decrease in acetylcholine release in the hippocampus and suggested that this may be a response to alterations to another physiological process. It is therefore possible that the entire cholinergic pathway may be in a constant stage of change as it corrects for mercury-induced dysfunction.

In addition to ACh levels, noradrenalin levels have been shown to be elevated following mercury exposure (Lakshmana et al., 1993). This is likely a result of inhibition of the monoamine oxidase (MAO) enzyme. MAO inhibition has been reported in adult male rats at a minimum dose of 5 mg/kg/day for 7 days and maximum dose of 7 mg/kg/day for 10 days (Chakrabarti et al., 1998). Accordingly, dopamine levels have been noted to be elevated, likely as a result of MAO activity (Faro et al., 1997; Faro et al., 2000). A more recent report by Faro *et al.* (2007) showed that MeHg and iHg had similar potencies as measured by dopamine levels but followed different mechanisms as seen by the onset, duration, and quantity of dopamine released.

From the data presented, it is evident that there is substantial variability in the literature for *in vivo* toxicity endpoints of mercury. One major issue is that few papers report internal doses associated with the brain region studied. Faro *et al.* (2007) noted that in their whole body perfusion, only about 17% of the mercury reached the brain. Of that, the authors estimated submicromolar concentration in the dopamine secreting cells. Accordingly, the reported doses cannot be compared to those of other reports.

Although literature exists in which internal doses are reported, finding similar endpoints in the same species is not possible nor can an interspecies comparison be made. As such, general observations are limited.

One interesting observation can be made, however, regarding the age of exposure. As observed by Peixoto *et al.* (2007), early stages of development (especially PD1-5 8-12) are most sensitive when iHg neurotoxicity exists. Maternal exposure to iHg in weaning rats showed neurotoxicity in neonates but not the mothers (Franco et al., 2007). This may be due to delayed closure of the blood brain barrier in the first week post parturition (Hirase et al., 1997). Additionally, weaning infants have different intestinal flora than those eating solid foods (Mackie et al., 1999). Intestinal flora is a main determinant in MeHg absorption and accumulation as bacteria can cleave the methyl group thus forming iHg (Rowland et al., 1980). Accordingly, a lack of intestinal flora can increase the likelihood of experiencing neurotoxicity.

Rate of Demethylation

It has been well established that MeHg can be converted to iHg in mammalian species (Charleston et al., 1996; Norseth & Clarkson, 1970; Watanabe, 2002; Yasutake & Hirayama, 2001). Evidence for this phenomenon resulted from the identification of iHg in the brain following exposure to MeHg in humans (Takeuchi & Eto, 1999). This has since

been evaluated *in vitro* in the liver (Suda & Hirayama, 1992; Yasutake & Hirayama, 2001) and brain (Syversen et al., 2006) of rats and *in vivo* through feeding trials in monkeys (Bjorkman et al., 1995; Charleston et al., 1996; Watanabe, 2002; Vahter et al., 1995).

MeHg dosing experiments in rats have evaluated the rate of demethylation *in vitro* through modification of oxygen radical levels in the liver and kidneys with limited work on the brain. Within 60 minutes, Suda and Hirayama (1992) showed significant demethylation from 10μM Fe(III)EDTA in liver microsomes. Yasutake and Hirayama (2001) showed the same effect in Hg-containing liver slices treated *ex vivo* with rotenone – a complex I inhibitor in the electron transport chain. Syversen *et al.* (2006) showed greater accumulation in neurons than astrocytes (75 and 47ng/mg protein, respectively) and determined that astrocytes but not neurons actively undergo demethylation. However, a relationship between demethylation rates cannot be established here since astrocytes were incubated for 3 weeks while neurons were only in culture for 1 week. This is also in contrast to previous findings both *in vitro* and *in vivo* (Charleston et al., 1995; Aschner et al., 2007).

In vivo studies evaluating rates of demethylation are limited. Magos and Butler (1976) treated rats with MeHg for up to 64 days (at 1 dose per day) in various organs but did not observe demethylation (constant at 3.2% iHg) in brains in the 64 day dosing period. Hirayama and Yasutake (1999) showed that significant demethylation occurs within 24 hours in the liver in the presence of 75mg/kg paraquat. To the best of our knowledge, this is the only study which has attempted to modify demethylation in the brain. Analysis of different brain regions in monkeys showed significant accumulation of iHg in the thalamus and pituitary following MeHg exposure (Vahter et al., 1995). Interestingly, iHg concentrations were several fold higher in the brains of MeHg exposed animals than those exposed to $HgCl_2$ (Vahter et al., 1995). Similar studies showed that in 6-12 months, iHg

accounted for 9% of total mercury in whole brain (Vahter et al., 1994). On the cellular level, microglia and astrocytes showed the highest levels of iHg following oral administration of MeHg after 6 months. Neurons did not show demethylation at that stage but eventually did after 18 months albeit in smaller quantities than in glia (Charleston et al., 1995). MeHg levels were comparable between a group dosed for 12 months with a 6 month clearance and a group analyzed immediately after a 6 month dosing period. Levels of MeHg reached a plateau after 12 months of dosing while iHg levels continued to rise (Charleston et al., 1996).

Davis *et al.* (1994) reported on a family in the United States who, for a period of 3 months, consumed pork products contaminated with MeHg. Following autopsy, the average proportion of iHg present in the brain (as quantified by brain region) in a 30 year old female, aged 8 years at the time of exposure, ranged from 82-100% of total mercury (Davis et al., 1994). However, this number does not necessarily quantify demethylation alone as elimination of MeHg from the brain would also increase this proportion.

The literature examining the rate of demethylation is limited. This may, in part, be attributed to a very slow rate of demethylation and mercury's complex biotransformation in the body. Although not well studied, demethylation is a significant concern with respect to the neurotoxicity of mercury. The next section deals with specific toxic endpoints from both inorganic and methyl-mercurial species.

Two mechanisms underlying the demethylation of MeHg have been proposed: cleavage of the methyl group by intestinal micro-organisms and by reactive oxygen species (ROS) (Suda & Hirayama, 1992). As previously mentioned, MeHg is freely absorbed while only a fraction (7-15%) of iHg passes biological membranes (National Research Council, 2000). Therefore, demethylation in the intestines would not account for the presence of iHg

in the brain (Yasutake et al., 1998). Moreover, Charleston *et al.* (1996) noted higher levels of iHg following oral MeHg exposure than intravenous (i.v.) injection of $HgCl_2$. Accordingly, the most significant source of iHg in the brain is most likely from MeHg demethylation in the brain. Findings that the proportion of brain MeHg decreases over time (Davis et al., 1994) support the theory that demethylation must occur at the site of iHg accumulation – at least in the case of the brain. Alternative theories for the presence of iHg in the brain involving binding to micronutrients have been proposed (Friberg & Mottet, 1989). The main support for this is the presence of iHg in the brain following inhalation of elemental mercury vapour (Bjorkman et al., 1995; Friberg & Mottet, 1989; Leong et al., 2001). However, accumulation in the brain may be the result of passive diffusion of Hg^0 across the blood brain barrier (Aschner & Aschner, 1990). It is evident that both demethylation of MeHg in the brain and passage of Hg^0 across the blood brain barrier exist. However, we will focus only on demethylation which occurs in the brain over time as it is the more toxicologically relevant mode of exposure with respect to community health and non-occupational exposure.

Implications for toxicity

The previous section illustrates the significance of the two species of Hg in the brain from a toxicological perspective. There is substantial evidence to support the theory that iHg is more toxic at the site of injury than MeHg in astrocytes – the most predominant cell in the brain. Furthermore, astrocyte dysfunction has been shown to mediate neuronal loss (Aschner et al., 2007). However, in order to reach significant levels of iHg in the brain, distribution of Hg in the form of MeHg must first occur. This illustrates the importance of understanding the role of demethylation in the brain. This section will address current knowledge of the mechanisms underlying demethylation.

As mentioned, Suda and Hirayama (1992) demonstrated significant demethylation of both methyl- and ethylmercury through the production of hydroxyl radicals by incubation with 10 μM Fe(III)EDTA, suggesting that ROS are involved in the demethylation of MeHg through cleavage of the C-Hg bond. This has been further supported in recent years (Yasutake & Hirayama, 2001). One inconsistency exists, however, between the two studies: the former claims that cleavage was driven by the hydroxyl radical since the presence of a hydroxyl radical promoter was found to accelerate demethylation. Additionally, the use of hydroxyl radical scavengers decreased demethylation in some instances (Suda & Hirayama, 1992). The latter speculates that superoxide is the species of interest since enhancement of hydroxyl radical production through iron chelators had no effect on demethylation nor did the use of hydroxyl radical scavengers (Yasutake & Hirayama, 2001).

In their report, Yasutake and Hirayama (2001) noted that the superoxide anion has an extremely low half life in aqueous solution but is more stable in organic solutions. This was extrapolated to looking at lipophilic regions of the cells as the sites of demethylation. Because of their extensive double membrane structures, mitochondria were evaluated to test superoxide-mediated demethylation. As expected, using isolated mitochondria increased demethylation when compared to whole cells (Aramaki et al., 2004); however, this may be due to disproportionate amounts of ROS production or types of membrane proteins, to name two associated issues. The electron transport chain, located on the inner membrane of the mitochondria, has been said to be a "leaky" system as electrons can readily escape and react with O_2 to form superoxide (Adam-Vizi, 2005). It is therefore reasonable to assume that mitochondrial ROS formation contributes to MeHg demethylation.

The current standard set by the Canadian Food Inspection Agency is 0.5ppm total mercury in fish (Health Canada, 2007). Based on the benefits of omega 3 fatty acids and

other important nutrients from vitamin D, it is recommended that adults consume at least two- 150g servings per week of fish (Health Canada, 2007). This would lead to an overall exposure of 200 μg of total mercury per week. Based on these values and the accumulation values generated in Table 1, one week's exposure would lead to 0.15 μg and 0.23 μg of mercury per gram of brain tissue for men and women, respectively.

Estimates obtained from acute data do not appear to accurately assess chronic exposure as modelled by *M fasicularis*. This is likely because diffusion of MeHg into the brain is limited by a concentration gradient. In a MeHg feeding trial, total mercury hit threshold at approximately 5.1 μg Hg/g wet weight in the brain following 12 months of exposure to 0.05mg/kg/day (Charleston et al., 1996). Interestingly, the 18 month dosing group had roughly the same total mercury content as the 12 month group. The problem, however, was that the 18 month group had double the iHg content as the 12 month group (Charleston et al., 1996). A clearance period of 6 months following 12 months exposure was used to compare mercury levels to the 12 month group that was sacrificed immediately following exposure. As expected, MeHg levels dropped (from 4.526 to 0.159 μg/g). However, iHg actually rose slightly from 0.608 to 0.616. Based on the toxicokinetics of iHg, the greater potency of iHg to MeHg *in vitro*, and experimental findings that demethylation is occurring in the brain, we recommend that the current recommendations for exposure limits be re-evaluated.

Conclusions

Mercury, a persistent pollutant, occurs naturally in the environment. Generally, the toxicity of mercury is modulated by the toxicokinetics of the mercurial species.

MeHg is readily absorbed while iHg absorption is limited (National Research Council, 2000). Similarly, the biological half-life of MeHg is roughly 70-80 days while the

rate of elimination of iHg is substantially slower (Aberg et al., 1969; Agency for Toxic Substances and Disease Registry, 1999; Miettinen et al., 1971). MeHg is more toxic than iHg when ingested orally due to its high absorption (Berntssen et al., 2003). In tissues such as the brain, most studies have found iHg to be the more toxic species (Basu et al., 2005b; Castoldi et al., 1996).

It has been shown that *in vitro* analyses can only be compared to *in vivo* findings when reported in terms of tissue levels rather than concentration of culture medium (Meacham et al., 2005). We may, however, infer that iHg and MeHg induce neurotoxicity via different mechanisms (Faro et al., 2007).Cell type has been shown to play a significant role in toxicity. For example, astrocytes and neurons show different toxicities to each mercurial species (see table 2) and display different toxicokinetic properties such as rates of absorption and metabolism (Aschner et al., 2007).

A review of the *in vivo* literature is not sufficient for direct comparisons of neurotoxicity by the two species of mercury due to issues of inconsistent reporting of internal mercury concentrations and differences in experimental conditions. As such, we have estimated internal doses based on accumulation data.

In lieu of cell differences and the existence of MeHg demethylation, we recommend that the toxicokinetics of both iHg and MeHg species be considered for chronic risk assessment and treatment.

Demethylation has been shown to occur in brain tissue following MeHg exposure (Bjorkman et al., 1995; Charleston et al., 1996; Eto, 2000; Watanabe, 2002). When considering all the above factors, a condition which would result in the greatest toxicity would involve the ingestion of MeHg, distribution to the brain, followed by conversion to iHg. Such is the case with oral exposure of MeHg. It is therefore essential that the

mechanism of this reaction be studied in greater detail in the brain as a means of controlling demethylation in vivo.

We are currently exploring the fate of MeHg in primary astrocyte cultures under different conditions of oxidative stress in mitochondria and cytoplasm. We hypothesize that reductions in oxygen radical levels will limit the rate of demethylation, thus limiting the amount of mercury which remains in the cells following exposure. Further understanding the trends in accumulation, the toxicity of mercurial species - both *in vivo* and *in vitro* - and the factors influencing demethylation will aid in developing novel approaches to limit mercury-induced toxicity in the central nervous system.

3 Interim Comments

The previous section exemplifies the importance of understanding the demethylation of MeHg in the brain. Two main conclusions can be drawn from the previous section. The first conclusion is that accumulation is severely limited and elimination occurs more rapidly with iHg compared to MeHg. The second is that the mechanism in which MeHg and iHg cause toxicity is different (see table 2). In the brain as a whole and in astrocytes, iHg is more potent a toxicant than MeHg. We may therefore deduce that demethylation in the brain is of detriment to humans. It is therefore prudent that we understand the mechanism in order to limit the toxicity of mercury. The following section attempts to understand the reaction and provides suggestions for reducing toxicity.

4 Publication II: Characterization of demethylation of methylmercury in cultured astrocytes

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Abstract

Mercury is a naturally occurring environmental contaminant that has been shown both experimentally and clinically to be neurotoxic. Mercury toxicity in organisms depends on the species of mercury exposed to the organism from the external environment, as well as the mercurial species that reach the target organs after distribution and metabolism. A steady emergence of inorganic mercury in the brain following chronic and accidental exposure to methylmercury has suggested that methylmercury undergoes demethylation. A better understanding of the mechanism through which demethylation occurs may aid in decreasing toxicity from mercury exposure. For the first time, we have demonstrated in an *in vitro* primary cell culture model that methylmercury is converted to inorganic mercury and the rate of demethylation is increased by oxidative stress. Astrocytes from neonatal rat pups cultured in the presence of the pro-oxidant rotenone and treated with methylmercury showed increased conversion to inorganic mercury (control vs. rotenone = -1.86±5.57% vs. 16.27±2.68%, $p<0.05$). The increase in demethylation was also accompanied by an increase in total mercury accumulation. These results indicate that not only is demethylation damaging because inorganic mercury had been shown to be a more reactive species intracellularly, but it is also harmful due to increased total mercury accumulation. Our results provide a useful model to study the determining factors for the rate of demethylation of mercury in astrocytes and to explore potential ways to protect an organism from mercury toxicity. With global mercury levels on the rise, the need to reduce the toxicity of environmental pollutants has become paramount for human health.

Introduction

Mercury is a widespread persistent environmental contaminant. For the purposes of this study, we will subdivide mercury into three species: elemental, organic, and inorganic. Elemental mercury (Hg^0) is a silver liquid substance that has applications in industrial practices and is a major component of dental amalgams (2001). Exposure to mercury in its elemental form occurs primarily from the inhalation of the vaporized liquid (Tang and Li, 2006). Since Hg^0 is uncharged, it may pass freely across the blood brain barrier (Aschner and Aschner, 1990). Inorganic mercury (iHg) exists in the environment either free in solution or bound to inorganic complexes (NRC2000). Methylmercury (MeHg), a common form of organic mercury, exists as the result of a conversion from iHg by methyltransferases present in bacteria (Siciliano and Lean, 2002; Compeau and Bartha, 1985), by UV radiation (Siciliano et al., 2005), and by methylating compounds in the environment (Celo et al., 2006). MeHg is the most toxicologically relevant form of mercury. It is able to accumulate and virtually pass freely across biological membranes because of its lipophilicity (Aschner and Aschner, 1990) and its perceived resemblance to methionine at the blood brain barrier (Hoffmeyer et al., 2006).

It is well understood that the degree of toxicity induced by exposure to mercury depends on which species of mercury is present (Basu et al., 2005; Carrier et al., 2001). For example, when iHg is consumed orally, approximately 7-15% is absorbed in the body; whereas, >95% of MeHg is absorbed by the same means of exposure (NRC 2000). However, it may be overlooked that the species of mercury found in the target organ may differ from its initial form, which each exhibit different toxicological properties. Hg^0 may undergo oxidation to form iHg in the environment through any number of reactions, while in living organisms this occurs via the hydrogen peroxidase-catalase pathway (U.S.EPA., 1997).

Similarly, MeHg has been shown to undergo demethylation (MeHg → iHg) through abiotic and biotic pathways. Abiotic factors generally involve UV radiation from the sun (Siciliano et al., 2005). This has been noted to cause photodegredation through the generation of a Hg radical, resulting in the heterolytic cleavage of the Hg-C bond (Gardfeldt et al., 2001). Certain bacteria – including those of the intestinal tract – have the ability to demethylate MeHg (Rowland et al., 1980). In addition to intestinal flora, mammals have the ability to cleave the C-Hg bond in organs such as the liver and brain (Friberg and Mottet, 1989; Hirayama and Yasutake, 1999; Suda and Hirayama, 1992; Syversen et al., 2006; Vahter et al., 1995; Yasutake and Hirayama, 2001). Ideally, in order to limit mercury toxicity, intestinal demethylation should be enhanced, thereby reducing absorption. Furthermore, demethylation within organs should be avoided, as the clearance rate for iHg from organs is slower than that of MeHg (Burbacher et al., 2005) and the cellular toxicity of iHg is greater than it is for MeHg in the brain (Allen et al., 2001; Basu et al., 2005).

Examination of gross brain pathology in victims of accidental Hg exposure in Minamata, Japan has revealed that the distribution of lesions depends on age of the person at the time of exposure (Takeuchi and Eto, 1999). Generally, fetal exposure resulted in non-specific lesions; however, in adults, lesions were specific to the cerebellum and the central gyrus and occipital lobe of the cerebral cortex (Takeuchi, 1968). These lesions were marked by neuronal loss and increases in microglia and astrocytes (Takeuchi and Eto, 1999).

Astrocytes have been shown to take up mercury preferentially over neurons (Charleston et al., 1996; Garman et al., 1975). Neurotoxicity following mercury exposure has been suggested to be, in part, due to failure of astrocytes to provide a suitable microenvironment to the neurons with which they interact (Allen et al., 2002). iHg has been shown to cause toxicity on various chemical endpoints with greater potency in astrocytes

while MeHg appears to be more potent in neurons (Allen et al., 2001; Castoldi et al., 1996; Gasso et al., 2001; Monnet-Tschudi et al., 1996). Taken together, this indicates that the study of demethylation is of greater consequence in astrocytes than neurons. Coincidently, preliminary data indicates that astrocytes undergo demethylation at a greater rate than neurons (Syversen et al., 2006, data not published).

Clinical and experimental findings have found that demethylation occurs in the liver as a result of the interaction of MeHg with the superoxide anion (Yasutake and Hirayama, 2001). However, despite clinical and *in vivo* support for demethylation, the mechanism has yet to be elucidated in the brain. The purpose of this paper is to understand the mechanism in which demethylation occurs in astrocytes by developing an *in vitro* system for its analysis. The hypothesis is that increased oxidation will increase the rate of demethylation of MeHg in astrocytes. An understanding of this mechanism may result in the development of methods to reduce demethylation, and thus lower mercury accumulation and toxicity.

Materials and Methods

Chemicals

MeHg was obtained from Alpha Aesar (Ward Hill, MA, USA). Rotenone, DNase I, L-buthionine sulfoximine (BSO), fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO), Poly-L-Lysine, protease, L-cysteine, and NaOH were obtained from Sigma Aldrich Canada (Oakville, ON, Canada). The Bradford protein assay was obtained from Biorad Laboratories (Hercules, CA, USA). Cell culture regents including Trypsin, Penicillin/streptomycin, Dulbecco's Modified Eagle Medium (DMEM), and TrypLE^{TM} express were obtained from Gibco/Invitrogen Inc. (Burlington, ON, Canada). Medical grade CO_2 was purchased from

Praxair Inc. (Prince George, BC, Canada). $HgCl_2$ standards, $CuSO_4$, and HCl were obtained from Fisher Scientific (Ottawa, ON, Canada).

Animals

Animal handling and experimental protocols were approved by the University of Northern British Columbia Animal Care and Use Committee. Seven week old adult Sprague Dawley rats (*Rattus norvegicus*) were obtained from the Charles River Laboratories (Montreal, Quebec, Canada) and housed in 18 X 33 X 30 inch cages for breeding, and 19 x 10.5 x 8 inch cages for holding and weaning at 22°C under a 12 hour light/dark cycle in accordance with the guidelines of the Canadian Council on Animal Care. Mating was accomplished by introducing two adult females into the male's cage. Females were removed from the breeding cages when they were visibly pregnant and relocated to the holding and weaning cages.

Isolation of rat neonatal cerebellar cells

On post-natal day (PD) 8, rat pups were first placed unconscious by CO_2 asphyxiation then decapitated using Mayo ToughCut scissors (Fine Science Tools, Vancouver, Canada). Cerebella were extracted from whole brain under a model 2600 dissecting microscope (Ken-a-vision, Kansas City, Mo, USA) and pooled together in 1X phosphate buffered saline (Fisher Scientific). *et al.*Tissues were finely diced with scalpels to maximize the surface area to volume ratio for chemical digestion in 0.25% trypsin and 5 kU/mL DNase I for 45 minutes at 37°C. The trypsin reaction was stopped using an aliquot of FBS to a final concentration of 1%. Tissues were then mechanically passed through a 100 micron filter. Cells were separated from non-dissociated tissues by centrifugation at 500xg for 10 minutes.

Purification of astrocytes from whole brain cells

Brain cells were resuspended in DMEM containing 10% FBS, 100 units/mL penicillin and 100μg/mL streptomycin, and plated on poly-L-lysine coated flasks at a density of approximately 10^6 cells/mL. Cells were allowed to incubate under high humidity at 37°C with 5% CO_2 until confluent. Selection was accomplished based on the ability of the astrocytes to divide in a culture with non-dividing contaminating cells; namely oligodendrocytes, microglia, neurons, and endothelial cells. Astrocyte purity was obtained by passaging cells three times prior to use. This method, when prepared with prenatal human brains, yields 90-100% purity at the third passage (Jack et al., 2005).

Determination of purity using FACS

In order to determine the purity of astrocytes isolated from whole brain tissue, cells cultures were analyzed using a BD FACSCalibur™ multipurpose flow cytometer (BD Biosciences) at the Biomedical Research Centre at the University of British Columbia (Vancouver, BC). Three cultures were used in the analysis: The human neuroblastoma line SH-SY5Y (American Type Culture Collection, Manassas, VA, USA), rodent astrocytes. Identification of cell type (neuron vs. astrocyte) was accomplished by comparing forward and side scatter from the emitting laser.

Quantification of protein content using the Bradford Protein Assay

Protein quantification was necessary in order to standardize the concentration of mercury in the samples. This was accomplished using the Bradford reagent assay kit (Bio-Rad Laboratories). Bovine serum albumen standards and cell samples were prepared in triplicate and were treated with 250 μL of the Bradford reagent, containing Coomasie

Brilliant Blue G-250 (Bradford, 1976). Following a 15 minute incubation period, the samples were read spectrophotometrically at 595 nm using a Multiskan EX™ plate reader (Thermo Electron). Sample absorbance values were compared to a calibration curve using bovine serum albumen standards to yield values of protein expressed in mg/mL.

Comparison of oxidative stress using DCFH

The purpose of monitoring ROS levels in the cells was to validate the claim that experimental demethylation was the result of oxidative stress rather than a side reaction with the treatment groups. ROS was quantified using the non-fluorescent dye 2,7-Dichlorofluorescin diacetate (DCFH) which, when reduced by ROS to 2,7-Dichlorofluorescein diacetate (DCF), yields a fluorescent emission at 525nm. Cell samples were dosed for 24 hours in the presence of a pro-oxidant. Rotenone, a complex 1 inhibitor in the electron transport chain, prevents the oxidation of nicotine adenine dinucleotide (NADH) leading to the production of the highly reactive superoxide anion in the mitochondria (Jacobson et al., 2005; Zeevalk et al., 2005). BSO, an inhibitor of glutamylcysteine synthetase - an enzyme essential for glutathione production (Griffith, 1982) - causes oxidative stress *in vitro* (Chen et al., 2005) by limiting the cell's antioxidant capabilities.

After incubating cells with pro-oxidants, the cultures were washed in PBS and incubated in PBS containing 5μM DCFH for 15 minutes. The DCFH solution was then aspirated off and replaced with clean PBS. Cells were lysed using a Model 100 Sonic Dismemberer (Fisher Scientific) and collected in microcentrifuge tubes. The tubes were then centrifuged for 10 minutes at 25000 x g, and the supernatants were collected and transferred in triplicate to a 96 well plate. Fluorescence was measured using a Varioskan Flash® spectral

scanning multimode reader (Thermo Electron Corporation, Waltham Ma, USA) with an excitation wavelength of 504nm and emission wavelength of 525nm. Since a calibration curve cannot be generated for ROS detection, all values are reported as percent of control.

Mercury Dosing

Rodent astrocytes prepared as described above were grown to approximately 80% confluency in 12 well culture plates (Corning) as visually determined using an inverted microscope (Fisher Scientific). Prior to dosing, the cells were washed once with PBS before the addition of pre-mixed solutions of DMEM in the presence or absence of treatment reagents for 24 hours. At the end of the dosing period, cell treatments were aspirated and replaced with PBS for 15 minutes to account for mercury adsorption on the side of the flask (see Figure 2). PBS was then removed and 200μL of a commercially available lysis buffer (R&D Systems, Minneapolis, USA) was added and incubated at 4°C for approximately 15 minutes. Samples were stored at 4°C in 500μL centrifuge tubes until ready for mercury and protein analyses.

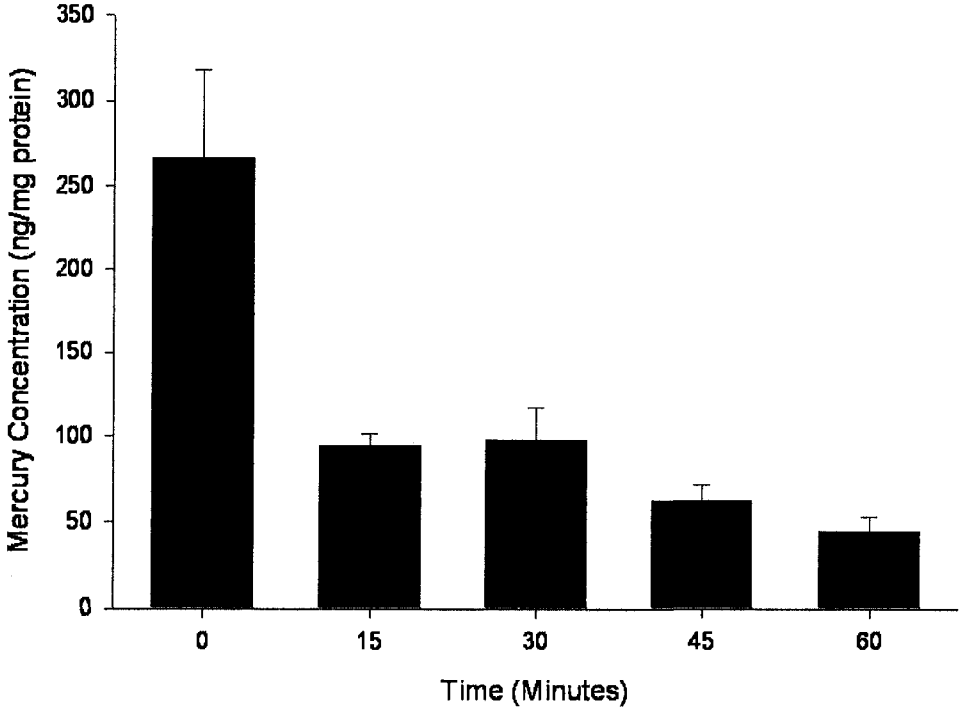

Figure 2: Quantification of total mercury (ng/mg protein) in astrocytes following 24 hour incubation with 1μM MeHg. Following incubation with mercury, cells were washed with PBS and media was replaced with mercury-free DMEM for the times indicated on the abscissa. Media was then aspirated and cells were collected in a commercial lysis buffer.

An *in vitro* concentration of 1μM MeHg was selected for all dosing experiments. This dose was chose in order to cause sufficient Hg accumulation in primary cultures of astrocytes without inducing cytotoxicity. A study in which primary cortical astrocytes were isolated using a similar method inhibited 45% of glutamine synthase activity with 10μM MeHg (Allen et al., 2001). Glutamate uptake in cerebellar astrocytes was inhibited by 50 μM MeHg (Syversen et al., 2003). In our experiments, under normal conditions, an 80% confluent astrocyte culture resulted in the detection of approximately 5 ng of Hg - about 1 000 fold greater than the published limit of detection (5 pg) (Nippon Instrument, Osaka, Japan).

Mercury analysis

Total mercury was measured using an oxygen combustion gold amalgamation mercury analyzer with a cold vapour atomic absorption spectrophotometer detector (Nippon Instruments, Osaka, Japan). 50μL of the 200μL sample were pipetted into a ceramic boat and treated as a liquid sample in accordance with the operations manual. All samples were tested in triplicate and accuracy was determined by measuring mercury in DOLT3 and DORM2 certified reference standards from the Canadian National Research Council (Ottawa, Ontario, Canada) with recovery within 10% of the reported values. Experimental quantities were normalized based on certified reference values.

Quantification of organic mercury

Because the particular mercury analyzer used was limited to determining the total mercury in a sample, a series of organic mercury extractions were performed according to a standard protocol developed by Callum *el al.* (1981). In brief, the samples were homogenized and processed to release protein-bound mercury with NaOH and $CuSO_4$ followed by the addition of sulphuric acid. Then, organic mercury was extracted using two liquid-liquid extractions with equal volumes of toluene. 4 mL (80%) of the extracted mercury was collected in a separate tube and mixed with sodium thiosulphate, which was then collected and placed directly in the mercury analyzer for analysis. Organic mercury was compared to DOLT3 and DORM2 certified reference materials from the Canadian National Research Council (Ottawa, Canada) for accuracy. Experimental quantities were normalized based on certified reference values.

Statistical Analysis

All statistical analyses were conducted using R statistical software (R Foundation for Statistical Computing, Vienna, Austria). Graphs were generated using Sigmaplot (Systat Inc. California, USA). Comparisons of treatment and mercury types were accomplished by analysis of variance (ANOVA) followed by pair-wise comparisons using Tukey's HSD test, and were adjusted by the Bonferroni Correction to control for inflated Type I error (false positives). All statistics are expressed as mean ± SE. Values were considered to be significantly different at $p<0.05$.

Results

Cell culture purity

Astrocytes and neurons were isolated as described above were analyzed for purity using FACS. Figure 3 shows the forward (FSC) and side scatter (SSC) plot for cultured astrocytes. Astrocytes were selected based on their size and granularity. The location of the major population in the neuroblastoma cell line (see Figure 3A) differed from the location of the astrocyte colony (Figure 3B). The astrocyte population has limited neuronal contamination as seen by the absence of events in the region where neurons appear.

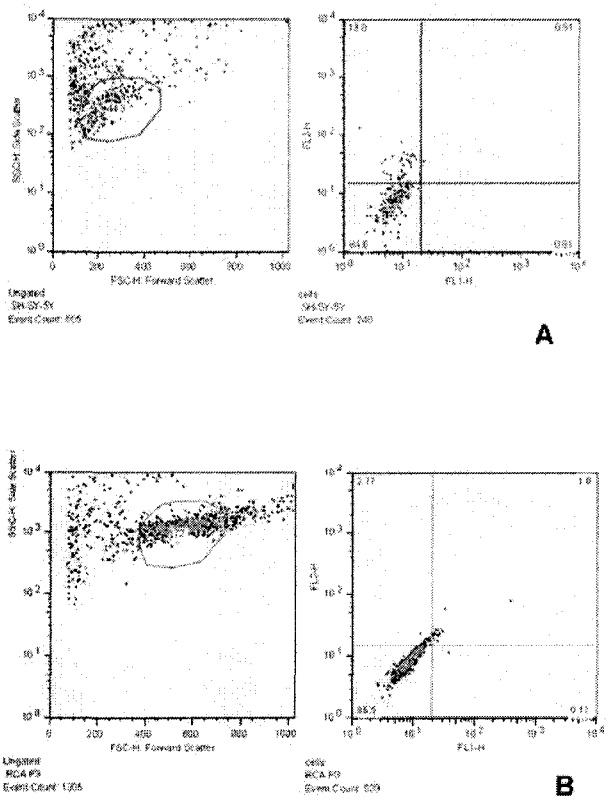

Figure 3: Forward and side-scatters of: (A) the human neuroblastoma line SH-SY-5Y and (B) rat cerebellar astrocytes as determined by FACS.

Protein Concentrations

All of the samples analyzed for mercury and ROS were standardized for protein content to control for variable cell concentrations in treatment wells. Figure 4 shows the mean protein levels from the three treatment groups for the mercury samples collected. BSO protein levels were significantly lower than those of the control (1.58 ±0.107 vs. 1.13±0.067 mg/mL, $p<0.001$). Rotenone protein levels were not significantly different from control (1.58 ±0.107 vs. 1.30±0.085mg/mL).

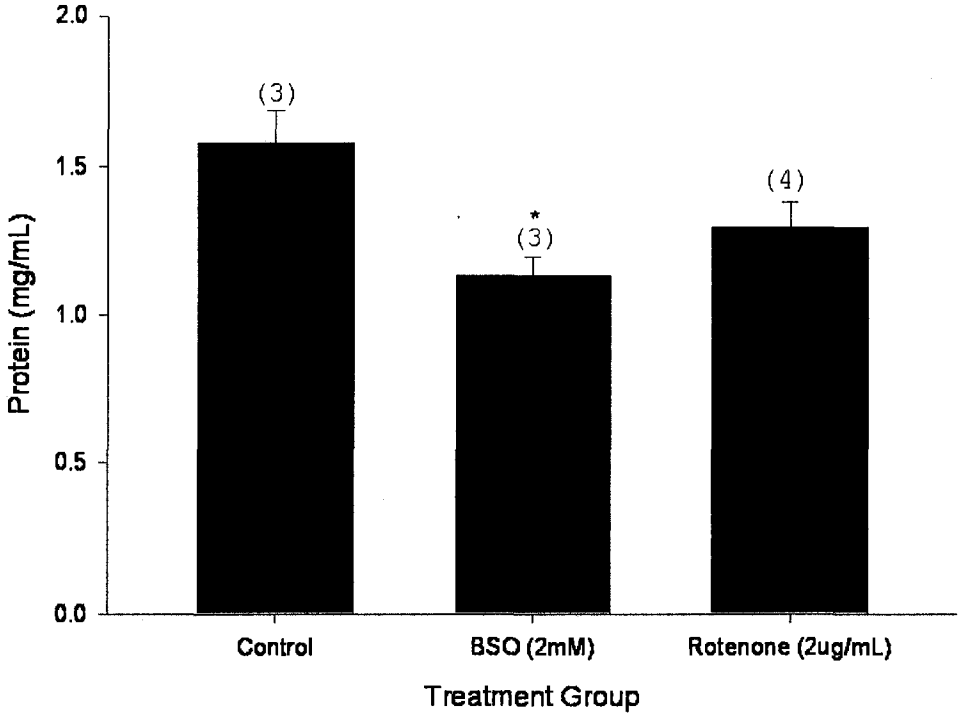

Figure 4: Comparison of protein concentrations in rat astrocytes following 24 hour incubation with 1μM MeHg in the control, BSO, Rotenone treatment groups. 5 μL samples of the 200μL cell lysate were comparted to 5μL bovine serum albument standards using the Bradford protein quantification assay. Numbers in parentheses represent the number of independent experiments done on that particular treatment group. *$p<0.001$ vs. control

Determination of Oxidative Stress

Since ROS are highly reactive, it is not possible to accurately obtain a quantitative standard curve. Accordingly, data are presented in Figure 5 in relative fluorescence units (RFU) as percent of control per mg protein. BSO produced a 21% increase in ROS content as compared to the control (control vs. BSO; 100±1.35 vs. 121±1.52 RFU/mg protein, $p<0.001$). Rotenone increased ROS levels significantly greater than the control (control vs. rotenone; 100%±1.35 vs. 207%±6.78 RFU/mg protein, $p<0.001$).

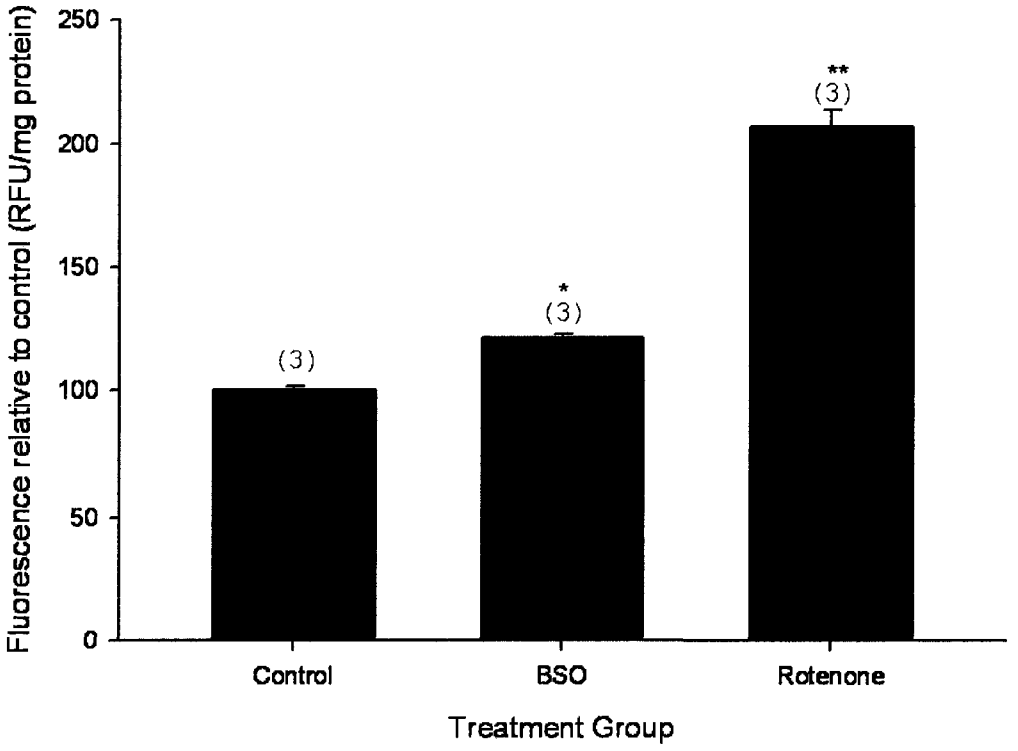

Figure 5: Evaluation of free radical production in astrocytes following 24 hour incubation of pro-oxidant treatment. Cultures were incubated with either rotenone or BSO for 24 hours. Culture medium was replaced with PBS containing 5 μM DCFH for 15 minutes. Cultures were then rinsed and lysed by sonication in 1mL PBS and fluorescence was measured. Since a standard curve could not be generated, a value of each treatment in arbitrary units was compared to that of the control to yield a relative fluorescence. Numbers in parentheses represent the number of independent experiments done on that particular treatment group. *$p<0.01$ vs. control. **$p<0.001$ vs. control.

Mercury Dosing

Following a series of trial runs, it became apparent that washing the cells with PBS was not sufficient to completely rid the culture wells of extracellular mercury. We hypothesized that mercury was adsorbing to the sides of the culture well. To test for this, treated cells were washed and placed in mercury-free culture media. The amount of mercury present following incubation in mercury-free solution for different time periods is listed in Figure 2. There was a sharp decline in mercury content between the 0 minute and 15 minute

groups, suggesting the removal of contaminating extracellular mercury. Mercury levels after 15 minutes declined at a much slower rate, indicating mercury loss by diffusion.

The BSO group, although slightly elevated from the control (control vs. BSO = 86.53±4.14ng/mg vs. 95.74±9.26ng/mg), did not significantly increase total mercury accumulation (see Figure 6). There were no differences in the accumulation rates for organic mercury between treatments. In contrast, rotenone increased the accumulation of total mercury (control vs. rotenone = 86.53±4.14ng/mg vs. 123.6±3.80ng/mg, $p<0.001$) but not organic mercury (control vs. rotenone = 88.55±3.48ng/mg vs. 103.15±3.82ng/mg).

Percent demethylation was expressed as the proportion of total mercury that was in the inorganic form relative to the total concentration (see Figure 7a for a sample calculation). An increase in demethylation for the BSO group was observed as compared to the control (control vs. BSO = -1.86±5.57% vs. 6.11±5.62%) (Figure 7b).Percent demethylation was significantly higher in the rotenone group than control (control vs. rotenone = -1.86±5.57% vs. 16.27±2.68%, $p<0.05$).

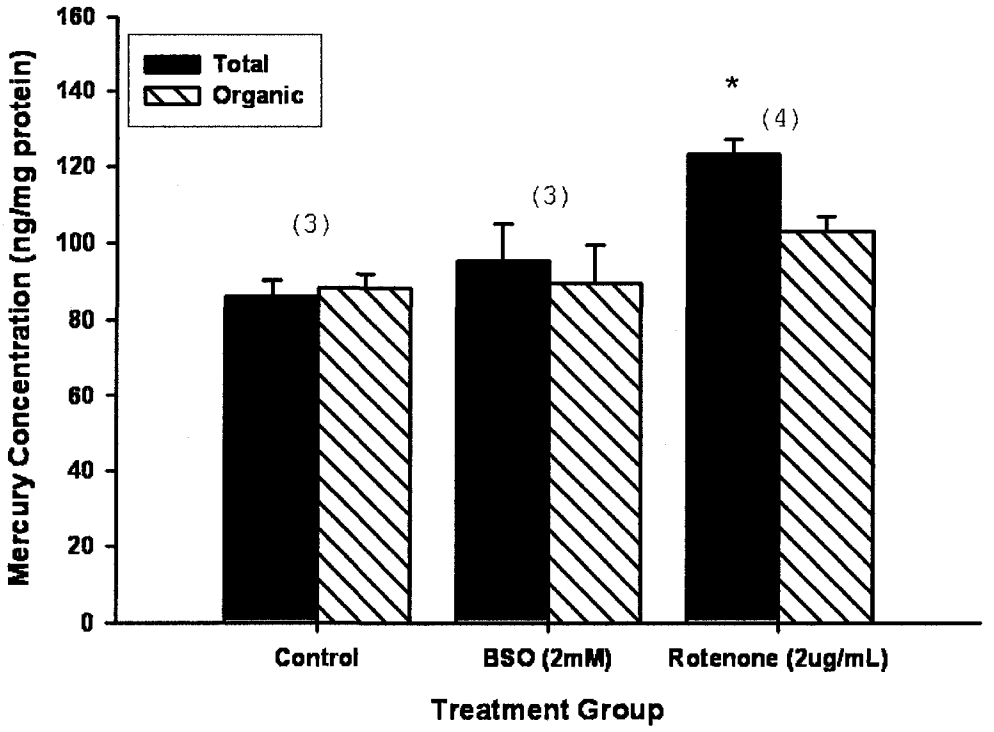

Figure 6: Effect of pro-oxidant treatment on mercury concentrations in cultured astrocytes. Total and organic mercury concentrations were collected following 24 hour incubation with 1μM MeHg *$p<0.001$ vs. control. Numbers in parentheses represent the number of independent experiments done on that particular treatment group.

A

$$\%Demethylation = \frac{Total\ Hg - Organic\ Hg}{Total\ Hg}$$

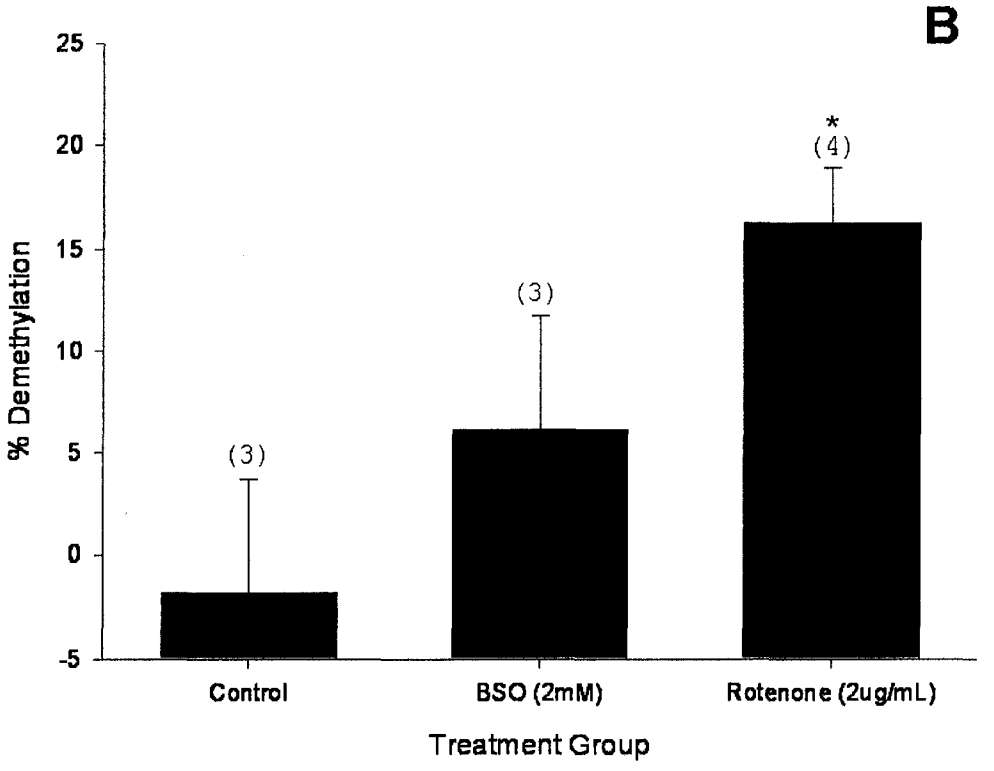

Figure 7: Effect of pro-oxidant treatment on mercury demethylation in cultured astrocytes. % demethylation was calculated according to the equation given (A).Total and organic mercury concentrations were collected following 24 hour incubation with 1μM MeHg (B). Numbers in parentheses represent the number of independent experiments done on that particular treatment group. *$p<0.05$ vs. control

Discussion

The present study demonstrates that, under specific conditions, demethylation can occur in astrocytes *in vitro*. This is consistent with a study conducted by Yasutake and Hirayama (2001) who successfully showed increased demethylation in rat liver slices incubated in the presence of rotenone.

As mentioned above, protein levels were quantified as a means of standardizing for variability in cell numbers between treatment groups. A reported disadvantage of the Bradford assay is that it underestimates protein concentration in the presence of certain detergents (Bradford, 1976) due to the disruption of hydrophobic and Van der Waals interactions (Compton and Jones, 1985). Interestingly, the protein content from stimulated rabbit muscle cells were underestimated by the Bradford assay in the presence of protein oxidation (Klebl et al., 1998). Accordingly, the decrease in observed protein content in the BSO group may be related to increased protein oxidation. The specificity of rotenone could have mitigated interference with the Bradford assay due to its localization in the mitochondria. There was concern that the significantly lower protein concentration in the BSO group would lead to an artificial skew in ROS detection and mercury concentration. The BSO group showed a small but significant increase in ROS production. While this is not surprising as BSO is a classic pro-oxidant, it may have been artificially increased by underestimation of protein content. It is therefore plausible that the detected ROS levels may be slightly elevated from the actual levels. Similarly, decreased protein levels could have significantly affected mercury accumulation levels in the BSO group. However, our results indicate no significant difference from the control. That said, the slight increase in the BSO group could be the result of increased reactive oxygen levels.

This theory is consistent with the results for the rotenone group which revealed a marked increase in mercury accumulation to accompany the high oxidative stress. There may be a correlation between oxidative stress and mercury accumulation. If this is the case, the accumulation of mercury may be exacerbated by the finding that mercury induces oxidative stress (Syversen et al., 2006; Aramaki et al., 2004) through inactivation of the ubiquinone cytochrome c reductase complex in mitochondria (Yee and Choi, 1996).

The rotenone group revealed a marked increase in mercury accumulation to accompany the high oxidative stress suggesting a correlation between oxidative stress and mercury accumulation. If this is the case, the accumulation of mercury may be exacerbated by the finding that mercury induces oxidative stress through inactivation of the ubiquitine cytochrome c reductase complex in mitochondria (Yee and Choi, 1996).

The reason for increased accumulation is not known; however, our results revealed a significant increase in total mercury in rotenone vs. control. Organic mercury levels were not statistically different from one another. This would imply that mercury accumulation depends on the degree of demethylation. It is possible that the conversion from organic mercury to inorganic mercury offsets the chemical equilibrium, thus causing an influx of mercury into the cells to re-establish the equilibrium of MeHg. Moreover, a number of previous studies have shown that iHg, at the site of injury, is the more potent species for toxicity (Aschner et al., 2007; Basu et al., 2005). These findings raise increasing concern about demethylation in the brain, as it appears to be a combination of both increased mercury accumulation and increased potency, thus substantiating the need to further understand and limit demethylation in the brain and other target organs.

In the control and BSO groups, a high degree of variability was seen with levels of demethylation while the rotenone group was relatively more consistent. It is conceivable that the variability could be due to the unpredictability of ROS production. Cultured rat astrocytes have been shown to express the majority of the cell's GSH content in the cytoplasm (27 ± 3 nmol/mg protein in cytoplasm vs. 0.49 ± 0.07 nmol/mg in mitochondria) (Muyderman et al., 2004). In contrast, rotenone, which showed a relatively low variability, is a specific mitochondrial pro-oxidant (Sherer et al., 2003). If correct, this hypothesis provides support the idea that demethylation occurrs in the mitochondria.

We have successfully established a novel *in vitro* system in which a greater degree of oxidative stress increased demethylation as well as the accumulation of total mercury in cultured cerebellar astrocytes. A necessary next step will be to evaluate whether or not demethylation and accumulation can be reversed in the absence of oxidative stress. Beyrouty and Chan (2006) noted that the co-consumption of vitamin E and selenium – two nutrients which are known antioxidants – decreased toxicity of mercury but had no apparent effect on accumulation. The difficulty with using antioxidants in a primary cell culture is that many antioxidants have been problematic due to poor water solubility while water soluble chemicals do not cross into cells. We are currently investigating enzymes conjugated to lipophilic reagents to aid in delivery to cells.

The observed effects in the rotenone group would suggest that demethylation is occurring in or around the mitochondria. An *in vitro* attempt has been made to culture purified mitochondria in a demethylation system (Aramaki et al., 2004; data not published); however, this study has yet to be published in a peer reviewed journal. Perhaps an easier method of analysis would be to determine the location of mercury using autometallographic staining on a transmission electron microscope similar to the studies performed *in vivo* by Charleston et al. (1995) and Pamphlett and Waley (1996). As mentioned, astrocytes take up MeHg in greater quantities than do neurons. Interestingly, astrocyte mitochondria have been found to be less tightly coupled to oxidative phosphorylation than those of neurons, indicating a greater degree of oxidative stress in astrocyte (Almeida and Medina, 1997).

With the amout of global mercury on the rise (Slemr and Langer, 1992), decreasing the potency of mercury neurotoxicity is becoming more important. The major concern is that source mercury pollution can disperse throughout the world by evaporation and deposition.

The complete characterization of demethylation in the brain by oxidative stress may prevent increased incidence of mercury toxicity in light of increasing global concentrations.

5 Summary

Mercury, a naturally occurring pollutant in the environment, exists in three different forms. These three forms have very different toxicological profiles not only because of their ability to enter and distribute throughout biological organisms, but also because of their specific effects at their respective sites of toxicity. A review of the available literature suggests that iHg and MeHg elicit toxicity through different mechanisms. This would explain why the toxicity endpoints for the two species are markedly different at certain sites. Specifically, iHg appears to preferentially target astrocytes while MeHg targets neurons. Since iHg does not penetrate the blood brain barrier, the presence of iHg in the brain following orally administered MeHg can only be explained by demethylation *in situ*. Findings such as these necessitate the characterization of demethylation in the brain. Accordingly, an *in vitro* system was developed which shows demethylation experimentally within 24 hours of exposure to MeHg. The pro-oxidants rotenone and BSO induced oxidative stress; however, only the rotenone group was able to induce demethylation in significant quantities ($p<0.05$ vs. control). The results from this study show that demethylation occurs in the brain and is driven by oxidative stress. Further understanding of this reaction may aid in decreasing toxicity in the future.

6 Future Directions

In order to complete the ultimate task of characterizing and subsequently controlling demethylation in the brain, the following issues must be addressed. Firstly, identifying the specific location of demethylation on an ultrastructural level is should be conducted so that a specific targeting system can be produced. Co-incubation of MeHg with rotenone, as outlined in section 4, indicates that demethylation may occur in mitochondria. Accordingly, autometallographic analysis of mercury under a transmission electron microscope, as

outlined by Charleston *et al.* (1995), should provide insight into the distribution of mercury in the brain under physiological conditions and under oxidative stress. Similarly, a chemical map on the same scale may be accomplished with x-ray absorption spectroscopy using a high powered synchrotron (7 GeV). Such a map would not only provide detail of mercury within the ultrastructure, but it would also indicate which species of mercury predominates by region.

The second aspect would need to confirm that demethylation is caused by oxidative stress rather than a side reaction involving rotenone. Ideally, to confirm that rotenone caused demethylation via oxidative stress, a system with low ROS should be established. An *in vitro* system containing rotenone in the presence of poly ethylene glycol (PEG) conjugated superoxide dismutase and catalase could confirm our conclusions.

Finally, the issue of why demethylation occurs in astrocytes but not appreciably in neurons should be addressed. As mentioned above, we believe that astrocytes have more mitochondria because they undergo division. Furthermore, astrocyte mitochondria have been shown to be coupled less tightly than those of neurons (Almeida & Medina, 1997) suggesting greater ROS production. Quantification of mitochondria in both cell types may aid in solving this quandary.

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Appendix A: Animal Ethics Protocol

UNBC UNIVERSITY OF NORTHERN BRITISH COLUMBIA

ANIMAL USE PROTOCOL FOR LABORATORY ANIMALS IN RESEARCH OR TEACHING

Office Use	
Protocol No.	_____
Category of Invasiveness	_____
Start Date	_____
End Date	_____

1. Administrative Information

a) Name and title of principal investigator

Laurie Chan, BC Leadership Chair, Professor

_____

b) Address for correspondence (include phone, fax and email)

Dr. Laurie Chan, UNBC, Community Health Program, 3333 University Way, Prince George, British Columbia V2N 4Z9, Canada, Phone: 960-5237, Fax: 960-5744 Email: lchan@unbc.ca

_____

c) Title of project/number and title of course

Toxic and biochemical effects of mercury compounds on primary rat neurons and astrocytes

_____

d) Expected date of: commencement __Sept 2006____ conclusion __Sept 2007_____

e) Indicate what year this represents for a multi-year project: 1

f) Type of project

X☐ Research ☐ Teaching

g) X☐ New ☐ Renewal of protocol no. _____

For renewed protocols, please provide a copy of the original ACUC approved application
and fill in sections 1 a) to 1 m) only.

h) If renewal,

☐ There have been no changes ☐ There will be changes

Provide justification for any proposed additions or changes.

i) If renewal, list the number and species of animals used in this project over the last year.

Indicate whether the numbers deviated from those targeted in the initial proposal.

Describe any morbidity or mortality experiences for target and non-target species.

N/A

j) Location:
Where will the study take place? (Name the closest town and the name of the laboratory)

Animal facility in the Northern Medical Building.

---

k) Permits

Permits applied for	Permits obtained (Y / N)	Permit Number

* Please provide photocopies of both sides of relevant licenses.

l) Emergency Contact:

Name: Eric Bayrd
Work Phone Number: 250-960-5260
Home Phone Number: 250-562-8417

ii) If this research / teaching / testing protocol has been approved by the Animal Care and Use
Committee of an agency / institution **other than** UNBC, please submit a filled-out and signed UNBC Animal Use Protocol form describing UNBC's involvement in the animal research/teaching/testing. In addition, please attach a copy of the other Agency's / Institution's authorized Animal Care and Use Protocol.

☐ This protocol is **not** governed by UNBC's ACUC approval and is therefore provided for
information only.
☐ A copy of the authorized research protocol is attached.

Name of the agency / institution whose ACUC authorized this research proposal:

_____

Declaration
All animals used in this research project will be cared for in accordance with the policies and guidelines
of the Canadian Council on Animal Care (http://www.ccac.ca) and the requirements of the relevant
international, federal, provincial/territorial and municipal legislation.

_____
Signature, Principal Investigator / Course Director Date

2. Source of Funding

a) Funding agency(ies) or commercial sponsor:

_____

b) X Grant approved, agency file number: NSERC Discovery grant, account #21204
c) ☐ Grant under review

3. Description of Use

(If you need more information, please refer to CCAC's website at www.ccac.ca)

a) Purpose of Animal Use (PAU):
Circle the number (1-6) below that best describes the purpose of animal use.

1. Studies of a fundamental nature in sciences relating to essential structure or function (e.g.
biology, psychology, biochemistry, pharmacology, physiology, etc.)

2. Studies for medical purposes, including veterinary medicine, that relate to human or animal
disease or disorders.

3. Studies for regulatory testing of products for the protection of humans, animals, or the environment.

4. Studies for the development of products or appliances for human or veterinary medicine.

5. Education and training of individuals in post-secondary institutions or facilities.

6. Other:

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b) Lay Summary:
Describe in terms understandable to the non-scientist how the proposed use of animals will contribute to the advancement of science, or to outcomes that can reasonably be expected to benefit humans, animals or the environment.

Many neurotoxic environmental contaminants accumulate in foods such as fish and marine mammals. These foods are also nutritionally rich in vitamins and other micronutrients. However, pregnant women who consume these foods are exposed to contaminants, like mercury, which can affect brain development of their unborn babies. The objective of this study is to better understand how this toxic damage occurs and how antioxidants may protect the developing brain cells.

We will isolate cells from the brain tissue of new-born rats. The cells will be grown in a culture-dish in the presence of mercury compounds. This cell culture technique is a widely accepted approach to understand how neurotoxic compounds can alter brain-cell development and function. We will then detect biochemical changes and signs of toxicity following exposure. Some chemical forms of mercury are less toxic to cells and so we will detect changes in mercury chemistry. We believe that some antioxidants, some of which are found in marine diets (i.e. vitamin E and selenium), are protective and may change mercury chemistry into a less toxic form.

c) Why is it necessary to use live animals, and what considerations has been given to the use of
alternative methods which do not involve the use of animals?

Before attempting this experiment on rats, we will be using commercially available brain "cell-lines" to optimize the experimental procedures. While the using commercial cell-lines do not require live animals, they are not considered a good model to understand brain development. Additionally, our technique does not require that animals be exposed to mercury compounds while alive. Using the cells harvested from euthanized rats eliminates the pain and suffering resulting from mercury exposure.

d) Provide rationale for the choice of species.

We have chosen the rat as it is generally accepted within the scientific community to serve as an model for this particular type of study (neurotoxicological bioassays).

e) Animals to be used:

Animal	Location	Number of animals required at a time	Annual total	Housing
Sprague-Dawley Rat	NMB animal facility	2 for mating, 14-16 pups (average number of pups per fertilization)	15 pups/2 weeks = 390/year +18 adults/year for mating = 408	2 females per female cage. 1 male per male cage

* Provide justification for numbers of animals to be used.

f) Agents to be administered:
Indicate all agents to be administered in the research protocol for each species. **None**

Species	Agent	Purpose	Route	Dosage	Frequency
Sprague-Dawley Rat	Carbon dioxide gas	Euthanize adult rats	Inhalation	100%	Once
Sprague-Dawley Rat	Carbon dioxide gas	Anaesthetize neonatal pups	Inhalation	100%	Once

g) Samples to be taken:
Indicate all samples to be taken for each species. **None**

Species	Type of sample	Site	Amount	Procedure	Frequency

h) Details of procedures to be performed on animals:

Descriptions must be sufficiently detailed to permit assessment of compliance with CCAC guidelines. Use terminology understandable to ACUC members with widely different backgrounds (including non-scientists). Indicate which members of the team will be carrying out which procedures. For killed specimens, describe the method to be used to destroy the animal. For complex projects with many procedures or those with routine procedures, it is easier to develop standard operating procedures (SOPs) in consultation with the ACUC. These can be attached to the application and referred to from this section.

i. Describe all procedures and manipulations performed on live animals for each species. If multiple procedures are to be performed, flow diagrams may be useful.

Mating

Male and female adult Sprague-Dawley rats will be acclimatize for three weeks in 12 h light:12 h dark cycle (12 L cycle starts at 7 am). Females will be introduced into the male's cage. Signs of mating (vaginal plug, swollen vagana) will be used as indicators of mating.

Neonatal Period

After birth, the offspring will be sacrificed 8 days after birth by decapitation using a guillotine by a trained researcher. According to the Canadian Council on Animal Care, this method of euthanasia is a virtually painless way to sacrifice animals without chemically contaminating tissues with anaesthesia. CO2 gas mixtures are not appropriate for use in neonatal rats, and barbiturates can alter brain cells and invalidate the study. Brain cells will be collected, processed and cultured for experiments. Although not suitable for euthanasia, CO2 gas will be used as a sedative prior to decapitation. Pups will be placed in small enclosures for 5 minutes under constant CO2 gas flow until adequately sedated at which point, they will be decapitated.

ii. For studies involving chemical restraint, detail the type of restraint chosen, provide details of immobilization agent used for chemical restraint, and describe all manipulations and precautions taken to protect the animal and investigator.

Not applicable.

iii. Provide details of marking, including potential long-term effects.

Since animals will be sacrificed prior to experimentation, there is no potential for long-term effects.

iv. Provide details of any surgical and medical procedures. Indicate where and under what conditions it will be performed, as well as by whom. Provide the name of the veterinarian where consultation is necessary.

Not applicable

v. Provide details for post-procedure monitoring animals: What is the frequency (specified times per day, duration (number of days) and type (specific parameters of post procedure monitoring) over which post procedure monitoring of the animals will be performed. If post procedure monitoring is not necessary / applicable, this should be stated and explained as necessary. Provide details on post procedure nutritional requirements.

Not applicable

- first 24 hours:
- second 24 hours:
- thereafter:

vi. Housing: Provide detail on the housing of the animals.

Females will be housed up to 4 per cage but minimum of 2 per cage (shoebox cages). Males will be kept one to a cage (shoebox cage) except when a female is placed in their cage for mating. Bottom of the cage will be covered with wood chips. Each cage will have sufficient feed and access to water as described by the CCAC guidelines. Cage will be cleaned frequently to minimize the buildup of ammonia.

4. Pain and Distress

(If you need more information, please refer to CCAC's website at www.ccac.ca)

a) Is any pain and / or distress likely to be associated with the procedures or manipulations?

According to the CCAC, decapitation causes the immediate loss of consciousness and is acceptable form of euthanasia without anesthesia. As such, animals are not likely to experience any pain. There may be mild distress when positioning the animals in the guillotine.

b) If animals encounter unanticipated pain and / or distress, what criteria will be used to terminate the
procedure / study and possibly euthanize the animal(s)?

If adult animals show any sign of distress, they will be euthanized by carbon dioxide asphyxiation. If rats less than 10 days old show signs of distress/pain, they will be euthanized by decapitation.

c) Indicate the category of invasiveness which best describes the protocol:

☐	A	Methods used on most invertebrates or on live isolates
☐	B	Methods used which cause little or no discomfort or stress
☐	C	Methods which cause minor stress or pain of short duration
X	D	Methods which cause moderate to severe distress or discomfort
☐	E	Procedures which cause severe pain near, at, or above the pain tolerance Threshold of un-anesthetized conscious animals.

5. Methods of Euthanasia

(If you need more information, please refer to CCAC's website at www.ccac.ca)

Provide details of method of euthanasia:

i. For species of interest, where necessary upon termination of the study;

Adult animals, once beyond their ability to produce viable offspring, will be sacrificed using carbon dioxide asphyxiation.

ii. For species of interest, where necessary due to unanticipated pain and/or distress;

Adult rats that show any signs of pain or distress will be sacrificed using carbon dioxide asphyxiation. Newborn rat pups will be sacrificed by decapitation if they are in pain/distress since asphyxiation is not considered appropriate at that age (less than 14days).

6. Disposition

(For further information, please refer to CCAC's website at www.ccac.ca)

Provide details of intended fate of the animals used in the study.

Animals in the study will be euthanized in order to harvest cells from specific regions of the brain. The cells will then be isolated, cultured and used in biochemical experiments. Mating adults will remain active until they are no longer able to produce viable offspring. Carcasses will be disposed of by incineration. Since they

will not be treated with anything prior to death, the carcasses will pose no harm chemically. Solid tissues not containing mercury will be discarded in normal biohazard waste containers. Tissues with mercury will be disposed of in appropriate mercury containers containing bleach.

7. Potential Hazards to Staff, Students and Visiting Researchers

(For further information, please refer to CCAC's website at www.ccac.ca)

List potential biohazards, chemical and any other hazards.

- Biohazards - Rat tissues
- Chemical – carbon dioxide
- Physical – guillotine blades

Please provide an emergency contact name and number:

Name: Laurie Chan.....

Position / Relationship: Professor/Principal Investigator

Phone Number: 960-5237.....

8. Qualifications and Experience

(For further information, please refer to CCAC's website at www.ccac.ca)

List names, positions and relevant training and experience of all individuals who will be working directly with the animals.
For Health & Safety Education and Training courses available at UNBC, please refer to the following website: http://www.unbc.ca/safety/training_programs.html

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Name of individual, course taken, course provider and date of course

Aaron Shapiro, BSc
- Rat Embryo Culture Training; "hands-on" training and experience from Jiu-Ni Liu, McGill University, 1st to 10th December 2005
- Animal Training Course (rodents and pigeons), Guelph University, 2004

Eric Bayrd, MSc.

- no animal training

Laurie Chan, PhD
- Small animal handling course at McGill several years ago.

I hereby certify that the above individual(s) is (are) qualified to conduct the procedures described and that they have read and initialled this application in person.

Signature of principal investigator

*** Copies ***
15 copies (i.e. signed original plus 14 copies), each containing all attachments, should be submitted to:
The Animal Care and Use Committee, Office of Research,
3333 University Way, Prince George, B.C. V2N 4Z9
(Telephone: (250) 960-5815, Fax: (250) 960-5746)
Email: koehlerj@unbc.ca

REVIEWED AT ACUC MEETING OF: _____

APPROVED (Authorization of Chair): _____

DATE APPROVED: _____

Certified Reference Material

Appendix B: Certificate of Analysis: DORM-3

National Research Council Canada | Conseil national de recherches Canada

DORM-3

Fish Protein Certified Reference Material for Trace Metals

The following table shows those elements for which certified values have been established for this reference material. Certified values are based on unweighted mean results from data generated at NRCC as well as results submitted by laboratories participating in an annual intercomparison. The expanded uncertainty (U_{CRM}) in the certified value is equal to $U = ku_c$ where u_c is the combined standard uncertainty calculated according to the ISO Guide [1] and k is the coverage factor. The value of u_c is calculated from the combined uncertainties of the various methods (u_{char}) as well as uncertainties associated with homogeneity (u_{hom}). It is intended that U_{CRM} accounts for every aspect that reasonably contributes to the uncertainty of the measurand [2]. A coverage factor of 2 was applied for all elements. The table below lists certified values for DORM-3 expressed on a dry mass basis.

TRACE ELEMENTS (milligram/kilogram)

Arsenic (d,g,h)	6.88	±	0.30
Cadmium (d,g,i,p)	0.290	±	0.020
Copper (d,i,p)	15.5	±	0.63
Chromium (d,g,i)	1.89	±	0.17
Iron (d,i)	347	±	20
Lead (d,g,p)	0.395	±	0.050
Mercury (c,d,p)	0.409	±	0.027
Nickel (d,g,i,p)	1.28	±	0.24
Tin (d,p)	0.066	±	0.012
Zinc (d,i,p)	51.3	±	3.1

Coding

The coding refers only to the instrumental method used for quantitation.

c - Cold vapour atomic absorption spectrometry.
d - Inductively coupled plasma mass spectrometry.
g - Electrothermal vaporization atomic absorption spectrometry.
h - Hydride generation atomic absorption, fluorescence or emission spectrometry.
i - Inductively coupled plasma atomic emission spectrometry.
p - Isotope dilution inductively coupled plasma mass spectromet

NRC · CNRC

National Research Council Canada | Conseil national de recherches Canada

DOLT-3

Dogfish Liver Certified Reference Material for Trace Metals

The following table shows those elements for which certified values have been established for this dogfish (*Squalus acanthias*) liver reference material. Certified values are based on unweighted mean results from data submitted by laboratories participating in an annual intercomparison. The expanded uncertainty (U_{CRM}) in the certified value is equal to $U = ku_c$ where u_c is the combined standard uncertainty calculated according to the ISO Guide [1] and k is the coverage factor. The value of u_c is determined from the combined uncertainties of the various methods (u_{char}) as well as uncertainties associated with homogeneity (u_{hom}).

It is intended that U_{CRM} encompasses every aspect that reasonably contributes to the uncertainty of the measurand [2,3]. A coverage factor of 2 was applied for all elements. The table below lists certifed values for DOLT-3.

TRACE ELEMENTS (milligram/kilogram)

Arsenic (d,g,h)	10.2	±	0.5
Cadmium (d,f,g,i,p)	19.4	±	0.6
Copper (d,i,p)	31.2	±	1.0
Iron (d,f,i)	1484	±	57
Lead (d,g,p)	0.319	±	0.045
Mercury (d,c,p)	3.37	±	0.14
Nickel (g,i,p)	2.72	±	0.35
Selenium (g,h,j)	7.06	±	0.48
Silver (f,g,i,p)	1.20	±	0.07
Zinc (d,i,p)	86.6	±	2.4

Coding

The coding refers only to the instrumental method of analyte determination.

c - Cold vapour atomic absorption spectrometry.
d - Inductively coupled plasma mass spectrometry
f - Flame atomic absorption spectrometry.
g - Electrothermal vaporization atomic absorption spectrometry (ETAAS).
h - Hydride generation atomic absorption spectrometry.
i - Inductively coupled plasma atomic emission spectrometry.
j- Hydride generation atomic fluorescence spectrometry.
p - Isotope dilution inductively coupled plasma mass spectrometry.

Appendix D: SH-SY-5Y Neuroblastoma Product Description

Cell Biology

ATCC® Number:	**CRL-2266™** Order this item	**Price:**	**$244.00**
Designations:	SH-SY5Y	**Depositors:**	JL Biedler
Biosafety Level:	1	**Shipped:**	frozen
Medium & Serum:	See Propagation	**Growth Properties:**	mixed, adherent and suspension
Organism:	*Homo sapiens* (human)	**Morphology:**	epithelial 

Source:	**Organ:** brain **Disease:** *neuroblastoma* **Derived from metastatic site:** bone marrow
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Cell Culture Products

Restrictions:	NOTE: SH-SY5Y was deposited at the ATCC by June L. Biedler, Memorial Sloan-Kettering Cancer Center. SH-SY5Y is distributed for academic research purposes only. Memorial Sloan-Kettering releases the line subject to the following: 1.) SH-SY5Y or its products must not be distributed to third parties. Commercial interests are the exclusive property of Memorial Sloan-Kettering Cancer Center. 2.) Any proposed commercial use of SH-SY5Y including any use by a for-profit entity must first be negotiated with Director, Office of Industrial Affairs, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; phone (212) 639-6181; FAX (212) 717-3439.
Isolation:	**Isolation date:** 1970 (SH-SY5Y is a thrice cloned (SK-N-SH -> SH-SY -> SH-SY5 -> SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH (see ATCC HTB-11) which was established in 1970 from a metastatic bone tumor.) [23032]
Applications:	transfection host (Roche FuGENE® Transfection Reagents technology from amaxa)
Antigen Expression:	Blood Type A; Rh+
DNA Profile (STR):	Amelogenin: X CSF1PO: 11 D13S317: 11 D16S539: 8,13 D5S818: 12 D7S820: 7,10 THO1: 7,10 TPOX: 8,11 vWA: 14,18
Cytogenetic	modal number = 47; the cells possess a unique marker comprised of a chromosome

Analysis:	1 with a complex insertion of an additional copy of a 1q segment into the long arm, resulting in trisomy of 1q
Age:	4 years
Gender:	female
Comments:	SH-SY5Y cells have a reported saturation density greater than 1 X 10(6) cells/sq cm. They are reported to exhibit moderate levels of dopamine beta hydroxylase activity [PubMed ID: 29704].
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is a 1:1 mixture of ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003, and F12 Medium. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0C **Atmosphere:** air, 95%; carbon dioxide (CO2), 5%
Subculturing:	**Protocol:** These cells grow as a mixture of floating and adherent cells. The cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites). Cells will aggregate, form clumps and float. Remove the medium with the floating cells, and recover the cells by centrifugation. Rinse the adherent cells with fresh 0.25% trypsin, 0.53 mM EDTA solution, add an additional 1 to 2 ml of trypsin solution, and let the culture sit at room temperature (or at 37C) until the cells detach. Add fresh medium, aspirate, combine with the floating cells recovered above and dispense into new flasks. **Subcultivation ratio:** A subcultivation ratio of 1:20 to 1:50 is recommended **Medium renewal:** Every 4 to 7 days
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Doubling Time:	48 hrs
Related Products:	recommended serum: ATCC 30-2020 parental cell line: ATCC HTB-11
References:	22554: Ross RA , et al. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J. Natl. Cancer Inst. 71: 741-749, 1983. PubMed: 6137586 23032: Biedler JL , et al. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res. 38: 3751-3757, 1978. PubMed: 29704