Mercury neurotoxicity and the development of peripheral biochemical markers of central nervous system function

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ABSTRACT

Methylmercury (MeHg) is a neurotoxic global pollutant that accumulates at high levels in predatory fish and marine mammals. The dietary intake of these animals is the main source of MeHg exposure in humans. At high levels, MeHg is known to damage the sensory and motor systems in both adults and children. Due to the complexity and inaccessibility of the central nervous system (CNS), early dysfunction is difficult to detect. Biochemical markers in the CNS have been used to identify MeHg neurotoxicity in animal models. Analogues of these biochemical targets are also present in peripheral blood tissue and may reflect early CNS dysfunction in human populations. The proposed peripheral biomarkers include 1) lymphocyte muscarinic acetylcholine (mACh) receptor, 2) serum cholinesterase (ChE) and 3) platelet monoamine oxidase (MAO). This thesis evaluates the effects of mercury (Hg) compounds on these CNS and peripheral biochemical markers in laboratory and epidemiological studies. In vitro studies showed that inorganic Hg ($HgCl_2$) and MeHg inhibited mACh receptor binding in human, rat, and mouse brain tissue. Additionally, studies demonstrated that a low-level gestational exposure to MeHg reduced MAO activity in the developing embryo and in adult female offspring. Combined, these studies provide a framework for the assessment of biochemical targets of Hg compounds in humans. A cross sectional study was conducted to evaluate the association between peripheral biochemical markers and MeHg exposure in fish-eating adults (n=129) from Lac St-Pierre, Quebec. Blood-Hg concentrations were used as a marker of exposure and ranged from 0.2 to 17.0 μg/L. Multiple linear regression analysis demonstrated that both blood-Hg (p=0.011) and heavy smoking (p=0.001) were associated with reduced platelet-MAO activity. However, neither lymphocyte mACh receptor nor serum ChE was related to blood-Hg. These results suggest that exposure to MeHg may result in reduced platelet-MAO activity, which may serve as an indicator of early CNS dysfunction. The use of peripheral biochemical markers may offer a novel strategy for risk assessment of neurotoxic pollutants and should be further investigated.

RÉSUMÉ

Le méthyle mercure (MeHg) est un polluant neurotoxique général qui s'accumule à des taux élevés chez les poissons prédateurs et les mammifères marins. Une diète alimentaire basée sur ces animaux constitue la principale source de MeHg chez les humains. On sait qu'à taux élevés le MeHg cause des dommages aux systèmes moteur et sensoriel chez l'adulte et l'enfant. Dû à la complexité et à l'inaccessibilité du système nerveux central (SNC), les dysfonctions précoces sont difficiles à déceler. Des marqueurs biochimiques dans le SNC ont été utilisés pour mesurer la neurotoxicité du MeHg chez des modèles animaux. Des analogues à ces cibles biochimiques sont aussi présents dans les tissus sanguins périphériques et peuvent refléter des dysfonctions précoces du SNC chez les populations humaines. Les bio-marqueurs périphériques proposés incluent 1) les récepteurs lymphocytaires muscariniques à acétylcholine (mACh), 2) la cholinestérase sérique (ChE) and 3) l'oxidase monoamine (MAO) des plaquettes. Cette thèse évalue les effets de composés du mercure (Hg) sur ces marqueurs biochimiques du SNC et périphériques tels que mesurés lors d'études de laboratoire et épidémiologiques. Des études *in vitro* ont montré que le Hg inorganique ($HgCl_2$) et le MeHg inhibent la liaison aux récepteurs mACh du tissu cérébral chez l'humain, le rat et la souris. De plus, des études ont démontré qu'une exposition à des taux faibles de MeHg durant la gestation réduisait l'activité de la MAO chez l'embryon en développement et chez les femelles adultes de la progéniture. L'ensemble de ces études fournit un cadre pour l'étude des cibles biochimiques des composés du Hg chez les humains. Une étude transversale à été conduite pour évaluer l'association entre les marqueurs biochimiques périphériques et l'exposition au MeHg chez des adultes (n=129) qui mangent du poisson du Lac St-Pierre, Québec. Les concentrations en Hg sanguin ont été utilisées comme mesure de l'exposition et variaient de 0.2 à 17.0 μg/L. Une analyse par régression linéaire multiple a démontré qu'à la fois le Hg sanguin (p=0.011) et une forte consommation de tabac (p=0.001) sont associés à une activité réduite de la MAO des plaquettes. Cependant, ni les récepteurs lymphocytaires à mACh ni le ChE sérique n'étaient reliés au Hg

sanguin. Ces résultats suggèrent que l'exposition au MeHg peut entraîner une réduction de l'activité de la MAO des plaquettes, ce qui pourrait être utilisé comme indicateur d'une dysfonction précoce du SNC. L'utilisation de marqueurs biochimiques périphériques peut offrir une nouvelle stratégie d'évaluation des risques associés aux polluants neurotoxiques, et devrait être étudiée de manière plus approfondie.

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CONTRIBUTION OF AUTHORS

All described studies in this thesis were successfully accomplished as a result of effective collaborative efforts among the co-authors. The contributions of the authors for each manuscript are described below.

The first manuscript entitled *"Biochemical markers of neurotoxicity in wildlife and human populations: Considerations for method development"* was co-authored by C. Stamler, N. Basu and H.M. Chan. Laboratory work related to wildlife samples was conducted by N. Basu, while laboratory work related to human samples was conducted by C. Stamler. The manuscript was written by the first author, C. Stamler. Manuscript was edited by the co-authors.

The second manuscript entitled *"An inter-species comparison of mercury inhibition on muscarinic acetylcholine receptor binding in the cerebral cortex and cerebellum"* was co-authored by N. Basu, C. Stamler and H.M. Chan. All authors contributed to the experimental design of the study. N. Basu (wild-life tissue analysis) and C.J. Stamler (rodent tissue analysis) contributed equally to laboratory work. M. Loua acquired human brain tissue from Douglas Hospital and provided technical guidance. The first draft of the manuscript was written by the first author, N. Basu, and edited by C.J. Stamler and H.M. Chan.

The third manuscript, entitled *"Methylmercury reduces monoamine oxidase activity in rat embryos and offspring"* was a collaborative research project. The idea for the study was from C.Stamler and H.M. Chan. The prenatal animal dosing experiment was conducted by ClinTrial BioResearch (CTBR) under the supervision of P. Beyrouty. Embryo culture and development was performed by J-N Liu. Protein and neurochemical markers in rat brain tissue were measured by C.J. Stamler. Measurement of MAO and protein in embryos, all data analysis, statistical analysis, interpretations and writing of the manuscript was performed by C.J. Stamler. Edits to the manuscript was made by H.M. Chan, J-N Liu, and S. Kubow.

The fourth manuscript entitled "Mercury exposure and biochemical markers of CNS function in fish consumers from the Lac St-Pierre Region,

Quebec, Canada" was co-authored by C.J. Stamler, N. Abdelouahab, C. Vanier, D. Mergler and H.M. Chan. The community field work and implementation of questionnaires was coordinated by N. Abdelouahab, C. Vanier, and D. Mergler. Biomarker measurement protocols and assays were developed by C. Stamler. All laboratory work involving the isolation of relevant blood cells, measurement of peripheral biochemical markers and serum ferritin, was performed by C.J. Stamler. Data and statistical analysis was conducted by C.J. Stamler. This manuscript was written by C.J. Stamler and edited by N. Abdelouahab, D. Mergler and H.M. Chan.

CONTRIBUTION TO KNOWLEDGE

While the effects of methyl mercury (MeHg) have been extensively studied for over 50 years, the most commonly used method for detection of neurotoxicity in epidemiology studies is through behavioural analysis. A useful biochemical marker of neurotoxicity has not been identified for MeHg exposure in humans.

The first chapter presents a comprehensive review of the neurotoxic effects of mercury (Hg) compounds on brain neurochemistry in animal models. The potential impacts of these changes on behavioural parameters and the potential interactions of Hg compounds with peripheral biochemical markers are described and their importance in risk assessment strategies is proposed.

The measurement of biochemical markers in brain and peripheral tissue has been previously described in controlled laboratory studies. The first manuscript (Chapter 2) describes the application of methodologies for measurement of biochemical markers in epidemiology studies. Proper storage conditions, including storage time and temperature, are systematically evaluated. Also, new high throughput methods are described to allow for the analysis of samples from large population studies. These results will be of interest to toxicologists and epidemiologists who are interested in measuring these biomarkers in human populations. The manuscript has published in the *Journal of Toxicology and Environmental Health A 68, 1414-29.*

Hg compounds are known to disrupt the function of the muscarinic acetylcholine (mACh) receptor in rat brain tissue; however the effects of Hg on receptors from other species, such as humans, have never been evaluated. Chapter 3 describes the in vitro effects of Hg compounds on mACh receptor binding in autopsied human, mouse, rat, mink and river otter cortex and cerebellum tissue. This study shows that effects of Hg are consistent across several species and provides a framework for future bio-monitoring studies in species at risk. This manuscript has been published in *Toxicology and Applied Pharmacology, Volume 205(1) Pages 71-76.*

The developing brain is particularly sensitive to the toxic effects of MeHg.

Monoamine oxidase regulates neurotransmitters and plays an important role in early brain development. Chapter 4 describes the effects of low-level prenatal exposure to MeHg on monoamine oxidase (MAO) activity in the various brain regions of rat offspring. While the inhibitory effects of MeHg on MAO activity have been reported previously, this is the first study to show that a chronic low prenatal dose can alter this biochemical marker in adult rat offspring. This study suggests that MAO activity may be a potential marker of the effects of MeHg neurotoxicity in utero. This data has been presented at the Annual Society of Toxicology Conference in Baltimore, 2004.

Animal studies have shown that Hg species can target biochemical markers such as the mACh receptor, cholinesterase (ChE) and MAO in the brain and peripheral blood tissue. However, interaction between Hg compounds and potential markers have never been explored in humans. Chapter 5 reports associations between potential biochemical markers and exposure to environmental pollutants in fish consumers from the Lac St-Pierre Region of Quebec. This is the first study to evaluate these associations and to provide potential evidence for an early biochemical marker for MeHg in a human population. This study was presented as a platform presentation at the International Conference of Mercury as a Global Pollutant, in Ljubljana, Slovenia in July 2004 and published as an extended abstract in the proceedings for this conference. These results provide important new information of the health effects of MeHg in fish eating communities. This manuscript has been submitted to Neurotoxicology for publication.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ATSDR	Agency for Toxic Substances and Disease Registry
B_{max}	maximum binding/receptor density
BMI	body mass index
BSS	balanced salt solution
ChAT	choline acetyltransferase
ChE	cholinesterase
CNS	central nervous system
d	day
DAT	dopamine transporter
EPA	Environmental Protection Agency
g	gram
Hg	mercury
$HgCl_2$	inorganic mercury
5-HT	serotonin
IC50	concentration required for 50% inhibition
Ki	inhibition constant
K_d	receptor-ligand affinity
K_m	Michaelis-Menten constant
LOEL	lowest observable effect level
mACh	muscarinic acetylcholine receptor
MAO	monoamine oxidase
MeHg	methyl mercury
min	minute
NRC	National Research Council
NMDA	N-methyl-D-aspartate
NOEL	no observable effect level
QNB	quinuclidinyl benzilate
PET	positron emission tomography
PND	post natal day
SD	standard deviation
SEM	standard error of the mean
V_{max}	maximum enzymatic velocity
WHO	World Health Organization

INTRODUCTION

Methylmercury (MeHg) is a widespread neurotoxic pollutant (ATSDR 1999). The dietary intake of contaminated fish and marine mammals is the primary route of exposure in human populations (Van Oostdam et al. 1999). These diets provide communities with an inexpensive source of protein, rich source of essential nutrients and cultural and traditional importance (Egeland and Middaugh 1997). MeHg is absorbed by the gastrointestinal tract and can cross the blood brain-barrier, disrupting nervous system protein function through high affinity interactions with sulfhydryl groups (Clarkson 1972). Accidental poisoning episodes in Japan and Iraq have shown that exposure to high-levels of MeHg leads to neurological abnormalities including ataxia, paresthesia, tremors, and visual and auditory impairments (Bakir et al. 1973; Harada 1995). Government and other agencies have set mercury (Hg) guideline exposure limits based on current epidemiological data. The guidelines for adults are based on studies where obvious severe clinical effects were observed in populations accidentally exposed to high doses of MeHg in the diet. Recent studies conducted in the Amazon Basin in Brazil, have suggested that chronic low-level exposure to MeHg is associated with impaired motor and visual neurofunction (Mergler 2002) at blood-Hg levels below the World Health Organization (WHO) low-risk exposure guideline (200 µg/L, Hg/blood) (WHO 1990). There is increasing interest to characterise the neurotoxic response at low MeHg exposure (National Research Council 2000). The ability to detect the neurotoxic effects of MeHg with greater sensitivity would provide a better understanding of health risks associated with the consumption of contaminated fish and marine mammals (Costa 1998).

While it is known that high exposure to MeHg can cause neuronal cell death (Takeuchi and Eto 1999), this may not fully explain the behavioural anomalies observed at low-level MeHg exposure levels. Biochemical changes in the nervous system generally occur prior to permanent damage and may be used to predict future neurotoxic outcomes (Costa and Manzo 1995; Manzo et al. 1996). MeHg exposure disrupts the function and transmission of the cholinergic (Hastings et al. 1975; Kobayashi et al. 1980; Levesque et al. 1992; Coccini et al.

2000) and monoaminergic nervous systems (Bondy et al. 1979; Oudar et al. 1989; Cagiano et al. 1990; Lindstrom et al. 1991; Faro et al. 1997; Dare et al. 2003). Some key markers of neuronal homeostasis and transmission in these systems, including monoamine oxidase (MAO, EC 1.4.3.4), muscarinic acetylcholine (mACh) receptor, and cholinesterase (ChE, EC 3.1.1.7), are molecular targets of Hg compounds (Hastings et al. 1975; Von Burg et al. 1980; Coccini et al. 2000; Basu et al. 2005; Basu et al. 2005). The identification of biochemical targets would help elucidate possible mechanisms of MeHg neurotoxicity and also lead to the development of new indicators to monitor these neurotoxic effects in human populations. However, due to the complexity and inaccessibility of nervous tissue, monitoring these biochemical markers remains difficult in humans.

Blood platelets and lymphocytes are unique non-neuronal cells, as they utilize similar cellular machinery as monoaminergic and cholinergic neurons, respectively (Reed et al. 2000; Tayebati et al. 2002). Specifically MAO, ChE and mACh receptors are present in peripheral blood (Costa et al. 1988; Chen et al. 1993) and interact similarly with neurotoxic compounds (Costa et al. 1990; Chakrabarti et al. 1998; Coccini et al. 2000). Chakrabarti et al showed that repeated oral exposure to MeHg reduced total MAO activity in cortex, striatum and platelet tissues in rats (Chakrabarti et al. 1998). In a similarly designed study, MeHg exposure resulted in an immediate increase in lymphocyte mACh receptor density, which was followed by a similar increase of mACh receptor density in the cerebellum and hippocampus (Coccini et al. 2000). Therefore, these animal studies suggest that monitoring peripheral biomarkers in non-neuronal tissue, may offer a method to screen for relevant biochemical perturbation following MeHg exposure. Additionally, human studies have demonstrated that platelet-MAO is reduced in heavy smokers (Whitfield et al. 2000; Berlin and Anthenelli 2001) and in industrial workers exposed to styrene and manganese (Checkoway et al. 1992; Checkoway et al. 1994; Smargiassi et al. 1995). PET (positron emission tomography) imaging studies have confirmed that MAO activity is also significantly reduced in brain, heart, kidneys and lung

tissue of heavy smokers (Fowler et al. 1996; Fowler et al. 2003), suggesting that platelet-MAO may serve as a surrogate marker for other inaccessible tissue. To our knowledge, the association between these potential peripheral biomarkers and MeHg exposure has not been reported in human populations. These markers have the potential to be used for detection of early stages of neurotoxicity and as a risk assessment strategy.

This thesis will evaluate the effects of Hg compounds on potential biochemical markers of CNS function; mACh receptor, ChE and MAO. These effects will be evaluated in 1) neuronal tissue to help understand the mechanism of MeHg neurotoxicity and 2) non-neuronal tissue for biomonitoring in human populations.

The thesis is separated into four specific objectives:

1. Determine optimal tissue storage conditions and develop high throughput methodologies for the measurement of surrogate biochemical markers in human populations.
2. Characterize the in vitro effects of Hg compounds (inorganic and organic Hg) on muscarinic acetylcholine receptor function in brain tissue (cerebellum and cortex) of several different animal species, including human.
3. Determine the effects of a low-level gestational exposure of MeHg on MAO activity in rat offspring and embryos, in order to highlight the relevance as a neurochemical marker in the developing brain.
4. Evaluate associations between the proposed peripheral biochemical markers and environmental Hg exposure in an adult fish-eating population living along the St-Lawrence River in Quebec, Canada.

Chapter 1: Literature Review

1.1 Mercury in the Environment

Mercury (Hg) is a persistent neurotoxic contaminant that is deposited in the atmosphere from both natural and anthropogenic sources (ATSDR 1999). Anthropogenic contributions, such as industrial manufacturing, fossil fuel combustion and waste incineration, are estimated to have doubled the global Hg levels during the last century (Mason et al. 1994). Hg can exist in one of three allotropic forms: elemental (Hg^{o}), inorganic ($HgCl_2$) and organic Hg. Atmospheric Hg accumulates in open bodies of water and settles in sediment where it is methylated by micro organisms into methylmercury (MeHg) (Morel et al. 1998). MeHg accumulates at the top of the food chain in predatory fish (i.e. walleye, lake trout, tuna, shark, swordfish), and fish-eating mammals (i.e. seals, whales, polar bears) (Muir et al. 1999). The dietary consumption of these top predators is the main source of human exposure to MeHg today (Van Oostdam et al. 1999). Due to the high global atmospheric residency time (6-24 months), Hg can be transported to regions located far from pollution sources (Fitzgerald et al. 1998). Therefore, due to the importance of country and traditional foods, remote and isolated communities may be at increased risk of Hg exposure (Van Oostdam et al. 1999).

1.2 Mercury and Human Health

Hg has been shown to affect the renal, cardiovascular, immune and reproductive systems in humans (National Research Council (NRC) 2000). However, it is clear that the nervous system is the most sensitive to the aberrant effects of Hg (ATSDR 1999). Absorption of MeHg by the gastrointestinal tract is estimated at 95%, compared to only 7-15% of $HgCl_2$ (NRC 2000). Once MeHg enters the blood circulation, it is transported throughout the body bound to proteins in red blood cells and plasma. A cysteine transporter on the blood-brain barrier facilitates the transport of a MeHg-cysteine complex into the brain (Aschner and Clarkson 1988). MeHg may undergo biotransformation to $HgCl_2$ in the target tissue (Vahter et al. 1995). Both MeHg and $HgCl_2$ are considered neurotoxic, however it is unclear which form has a greater impact on brain

function. The subsequent damage to the brain is usually permanent, due to the inability of most neurons to efficiently remove Hg compounds, and repair the damage (Rice 1999). MeHg can also cross into the placenta causing damage to the developing fetus and fetal brain (Kajiwara et al. 1996). The fetal brain is particularly sensitive, as Hg compounds can disrupt neuronal migration, mitosis, and cellular signalling processes resulting in improper brain development (Castoldi et al. 2001).

1.3 High Dose Poisoning Episodes

The majority of knowledge regarding the toxic effects of MeHg on human populations is from catastrophic episodes of high-dose poisonings. The first documented disaster occurred during the 1950s in Minamata Bay, Japan, when inhabitants consumed fish from water severely polluted with Hg by a local industry (Harada 1995). Another major poisoning occurred in Iraq in the early 1970s from the consumption of bread made from grain treated with an organomercury fungicide (Bakir et al. 1973). These events resulted in severe neurological damage, and in many cases fatalities.

Reports from these incidences highlighted the sensitivity of the developing nervous system to Hg exposure. Children who were exposed to MeHg *in utero* showed severe congenital neurological dysfunctions and developmental abnormalities (Amin-Zaki et al. 1974; Harada 1995). MeHg increased the incidence of cerebral palsy, blindness, deafness, and severe mental retardation in children exposed during gestation (NRC 2000). Autopsies revealed severe damage to the neurons throughout the entire brain cortex (Takeuchi and Eto 1999). Children born with less severe effects of MeHg exposure in Iraq were evaluated for abnormalities in central nervous system (CNS) function (NRC 2000). From these developmental observations, a toxic effect threshold level was estimated at approximately 10μg/g (Hg/hair) or 50μg/L (Hg/blood) for abnormal CNS outcomes (Cox et al. 1989).

Adults exposed to high MeHg concentrations suffered from paresthesia tremor, muscle weakness, visual abnormalities, sensory impairment, convulsions,

and mental deterioration (Bakir et al. 1973; Harada 1995). Pathological analysis of the brain indicated that MeHg was regionally specific, consistently damaging areas in the cerebral cortex and cerebellum, while to a lesser extent affecting the brain-stem and spinal cord (Takeuchi and Eto 1999). More specifically, the calcarine sulcus of the visual cortex was frequently damaged. The damage to these brain regions are related to the clinical symptoms (visual and motor dysfunction). The cerebellum facilitates basic motor function such as balance and coordination of movement (Harry 1999). Generally these regions show loss of neurons due to the initiation of apoptotic and necrotic cellular death pathways (Takeuchi and Eto 1999). Increased glial cell proliferation (gliosis) can be observed in the cortex as a consequence of neuronal cell loss. Data from the Hg disaster in Iraq was used to set the low risk guideline for developing paresthesia in adults (WHO 1990).

1.4 Effects of Low-Level Chronic Exposure to MeHg

While the toxic effects of high exposure to MeHg are well known, current research is attempting to elucidate the risks posed by chronic low dietary ingestion of MeHg (NRC 2000). Communities that rely on fish and aquatic animals as an important part of their diet, may be at risk of chronic Hg exposure (Van Oostdam et al. 1999; Chan and Receveur 2000; Chan et al. 2003). In addition, pregnant women who consumed marine mammals and fish must be particularly conscious of Hg intake, as even low-levels may be neurotoxic to the developing fetal brain (ATSDR 1999). It has been estimated that in 2000, 300,000 women in the United States consume enough Hg to harm their unborn baby (Mahaffey et al. 2004). In order to evaluate these early effects of low Hg exposure, sensitive behavioural tests are performed to evaluate cognitive, sensory and motor function skills in controlled animal and in epidemiological studies.

1.4.1 Low-Level Exposure in Animals

Animal studies have simulated low-level chronic MeHg exposure and observed patterns of behavioural related neurotoxic effects. A series of controlled laboratory studies performed with monkeys highlighted that neurobehavioural damage was related to the visual, auditory and somatosensory system following developmental and adult exposure (Rice and Gilbert 1990; Rice 1996). Monkeys exposed to low-levels of MeHg during developmental stages showed a restriction in visual fields and reduction in contrast sensitivity compared to control animals (Rice 1996). These observations were similar to characteristics of human poisoning episodes in Minamata Bay and Iraq (ATSDR 1999). Interestingly, the onset of somatosensory impairments was delayed and appeared years after the final dose was administered to the monkeys (Rice 1996). However, rats are the most widely used animal model to evaluate the toxic effects of Hg compounds. While rodent studies have shown less conclusive findings, impaired motor function is the most consistently observed behavioural dysfunction (Rice 1996). The absence of damage to the visual and auditory systems in rats may be related to lower brain complexity when compared to primates (Rice 1996). Toxicokinetics may also explain these differences as monkeys and humans have higher brain:blood Hg ratios (2-5) when compared to rats (0.06) (Rice 1996). Despite these differences observed among species, the rodent model is still extensively used to elucidate the mechanism of MeHg neurotoxicity.

1.4.2 Prenatal Low-Level Exposure in Humans

Unlike high exposure levels, which result in definitive clinical symptoms, chronic low exposure levels are associated with non-specific neurological impairments that cannot be directly linked to MeHg exposure in individuals. At the present time, only studies in large populations are able to identify associations between low-level MeHg exposure and subtle changes in CNS function (Grandjean et al. 1997; Mergler 2002; Myers et al. 2003). Two large (n>700 mother/child pairs) longitudinal studies examined the effects of low-dose prenatal Hg exposure on various neurobehavioral endpoints in children

(Grandjean et al. 1997; Davidson et al. 1999; Myers et al. 2003). Two cohorts were chosen because of their homogeneous populations and because of elevated exposure to Hg through dietary dependence on marine animals. The data from the Faroe Islands suggested a relationship between prenatal exposure to MeHg (mother hair exposure) and children's performance on neurobehavioural tests at 7 years of age (Grandjean et al. 1997; Murata et al. 1999). The observed neurofunctional impairments related to MeHg exposure in the Faroe Island cohort were similar to those observed in studies performed in non-human primates (Rice 1996). MeHg exposure was associated with reduced visual, auditory and impaired cognitive performance (Murata et al. 1999). Milestone development and neurobehavioural analysis was assessed at 0.5, 1.5, 2.5, 5.5, and 9 years of age in the children from the Seychelles Island (Davidson et al. 1999; Myers et al. 2003). Despite having similar exposure levels, MeHg related behavioural impairments were not observed in the Seychelles Island cohort (Myers et al. 2003). Reasons for these inconsistencies are not known. Several hypotheses include differences in exposure pattern, genetics, intake of other dietary nutrients and other contaminants as factors (NRC 2000).

1.4.3 Low-Level Exposure in Adults

Several studies performed in adults suggested an association between chronic exposure through fish consumption and clinical neurotoxicity (NRC 2000). In 1990, a World Health Organization (WHO) report suggested exposure to dietary MeHg resulting in blood-Hg concentrations above 200 µg/L were associated only with a low-risk of neurological damage in adults (WHO 1990). However, six years later a meta-analysis of 13 studies evaluated the effects of Hg toxicity in adults and suggested that the WHO guideline level was not an appropriate lowest observable effect level (LOEL) for adults (Kosatsky and Foran 1996). This report further suggested that the effects of low-level exposure be more thoroughly evaluated (Kosatsky and Foran 1996). In response to this meta-analysis, several studies examined neurotoxic effects of Hg in adults living in the Brazilian Amazon using highly sensitive tests to quantitatively evaluate motor,

cognitive and sensory performance (Lebel et al. 1998; Dolbec et al. 2000; Mergler 2002; Yokoo et al. 2003). The Brazilian populations were selected because of their dependence on fish for sustenance and subsequent high MeHg exposure levels. Three studies examined MeHg exposure and neurobehavioural tests in 233 adults (Mergler 2002). MeHg exposure was relatively high with 80% of the participants above the 20µg/L guideline (increasing risk) level for blood-Hg (Mergler 2002). In these populations, MeHg exposure was associated with impaired motor function as evaluated by a grooved pegboard test, finger tapping test, and disorganized arm movement. Impaired visual contrast and colour sensitivity were also associated with increased MeHg exposure. These results are in agreement with the previously mentioned controlled monkey feeding studies (Rice 1996; Mergler 2002). Analysis of these populations revealed significant Hg related impairments at concentrations below the WHO 1990 guideline level for adults (Lebel et al. 1998).

1.5 Hg Guidelines

National and international agencies have set guidelines for safe Hg exposure limits in humans. These guidelines were set to protect the most sensitive individuals in the population. Previous observations in human populations were used as a basis for guideline limits, however discrepancies arise due to interpretation and application of uncertainty and safety factors. Health Canada's current "increasing risk range" is from 20 to 200 µg/L blood-Hg (or 6 to 50 ppm for hair Hg), while the US-Environmental Protection Agency's (EPA's) upper safety limit is currently several times lower (5.8 µg/L blood-Hg). The EPA used the LOEL from the Faroe Island study (blood-Hg; 58 µg/L) in conjunction with a 10-fold dilution safety factor to achieve the upper safe limit (Budtz-Jorgensen et al. 2000). This level compares to a dietary reference dose intake of 0.1 µg MeHg/kg body weight/day. Under the EPA guideline, a large percentage of pregnant women in fish eating communities (i.e. Nunavik) may be at or above these levels (Muckle et al. 2001).

It is clear that setting low guideline limits is important to protect potential

sensitive subsets of the population and to increase public concerns of Hg pollution. However, the consequence of excessively low guideline levels can negatively affect those populations that are dependent on fish and marine mammals for sustenance (Kuhnlein and Chan 2000). Aquatic mammals and fish are an excellent source of essential nutrients including vitamin E, selenium, calcium, and omega-3 and omega-6 fatty acids, which are also required for proper brain development (Grandjean et al. 2001; Mahaffey 2004; Sakamoto et al. 2004). Fish also provide a source of high-quality protein and have low-levels of saturated fats (Kuhnlein 1995). Therefore, dramatic reduction in marine diet may have a severe impact on nutrient intake (Kuhnlein 1995; Egeland and Middaugh 1997). In addition to providing an affordable source of nutrients, dietary consumption of aquatic animals has cultural importance in these communities (Kuhnlein 1995; Kuhnlein and Chan 2000). In many cases the alternative to the traditional diet is market foods which tend to be more expensive especially in remote rural locations (Kuhnlein and Receveur 1996; Kuhnlein and Chan 2000). In theory the intake of these market foods may increase the prevalence of obesity and diseases such as diabetes, heart disease, and stroke (Kuhnlein et al. 2004).

Furthermore, studies performed in adults have shown that neurotoxic effects of MeHg can be observed when blood-Hg concentrations are below 200 µg/L (Mergler 2002). However, little is known about the lower end of the dose-response curve in adults, and guidelines have been made based on extrapolations. Therefore, in order to better inform the public on health concerns related to eating fish, it is important to understand the earlier stages of neurotoxicity by developing more sensitive detection methods. The identification of sensitive populations would allow the implementation of a targeted approach to address specific health needs of a community.

1.6 Risk Assessment and Detection of Neurotoxicity

Due to the complexity and inaccessibility of the central and peripheral nervous system, the diagnosis of neurotoxicity remains difficult in humans. A simple risk assessment approach is to measure and compare markers of Hg

exposure in a community (i.e. hair-Hg or blood-Hg) with the established exposure guideline levels (Agusa et al. 2005; Legrand et al. 2005). Therefore, this approach is limited as it only examines exposure, and not specific effects of neurotoxicity.

When evaluating the risks of more common diseases in a population, such as cancer, risks can be determined from population databases (Gaudette et al. 1996). During the poisoning episode in Japan and Iraq, the primary effects of Hg exposure were observed as obvious clinical and physiological symptoms detected at the individual level (Bakir et al. 1973; Harada 1995). However, in a small community exposed to low ranges of MeHg, these clinical neurotoxic illnesses would be extremely rare and the assessment of risk would be difficult to evaluate. Therefore, in order to asses the risk of MeHg neurotoxicity, it is important to use sensitive neurofunctional tests designed to quantitatively detect minor changes in motor, cognitive and sensory performance skills (Mergler 2002). However, these tests can be time consuming for the researcher and the participants. Moreover, in early stages of chronic low-level exposure, MeHg may cause a disruption of biochemical and neuron function, without manifesting a neurophysiologic or behavioural effect (Castoldi et al. 2001). Therefore, identification of these specific biochemical alterations in the brain following exposure to MeHg could potentially be used to monitor the early stages of neurotoxic effect at the lower end of the dose-response curve (Manzo et al. 1995). Therefore, in order to better evaluate the health risks and impacts of a particular environmental contaminant, it is critical to understand the underlying biochemical mechanism of its neurotoxicity (Manzo et al. 1995; Costa 1998).

1.7 Neurotoxic Mechanisms of Exposure to Hg Compounds

The mechanisms by which Hg compounds act to disrupt neuronal function are difficult to elucidate because of its non-specific action and due to brain complexity (NRC 2000). Generally speaking, Hg compounds have high affinity for sulphur containing amino acids, such as cysteine, which are important for the structure and function of proteins (Clarkson 1972). Hg can interfere with a

number of neuronal parameters including enzyme activity, receptor binding, transporter function and protein synthesis (Costa 1988). Following neurotoxic exposure to Hg, disruption of neuronal function may result, causing behavioural or physiological effects. If Hg exposure exceeds the capacity to maintain homeostasis, the initiation of apoptotic or necrotic pathways may occur, resulting in cellular loss and permanent damage (Castoldi et al. 2000).

Hg compounds have been shown to depolymerise microtubules, resulting in altered structure of the cytoskeleton (Miura et al. 1999; Heidemann et al. 2001). Microtubules are also important for axonal growth and synapse formation during development and in turn play a role in transport of proteins, organelles and other vesicles throughout the neuron. Cytoskeleton disruption is thought to cause alterations in the developing and adult brain structure. Another proposed mechanism suggests that MeHg blocks the reuptake of the neurotransmitter, glutamate, by astrocytes at the synapse (Aschner et al. 2000). Accumulation of extracellular glutamate binds and activates the N-methyl-D-aspartate (NMDA) receptor. The NMDA receptor facilitates the increase in cellular calcium and subsequent activation of apoptotic pathways. MeHg may also act to increase the reactive oxygen species which can induce oxidative stress resulting in cellular damage (Yee and Choi 1996). While many targets for MeHg toxicity have been identified in the CNS, it is important to identify sensitive targets of MeHg responsible for behavioural impairments at the whole organism level (Castoldi et al. 2001).

Brain sensory, cognitive and motor signals are mediated throughout the brain and body via synaptic communication between neurons. The synapse is the point through which neurons communicate, and is pivotal in behavioural processes (Harry 1999). Neuronal synaptic transmission is dependent upon a series of electrical and/or chemical processes. For example, the presynaptic release of transmitters into the synapse and their binding to specific postsynaptic receptors requires a series of enzymes, transporters and receptors for proper function (Schwartz 2000). Any disruption of the key components in neuronal communication may result in behavioural alterations (Harry 1999). In some

cases following neurotoxic exposure, the neuron may be capable of maintaining homeostasis through compensatory mechanisms (Harry 1999). While these compensations may mask overt behavioural impairments in the whole organism, the detection of these mechanisms can serve as early warning markers of brain dysfunction (Costa and Manzo 1995). These neurotransmitter systems also play an important role in the regulation of brain development, suggesting that disruption *in utero* may be a potential mechanism to explain MeHg neurotoxicity (Buznikov et al. 1999; Herlenius and Lagercrantz 2004). Several different neurotransmitter systems have been identified to play a role in specific physiological functions. Some of these neurotransmitter systems are affected by Hg compounds and include the cholinergic (acetylcholine) and the monoaminergic (dopamine, noradrenalin and serotonin) systems.

1.7.1 Cholinergic System

The cholinergic system utilizes the neurotransmitter, acetylcholine (ACh), which binds and activates either the nicotinic or muscarinic acetylcholine (mACh) receptor on the postsynaptic cell. Acetylcholine is synthesized through a single enzymatic reaction catalyzed by choline acetyltransferase (ChAT, EC 2.3.1.6) in neurons. The degradation and inactivation of the transmitter occurs in the synapse by the enzyme cholinesterase (ChE) (Schwartz 2000). Both isoforms of ChE, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8), hydrolyze acetylcholine (ACh) into choline and acetate. The physiological and behavioural roles of the cholinergic system have been suggested through various studies designed to selectively disrupt or inhibit the individual components. Acetylcholine neuronal transmission is involved in cognitive, learning, memory, motor and thermoregulation (Chrobak et al. 1988; Wess 2004) (Sims et al. 1980; Bymaster et al. 2003; Wess 2004).

The mACh receptor is expressed in cholinergic neurons throughout the cortex. In vitro studies have shown that Hg compounds inhibit the mACh receptors binding in rat brain tissue preparations (Eldefrawi et al. 1977; Bondy and Agrawal 1980; Von Burg et al. 1980; Castoldi et al. 1996). It has been

proposed that Hg can competitively bind to the cysteine residues localized in the binding pocket of the mACh receptor (Cagiano et al. 1990). However, an in vivo study showed that long term ingestion of MeHg by rats actually increased the density of the muscarinic receptor in the hippocampus and cerebellum by 20-40% (Coccini et al. 2000). These effects were only observable 2 weeks after the final dose. This up-regulation of the mACh receptors in the brain may be a compensatory mechanism for disrupted ACh binding and reflect early stages of neurochemical impairment.

Experiments have also shown that Hg can alter the release of ACh from the neuronal synapse. Hg inhibits the nerve evoked release of ACh, while increasing the spontaneous release from the nerve terminal (Kostial and Landeka, 1975; Atchison, 1986; Traxinger and Atchison, 1987) in a dose dependent fashion (Minnema et al. 1989). In a carefully designed study, the MeHg induced release of ACh could be blocked by a drug that inhibits calcium release from the mitochondria transition pore (Levesque and Atchison 1988). This suggests that MeHg exposure results in 1) increased calcium release from intracellular organelles, 2) neuronal depolarization and 3) release of ACh into the synapse (Levesque and Atchison 1988). Earlier studies indicated that repeated doses of MeHg decreased acetylcholine in the cortex and striatum of mice (Kobayashi et al. 1980). Therefore, continuous spontaneous release of ACh from neurons may explain reduced brain concentrations.

Additionally, several enzymes involved in ACh metabolism are impaired following MeHg exposure, including ChAT (Kobayashi et al. 1979) and ChE (Hastings et al. 1975; Lakshmana et al. 1993). Due to the numerous cholinergic markers that change as a result of exposure to Hg compounds, it is difficult to identify the primary target. It is known that the mACh receptor, ChE, ChAT and ACh are maintained in homeostasis (Erb et al. 2001). Therefore, the observed changes in the biochemical components of the cholinergic system following Hg exposure may represent compensatory mechanisms and could be used as a marker of early neurotoxicity.

1.7.2 Dopaminergic System

Dopamine is synthesized from the essential amino acid, tyrosine, through a series of enzymatic reactions which involve the rate limiting enzyme, tyrosine hydroxylase (EC, 1.14.16.2) (Schwartz 2000). Once released into the synapse, dopamine binds to dopamine receptors (D1 and D2). Re-uptake is facilitated by the dopamine transporter (DAT) on the surface of astrocytes and neurons. Intracellular dopamine is oxidatively deaminated by monoamine oxidase (MAO, EC 1.4.3.4) an enzyme located on the surface of mitochondria (Schwartz 2000). Dopaminergic neurotransmission has been shown to be important for motor function (Zhang and Creese 1993; Harry 1999), learning and memory (Zhang and Creese 1993) and in visual function (Gobba 2000). Specific damage to dopamine neurons in the nucleus nigrostriatal tissue results in motor and tremor impairments similar to those observed in Parkinson's disease (Harry 1999).

In order to evaluate the effects of low-level prenatal exposure to MeHg on the dopaminergic system in rat offspring, a series of studies were conducted to assess motor performance before and after drug induced stimulation of dopamine pathways (Rossi et al. 1997; Gimenez-Llort et al. 2001; Dare et al. 2003). Apomorphine, d-amphetamine, and a D2 receptor agonist (U91356A) were used to stimulate the dopaminergic system. The observed spontaneous motor activity in MeHg treated offspring was altered following administration of these drugs when compared to controls, suggesting altered dopamine transmission.

Hg compounds are known to alter various components of the dopaminergic system. In vitro studies have demonstrated that both $HgCl_2$ and MeHg inhibit D2 receptor binding in synaptosomes of rat brain (Scheuhammer and Cherian 1985). However, *in vivo* studies have observed both increases and decreases in D2 receptor density of rat offspring exposed to MeHg (Cagiano et al. 1990; Dare et al. 2003).

Similar to the effects of Hg compounds on ACh release, MeHg stimulates the spontaneous release of dopamine from various brain tissue preparations (Bondy et al. 1979; McKay et al. 1986). To determine the effects of chronic MeHg exposure on dopamine release from the striatum in rats, microdialysis

techniques have been used to directly detect changes in brain transmitter levels (Faro et al. 1997). This study observed increases in extracellular dopamine concentrations in response to exposure to low-levels of MeHg (Faro et al. 1997). A similar in vivo study also observed that administration of intra-striatal MeHg increased dopamine concentrations in the caudate-putamen region of the brain, with a paralleled decrease in dopamine metabolites (Faro et al. 2003). Changes in dopamine concentrations could be explained by increased release, increased synthesis, reduced metabolism or reduced uptake transport of the neurotransmitter.

MAO is flavin-bound enzyme present on the outer surface of mitochondria in neurons and astrocytes. The enzyme is involved in the oxidative deamination of several biogenic amines including serotonin, dopamine, and noradrenalin (Shih 2004). Early studies have suggested that Hg compounds can bind to critical cysteine residues and thereby impair MAO function (Gomes et al. 1969; Gomes et al. 1976). Repeated MeHg dosing studies performed in rats were shown to reduce MAO activity equally in several different brain regions (Chakrabarti et al. 1998) and in other tissues (Fowler and Woods 1977; Fowler and Woods 1977). Hg does not appear to alter tyrosine hydroxylase activity, suggesting that rates of dopamine synthesis are not affected. MeHg inhibits the uptake of dopamine into rat brain synaptosomes and cells suggesting that the dopamine transport may be blocked (Rajanna and Hobson 1985; Faro et al. 2002). It has been suggested that altered neurotransmitter degradation and reuptake may also explain the observed increases in dopamine concentration in the brain tissue (Faro et al. 2002).

1.7.3 Serotonergic System

Serotonin is an indolamine transmitter that is synthesized from tryptophan by the rate limiting enzyme tryptophan hydroxylase (EC 1.14.16.4) (Schwartz 2000). Reuptake of serotonin occurs through the monoamine transporter on the cell surface of astrocytes and neurons, and can then be oxidized by MAO at the mitochondrial surface (Schwartz 2000). The serotonergic system has been

implicated in neurobehaviour processes including, locomotion, learning, memory and emotion (Buhot et al. 2000; Sillar et al. 2002; Overstreet et al. 2003). The majority of current research focuses on the role of serotonin in mood disorders (Overstreet et al. 2003). Additionally, serotonin has been connected with learning and memory paradigms in studies conducted with genetically modified mice (Buhot et al. 2000). Serotonergic neurons are also known to innervate several regions in the brain including the visual cortex and cerebellum (Harry 1999).

Studies have shown that long-term treatment with MeHg (0.4 and 4.0 mg/kg/d) decreased serotonin concentrations in the brain-stem of rats (Lakshmana et al. 1993). Hg compounds increase the uptake and release of serotonin from isolated synaptosomes in vitro (Komulainen and Tuomisto 1982). Studies using primary cultured astrocytes suggest that MeHg also inhibits cellular uptake of serotonin (Dave et al. 1994). Additionally, the previously mentioned inhibition of MAO by MeHg could alter the balance of serotonin in the neurons and therefore also contribute to the disruption of this pathway (Chakrabarti et al. 1998). The effects of Hg compounds on other serotonin receptors or its catabolic enzymes have not been reported.

1.7.4 Noradrenergic System

The catecholamine, noradrenalin, is synthesized from dopamine precursor through the actions of dopamine-beta-hydroxylase. The neurotransmitter is inactivated through oxidation by MAO or methylation by catecholamine-O-methyltransferase (COMT, EC 2.1.1.6). Noradrenergic neurons are innervated in several key regions of the brain involved in arousal, learning and memory (Murchison et al. 2004). Noradrenalin also plays an important role in the sympathetic nervous system which regulates heart rate, blood pressure and fight or flight responses (Harry 1999).

There is evidence to suggest that the noradrenergic system is also affected by exposure to Hg compounds. Prenatal exposure to MeHg increased cerebellum noradrenalin concentrations in 50 day old rat pups by two-fold (Lindstrom et al. 1991). Long-term dosing studies in developing rats showed that

noradrenalin concentrations increased in the olfactory bulb, brainstem, and visual cortex after 60 days exposure to MeHg when compared to control animals (Lakshmana et al. 1993). These observations may be explained by decreased MAO activity observed following MeHg exposure (Chakrabarti et al. 1998). Additionally, both $HgCl_2$ and MeHg compounds stimulate noradrenalin release from rat hippocampus slices (Gasso et al. 2000). There have been no reported effects of Hg compounds on noradrenalin receptors, or its anabolic enzymes.

Table 1.1: Summary of interactions between in vivo exposure to MeHg and neurochemical changes

Neurochemical System	Associated Behaviour	Proposed marker	Observed change	Reference
Cholinergic	Motor, learning	ChAT	Decreased activity	(Kobayashi et al. 1979)
		ChE	Decreased activity	(Hastings et al. 1975)
		mACh receptor	Increased density	(Coccini et al. 2000)
		ACh	Decreased levels	(Lakshmana et al. 1993)
Dopaminergic	Locomotion, learning, memory	MAO	Decreased activity	(Chakrabarti et al. 1998)
		Transporters	Inhibition	(Faro et al. 2002)
		D2 receptor	Increased density	(Cagiano et al. 1990)
		Dopamine	Increased levels	(Faro et al. 1997)
Serotonergic	Emotion, memory, learning	MAO	Decreased activity	(Chakrabarti et al. 1998)
		Transporters	Inhibition	(Dave et al. 1994)
		5-HT	Increased levels	(Lakshmana et al. 1993)
Noradrenergic	Sympathetic system, learning and memory	MAO	Decreased activity	(Chakrabarti et al. 1998)
		Noradrenalin	Increased levels	(Lindstrom et al. 1991)

1.8 Development of Surrogate Biomarkers of Nervous System Function

Due to the inaccessibility of human brain tissue, the study of neurochemical changes in individuals exposed to neurotoxic compounds remains difficult. Technology exists to allow the in vivo detection of neurochemical markers in the brain through the use of positron emission tomography (PET) techniques (Fowler et al. 1996; Bohnen et al. 2003). However, the use of PET imaging is costly, time consuming and is therefore not practical for explorative population studies. Another possible technique to assess neurochemical changes in humans involves the use of biochemical markers or biomarkers (Manzo et al. 1995). Biomarkers are indicators of changes in function, structure or quantity of a biological or cellular component. These indicators are measurable in samples from organisms or can be taken from the organisms directly. Biomarkers can be divided into three classes, based on their identification of exposure, effect, or susceptibility.

There are no established neuronal biochemical markers of biological effect for MeHg neurotoxicity in humans. As described previously, neurobehavioral tests are currently used to address the relationship between MeHg exposure and neuronal damage in humans. The development of biochemical markers to detect neurotoxic effects that could be easily and ethically measured, may provide a new opportunity to further understand the effects of MeHg in humans (Costa and Manzo 1995). Neurochemical related targets of MeHg have been tested in animal models, and involve the examination of neurotransmitter concentrations, receptors and enzymes. While the target tissue for Hg is inaccessible in humans, some biochemical markers have been identified in non-neuronal blood cells and plasma (Costa and Manzo 1995). Therefore, the potential exists for these markers to be used as surrogate biomarkers of brain neurotoxicity. Research has shown some biochemical markers can reflect equivalent changes in similar neurochemical parameters in brain tissue (Costa, 1998). Generally these biomarkers are measured in isolated blood components including, red blood cells, platelets, lymphocytes and plasma (Table 1.2).

Table 1.2: Summary of some potential blood biochemical markers of central nervous system function

Blood Tissue	Biochemical Marker
Erythrocytes	Acetylcholinesterase
Platelets	Serotonin Monoamine oxidase Vesicular monoamine transporter Serotonin Receptor
Plasma/Serum	Dopamine-β -Hydroxylase Butyrylcholinesterase
Lymphocytes	Muscarinic Acetylcholine Receptor Acetylcholinesterase Choline Acyltransferase Choline transporter Acetylcholine Dopamine Receptor

1.8.1 Non-Neuronal Cholinergic System

Peripheral lymphocytes are a type of white blood cell involved in immune system function. These non-neuronal cells contain similar components to the cholinergic neurons, including acetylcholine, ChAT and ChE (Tayebati et al. 2002; Kawashima and Fujii 2003). Functional mACh receptors have been identified on the surface of isolated rat lymphocytes (Costa et al. 1988; Costa et

al. 1990) and human lymphocytes (Adem et al. 1986; Bronzetti et al. 1996). As a whole, these components in lymphocytes work to regulate immune system function. However, it has been proposed that lymphocytes may reflect changes in cholinergic nervous system following toxic exposure to environmental pollutants. In the study performed by Coccini et al, the repeated low doses of MeHg (2 mg/kg/d) increased mACh receptor density in the hippocampus and cerebellum, which was preceded by a marked increase in density of mACh receptors on peripheral blood lymphocytes (Coccini et al. 2000). These results suggest that peripheral lymphocytes may represent a sensitive target to study the interaction of MeHg with mACh receptors and may be predictive indicators of neurotoxicity. These assumptions are strengthened by studies that show individuals with neurological disorders, such as Parkinson's disease (Rabey et al. 1990; Rabey et al. 1991) and Alzheimer's disease (Adem et al. 1986; Rabey et al. 1986; Smith et al. 1988) can have altered mACh receptor binding and expression in blood lymphocytes. These studies suggest that lymphocytes may reflect neuronal cholinergic system function and provide a strategy to monitor neurotoxicity in humans.

Several biochemical markers of CNS function can be measured in erythrocytes and plasma. One of the most studied of these biomarkers is the enzyme, ChE (Wilson et al. 1997). Two classes of pesticides, organophosphates and carbamates, act to specifically inhibit ChE activity which results in synaptic ACh accumulation and neurological symptoms (Wilson et al. 1997). Blood ChE monitoring serves as an inexpensive method to track exposure and is required for workers who handle these pesticides (Wilson et al. 1997). ChE activity in blood has shown to mimic enzyme activity in the CNS of animals exposed to these pesticides (Costa 1988; Manzo et al. 1995). While direct inhibition of blood ChE by industrial Hg exposure have not been observed in humans (Zabinski et al. 2000), reductions have been observed in brain tissue of MeHg exposed animals (Hastings et al. 1975).

1.8.2 Non-Neuronal Monoaminergic System

Platelets are secretory cells that play an important role in hemostasis, thrombosis, vascular remodelling and repair. These cells are considered to be a peripheral model of serotonergic nerve endings, as they utilize similar transmitters, enzymes, receptors and transporters for cellular communication (Reed et al. 2000; Polgar et al. 2002). Specifically, platelets contain serotonin, serotonin transporters and MAO, which are homologous to forms expressed in neuronal cells (Chen et al. 1993; Billett 2004). In vitro studies suggested that serotonin uptake in rat hypothalamic synaptosomes and human platelets were similarly inhibited by incubation with MeHg (Tuomisto and Komulainen 1983). Chakrarbarti et al has shown that repeated oral exposure to MeHg in rats inhibits MAO activity in both platelets and brain tissue (Chakrabarti et al. 1998). These studies suggest that platelet serotonin transporters and MAO activity may act as a surrogate for neuronal effects of MeHg exposure. The level of serotonin in the blood platelets may also be used as a surrogate biomarker to reflect changes in concentrations in the brain due to exposure of neurotoxic compounds such as MeHg. There is evidence that MAO and serotonin levels in platelets may be related to neurological diseases, including Parkinson's, Alzheimer's and Schizophrenia (Bond et al. 1979; Jossan et al. 1991; Jarman et al. 1993; Fitzgerald et al. 1996; Zhou et al. 2001), however some evidence is inconsistent (Konings et al. 1995; Simpson et al. 1999). The application of these platelet biomarkers have been measured in industrial workers exposed to neurotoxic compounds, including manganese and styrene. In these cases, exposure has been associated with decreases in platelet-MAO (Checkoway et al. 1992; Smargiassi et al. 1995) suggesting a potential neurotoxic effects. Therefore, blood platelets may offer an ethical way to evaluate serotonergic neuronal function in human populations (Costa and Manzo 1995).

1.9 Research Perspectives

There are several stages involved in the selection, development and validation of potential biochemical marker of CNS function. The first stage

involves basic laboratory studies to develop biochemical assays, and to test the neurotoxicant/biomarker relationships in animal and in vitro systems. These preliminary investigations are followed by transitional studies designed to evaluate the feasibility and application of measuring these biomarkers in humans. Sample collection, transportation, storage and processing concerns for field application are also addressed at this stage. Subsequently, applied epidemiological studies evaluate the relationship between neurotoxic exposure and biomarker in a select population. However, these applied studies cannot prove a relationship between the biomarker and CNS function. If initial studies suggest that a proposed biomarker correlates with exposure in a population, further studies would be required to understand the detailed nature of this response and what possible physiological consequence these biomarker changes represent.

Several receptors and enzymes in the nervous tissue have been proposed as biomarkers that may indicate the neurotoxic effects of MeHg exposure (Costa 1988). Studies in animals have shown that biochemical markers (i.e. mACh receptors, and MAO activity) in the target nervous tissue and in accessible non-neuronal tissue interact similarly with MeHg (Chakrabarti et al. 1998; Coccini et al. 2000). These transitional studies suggest a proposed strategy to measure these biomarkers in human populations. Laboratory techniques are available to isolate the relevant non-neuronal tissues from blood samples for biochemical analysis. Human studies have identified factors, such as age, sex, and smoking habits that may influence these biomarkers (Costa and Manzo 1995; Whitfield et al. 2000). Therefore, there is sufficient evidence to suggest that applied studies should begin to evaluate the associations between MeHg exposure and these proposed peripheral biomarkers.

Connecting Paragraph

While methodologies for measurement of the proposed biochemical markers have been previously reported in small scale laboratory studies, the application towards epidemiological studies may have limitations. This next chapter describes new high throughput microplate techniques and optimal blood storage conditions for the measurement of biomarkers of neurotoxicity in large community studies. This manuscript describes an ecosystem approach to evaluate neurotoxic risks of pollutants, and therefore an additional component discusses the feasibility of using wildlife brain tissues obtained from the field for biomarker measurement. The wildlife component of this manuscript is the focus of N. Basu's PhD thesis.

Chapter 2: Biochemical markers of neurotoxicity in wildlife and human populations: Considerations for method development

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Abstract

Disruption of neurochemical parameters in blood and brain tissues can be used as early biomarkers of neurotoxicity in human and wildlife epidemiological studies. To investigate the feasibility of biomarker measurements in field samples obtained from remote locations, tissue storage limits were determined with human blood and mink cortex tissue using efficient and cost-effective microplate assays. Results show that isolated blood platelets and plasma can be stored at 4°C for 4 weeks before measurement of monoamine oxidase (MAO) and cholinesterase (ChE) activities, while human lymphocytes can be stored at 4°C for up to 2 days before muscarinic acetylcholine (mACh) receptor binding analysis. Blood cells stored frozen resulted in decreased MAO activity and mACh receptor function. These data suggest that mink brain tissue obtained from field samples can be stored at various temperatures without affecting dopamine (D2) and mACh receptor densities, however MAO and ChE activities were most stable in samples stored in a –20°C domestic freezer or at 4°C. Multiple freeze/thaw cycles alters mACh and D2 receptors and MAO activity in mink cortex samples and should therefore be minimized. In conclusion, these neurochemical biomarkers can efficiently be measured in large human and wildlife neurotoxicity studies provided proper storage conditions are maintained.

2.1 Introduction

Several hundred chemicals present in the environment have been identified as neurotoxic to humans (Rice 1999) and wildlife (Hoffman et al. 2003). A common method for the evaluation of neurotoxic risk to humans is a cross-sectional epidemiological study that evaluates the relationship between chemical body-burden and observable outcomes obtained from clinical evaluations or neurobehavioural tests (Lebel et al. 1996; Stewart et al. 2003; Bellinger 2004). While these tests provide necessary exposure-response information, they are generally labour intensive, expensive to perform, and may not be sensitive enough to detect some early pre-clinical adverse effects. It is established that changes in brain chemistry often precede irreversible behavioural alterations following neurotoxic exposure (Costa and Manzo 1995; Manzo et al. 1996). Monitoring these neurochemical changes (i.e. neurotransmitter levels, metabolizing enzyme activities, and receptor functions) can be used as biomarkers of effect to assess the risks posed by neurotoxicants on susceptible populations (Manzo et al. 2001).

Monitoring wildlife health is often overlooked as a risk assessment strategy to evaluate the impact of environmentally relevant concentrations of neurotoxicants on humans and ecosystems (Fox 2001). Wildlife, such as mink, pigeons, bald eagles, and polar bears can be used as sentinel species due to their susceptibility to bioaccumulate neurotoxicants from the local environment (Roperto and Galati 1998; Chan et al. 2003; Hoffman et al. 2003). For example, chronic low-level chemical exposure from contaminated fish may have deleterious effects on wild mink and river otter populations in the Great Lakes basin (Wren 1991). These effects may be a result of exposure to organochlorines and mercury (Hg), which are well known to impair normal behaviour in laboratory animals (Rice 1996; Newland and Paletz 2000; Roegge et al. 2004). While neurobehavioural tests are impractical to perform on wildlife, biological tissues are routinely collected and concentrations of toxicants in various organs can easily be measured to provide an indication of environmental contamination on a spatial and temporal scale. Therefore, studying the

neurochemical responses in wild animal tissues represents a novel tool to assess the potential neurotoxic health risks in human populations that inhabit similar ecosystems.

During the past 30 years, laboratory studies have characterized neurochemical changes in animal models following exposure to a variety of environmental neurotoxicants such as heavy metals, pesticides and solvents (Costa 1988; Tilson and Kodavanti 1997; Castoldi et al. 2001; Jarry et al. 2002). Disruptions of these neurochemical receptors and enzymes in the cholinergic and dopaminergic nervous system have been linked to specific behavioural changes in laboratory animals (Zhang and Creese 1993; Shih 2004; Wess 2004). Recently, studies demonstrated that significant mercury exposure-related changes exist in muscarinic acetylcholine (mACh) receptor and dopamine-2 (D2) receptor binding characteristics in the cortex of wild mink collected across Canada (Basu et al. 2005). While epidemiological studies of this nature are possible using brain tissues collected from wild mammals, the human nervous system is not accessible, and therefore direct neurochemical measurements cannot be easily performed in humans. However, surrogate neurochemical biomarkers in blood have been shown to reflect changes in the brain in response to neurotoxicants and offer an ethical way to evaluate neurotoxicity in human populations (Manzo et al. 1996). For example, cholinesterase (ChE) activity in blood is currently used as a surrogate biomarker to monitor the toxic effects and degree of organophosphate and carbamate exposure in agricultural workers who frequently use pesticides (Wilson et al. 1996; Wilson et al. 1997). Other studies have linked manganese and styrene exposure in industrial workers with decreased monoamine oxidase (MAO) activity in peripheral blood platelets (Checkoway et al. 1992; Smargiassi et al. 1995; Bergamaschi et al. 1997; Cohen et al. 2002). Additionally, studies have related mACh receptors on peripheral lymphocytes and MAO activity in platelets to clinical central nervous system disease states such as schizophrenia, Parkinson's and Alzheimer's disease (Bond et al. 1979; Fitzgerald et al. 1996; Tayebati et al. 2001; Zhou et al. 2001). The potential application of these biomarkers as markers of neurotoxic effects

requires further investigation.

Special considerations for biological sample collection and analysis are required in human and wildlife studies. Frequently, populations at risk of neurotoxic exposure are not in close proximity to adequate laboratory facilities and therefore samples may require longer storage times under limited storage temperatures. Factors such as storage temperature, length of storage time, and freeze/thaw cycles prior to laboratory acquisition may affect biomarkers from both human and wildlife tissue samples. In this paper, optimal collection and storage procedures are outlined for measurement of biomarkers in tissue samples acquired from human and wildlife populations. While several methods have been developed for measurement of biochemical markers of neurotoxicity, they have primarily been used to assess neurochemical changes in laboratory animals or in small-scale human clinical studies (Costa 1998). Therefore, high-throughput and cost-effective methods using microplates for the measurement of cholinergic and dopaminergic biomarkers, including two neurotransmitter metabolizing enzymes, ChE and MAO and two neurotransmitter receptors, mACh and dopamine-2 (D2) receptors are described.

2.2 Materials and Methods

2.2.1 Chemicals

[^{3}H]-Quinuclidinyl benzilate ([^{3}H]-QNB; specific activity, 42 Ci/mmol) and [^{3}H]-spiperone (specific activity, 15.7 Ci/mmol) were obtained from NEN/Perkin Elmer (Boston, MA, USA). Acetylcholine, atropine, bovine serum albumin, (+)-butaclamol, choline oxidase, horseradish peroxidase, ketanserin, polyethylenimine, resorufin, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade or higher. 10-Acetyl-3, 7-dihydroxyphenoxazine (Amplex Red) was purchased from Molecular Probes, Inc (Eugene, OR, USA).

2.2.2 *Blood*

Blood samples were obtained from 6 healthy volunteers (25 to 55 years of age, 3 females) into 10 ml Vacutainer-Tubes containing EDTA-K_2 by a registered nurse. Plasma was isolated by centrifugation of whole blood (0.5 ml) at 200 x g for 10 min at 4°C. Lymphocytes were isolated based on the method described by Boyum (Boyum 1968) with modifications. Briefly, blood samples were diluted 1:1 with balanced salt solution (BSS) (0.01% anhydrous D-glucose, 5 µM $CaCl_2$, 98 µM $MgCl_2$, 0.54 mM KCl, 14.5 mM Tris base, 0.126 M NaCl; pH 7.6), layered on Ficoll-Paque Plus reagent (Amersham Biosciences, Brown Deer, WI, USA), and then centrifuged at 400 x *g* for 40 min at 18°C. The cell layer was washed with BSS followed by centrifugation at 400 x g for 15 min at 18°C. The platelet rich supernatant was removed and set aside, and the resulting lymphocyte-pellet was washed, re-centrifuged, and suspended in NaK buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) at 1 mg protein/ml. The platelet rich supernatant was centrifuged at 2500 x *g* for 25 min at 4°C and the resulting pellet was washed, re-centrifuged, and suspended in NaK buffer at 1.5 mg protein/ml. The concentration of protein in lymphocytes and platelets was determined by the method described by Bradford (Bradford 1976) using bovine serum albumin as the standard, and cell numbers were counted with a hemocytometer.

2.2.3 *Tissue*

Brain tissues from mink were obtained from licensed fur trappers (Watson Lake Region, Yukon Territory, Canada) or from a ranch-bred strain housed at Michigan State University's Experimental Fur Farm (East Lansing, MI, USA). Membrane preparations for receptor binding studies were prepared by homogenization of brain cortex for 30 sec in cold NaK buffer followed by centrifugation of the homogenate at 32,500 x *g* for 15 min at 4°C. The resulting pellet was washed twice under the same conditions and the final pellet was re-suspended in NaK buffer and immediately frozen in liquid nitrogen and stored at –80°C until required. For comparative purposes, membranes were prepared in a similar manner from rat cortex and striatal tissue obtained from adult Sprague-

Dawley rat weighing 250-300 g (Charles Rivers, Montreal, QC, Canada). To create enzyme preparation, mink cortex tissues were sonicated for 30 sec (Sonic Dismembrator, Model 60, Fisher Scientific, Pittsburgh, PA, USA) in cold NaK buffer and 0.1% Triton X-100, and centrifuged at 15,000 x g for 10 min at 4°C. The concentration of protein in the membrane preparation and enzyme supernatant were determined as described earlier.

2.2.4 MAO Assay

MAO activity was measured based on the method described by Zhou and Panchuk-Voloshina (Zhou and Panchuk-Voloshina 1997) with modifications. Human platelets were sonicated for 30 sec while submerged in an ice bath. Samples were diluted with NaK buffer to a concentration in the linear detectable range of the MAO assay (human platelets, 25 µg protein/100µl buffer; ranch-bred mink cortex supernatant, 20 µg protein/100 µl buffer). Diluted samples (100 µl/well) were added to a 96-well microplate. Reaction was initiated with the addition of 100 µl/well of NaK reaction buffer (100 µM Amplex Red, 2 U/ml horseradish peroxidase, and 4 mM tyramine). The reaction co-product, H_2O_2, is detected by a horseradish peroxidase coupled oxidation of Amplex Red, forming the fluorescent product resorufin. Fluorescence was measured every 5 min between 30-90 min at 540/590 by a fluorometric plate reader (Wallac Victor 2, Perkin Elmer, Boston, MA) at 23 °C. The concentration of resorufin was determined from a standard curve (0.5 - 5 µM resorufin), and specific activities of samples were expressed as pmol of resorufin per min per mg of protein. NaK buffer was used as a sample blank and 2.5 µM H_2O_2 was used as a positive control.

2.2.5 ChE Assay

ChE activity was measured based on a modified version of the previously described methods (Zhou et al. 1997; Zhou et al. 2000). Human and ranch-bred mink tissue samples were diluted with NaK buffer to a concentration in the linear

detectable range of the ChE assay (human plasma, 20 nl plasma/100µl buffer; mink cortex supernatant, 1 µg protein/100 µl buffer). Diluted samples (100 µl/well) were added to a 96-well microplate. Reaction was initiated with the addition of 100 µl/well of NaK reaction buffer (100 µM Amplex Red, 2 U/ml horseradish peroxidase, 0.2 U/ml choline oxidase and 100 µM acetylcholine). The initial reaction co-product, choline, is oxidized by choline oxidase, producing H_2O_2 as a co-product which is detected as described above. Fluorescence and specific activity was detected and calculated as described above and expressed as nmol of resorufin per min per mg of protein or ml of plasma.

2.2.6 mACh Receptor Binding Assay

The receptor binding assay for the mACh receptor was performed on a 96-well 0.22 µM GF/B glass filter system (Millipore, Boston, MA, USA). Human lymphocytes (20 µg protein/well) or cortex membrane preparations (20 µg protein/well) were pre-incubated in NaK buffer for 30 min at 25°C. Samples were incubated with [^{3}H]-QNB (0.01 nM - 3.2 nM for cortex samples and 1.0 nM – 100 nM for lymphocytes (Costa et al. 1990)), a mACh receptor specific radioligand, for 60 min at 25°C under constant agitation in a total volume of 200 µl. The incubation was terminated by rapid vacuum filtration and the filters were washed three times with NaK buffer. Freeze/thaw and storage temperature experiments with mink membrane preparations were performed in culture tubes and the total volume of the sample in each tube was 1 ml. The incubation was terminated by rapid vacuum filtration through 0.22 µM GF/C glass filters (Millipore Inc., Boston, MA, USA) and the filters were washed three times with ice-cold NaK buffer. All extracted filters were soaked overnight in liquid scintillation cocktail. Radioactivity retained by the filters was quantified by a liquid scintillation counter (LKB Wallac 1209 Rackbeta) with approximately 65% counting efficiency. Specific binding was defined as the difference in [^{3}H]-QNB bound in the presence and absence of 100 µM atropine.

2.2.7 *D2 Receptor Binding Assay*

The receptor binding assay for the D2 receptor was modified for a 96-well 0.22 μM GF/B glass filter system (Millipore, Boston, MA, USA). Brain membrane preparations were pre-incubated in Tris buffer (50 mM Tris, 5mM KCl, 2mM $MgCl_2$, pH 7.4) for 30 min at 25°C. Samples (20μg protein) were incubated with various concentrations (0.1 nM - 5.6 nM) of [^{3}H]-spiperone, the D2 specific radioligand, for 90 min at 25°C under constant agitation in a total volume of 200 μl. The termination of the incubation and subsequent quantification of radioactivity retained was completed as described earlier. Specific binding was defined as the difference in [^{3}H]-spiperone bound in the presence and absence of 100 μM (+)-butaclamol. To reduce non-specific binding of the radioligand, filters were soaked for 30 min in 0.5% (*w/v*) polyethylenimine prior to use, and 50 μM ketanserin (5-HT_2 receptor antagonist) was added to each well to prevent binding of [^{3}H]-spiperone to 5-HT_2 receptors.

2.2.8 *Effects of Storage Time and Temperature*

The effects of storage time and temperature on receptor binding characteristics and enzymatic activity were evaluated. Ranch-bred mink cortex samples, and human platelet, lymphocyte, or plasma samples were stored at +4, -20, or –80°C for 2, 7, or 28 days prior to analysis. In addition, samples were stored in a second –20°C freezer, equipped with an automated defrost cycle. Wild mink cortex samples used to evaluate the effects of storage temperature on receptor binding characteristics were stored for 7 days. All values were converted to % of biomarker function of the respective time-zero tissue samples. Receptor binding and enzymatic assays were conducted as described above.

2.2.9 *Effects of Freeze/Thaw Cycling*

To assess effects of multiple freeze/thaw cycles, cortex tissues from mink were subjected to 1, 2, or 3 freeze/thaw cycles (-80°C to 4°C for 24 hr) prior to membrane or cell lysate preparation. Wild mink cortex tissues were used in

receptor studies and ranch-bred mink cortex tissues were used in enzyme studies. All values were normalized to % of the biomarker function of the respective time-zero tissue samples. Receptor binding and enzymatic assays were conducted as described above.

2.3 Statistical Analysis

A p-value less than or equal to 0.05 was considered statistically significant in all analyses. A two-way repeated-measures analysis of variance (ANOVA) was used to examine the effects of storage temperature and time on biomarkers. A one-way repeated-measures ANOVA was used to assess the effects of freeze/thaw cycling on biomarkers. When an overall significance resulted, post-hoc pair-wise comparisons were performed using Tukey-Kramer test. An unpaired *t*-test was used to compare receptor binding characteristics in ranch-bred and wild mink. Data from all receptor-binding studies were curve fitted using GraphPad Prism (Version 3.02, GraphPad Software Inc., San Diego, CA, USA) to calculate receptor density (B_{max}) and ligand affinity (K_d). The relationship between calculated B_{max} or K_d values and specific binding at a single radioligand concentration in wild mink cortex (n=39) were determined using Pearson correlation on mACh receptor raw data previously reported by Basu et al (Basu et al. 2005). All statistics were analyzed using SigmaStat Statistical Software (Version 2.03, SPSS Inc. San Rafael, CA, USA).

2.4 Results

2.4.1 Isolation of Platelet and Lymphocytes

Recoveries of blood cells were determined from each blood tube. A 10 ml tube of human blood yielded an average of $4.6 \times 10^8 \pm 1.0 \times 10^8$ platelets (1.9 ± 0.3 mg of protein; n = 6) and $6 \times 10^6 \pm 2 \times 10^6$ lymphocytes (0.54 ± 0.2 mg of protein; n = 6).

2.4.2 *Enzyme Activity*

The MAO assay was optimized for human platelet concentrations and the activity was shown to be linear between 2.5 - 50 µg of protein per well. MAO activity in mink cortex supernatant was shown to be linear between 10 - 60 µg of protein per well. Mean MAO specific activities were 246.9 ± 64.0 pmol/min/mg and 207.9 ± 17.8 pmol/min/mg for human platelet and mink cortex supernatant, respectively.

The ChE assay was optimized for amount of tissue required and activity was linear between 6 – 50 nl of human plasma per well and between 0.5 – 4 µg of mink cortex supernatant protein per well. Mean ChE specific activities were 322.9 ± 64.0 nmol/min/ml and 6.6 ± 1.4 nmol/min/mg for human plasma and mink cortex, respectively. The intra-assay variation was consistently less than 6% for both enzyme assays.

2.4.3 *Receptor Binding*

Saturation binding curves for specific [^{3}H]-QNB binding to the mACh receptor in human lymphocytes, wild mink cortex and rat striatal samples are shown (Figures 2.1A and 2.1B, respectively). Analysis of mACh receptor binding in human lymphocytes resulted in a mean B_{max} of 370.8 fmol/mg protein and a K_d of 49.3 nM. Analysis of cortex mACh receptor binding data from wild mink resulted in a mean B_{max} of 721.5 ± 227.2 fmol/mg protein and K_d of 0.11 ± 0.02 nM. These values were not significantly different from values previously reported in ranch-bred mink cortex samples (B_{max}, 801.8 ± 107.8 fmol/mg protein; K_d, 0.14 ± 0.05 nM) (Basu et al. 2005). Non-specific binding, as determined by incubation of samples with atropine, was 65-75% total binding at 10 nM [^{3}H]-QNB for lymphocytes and consistently less than 4% of total binding at 0.1 nM [^{3}H]-QNB for mink and rat cortex samples. Intra-assay variations were determined from triplicate analysis and were consistently less than 6%.

Experiments were conducted to characterize the high-affinity D2 binding site and a typical saturation-binding curve using 6 concentrations of [^{3}H]-

spiperone (0.1-5.6 nM) is shown for mink cortex and rat striatum (Figure 2.2). Analysis of cortex D2 receptor binding data from wild mink resulted in a mean B_{max} and K_d of 112.2 ± 32.8 fmol/mg/protein and 1.64 ± 0.33 nM, respectively. D2 receptor densities in cortex from ranch-bred mink (96.5 ± 39.1 fmol/mg/protein, n=6) were not significantly different than mean values from wild mink. Non-specific binding, as determined by incubation of samples with (+)-butaclamol, was 45-55% of total binding at 1.8 nM [^{3}H]-spiperone.

In order to determine if a single concentration of radioligand can predict B_{max} and K_d values in cortex tissue of wild mink populations, Pearson correlations were performed. For the mACh receptor, specific binding at 1.0 nM ^{3}H-QNB correlated with B_{max} (n=39, R = 0.986) but not K_d (n=39, R=0.298). D2 receptor B_{max} correlated with specific binding at 3.2nM ^{3}H-spiperone (n=39, R=0.80), while correlations with K_d were weaker (n=39, R=0.373).

2.4.4 Effect of Storage Temperature and Time

Both storage temperature and time had a significant effect on MAO activity in human platelets (Figure 2.3A). Platelet-MAO activity was unaffected following storage up to 4 weeks at 4°C. MAO activity decreased by 30 and 55% following storage for 2 days, at –80°C and –20°C, respectively when compared to fresh samples. Platelet-MAO activity continued to decrease while stored at –20°C, however activity did not decrease further while stored at -80°C for 4 weeks. No significant effects of storage temperature or time on ChE activity in plasma samples were observed (Figure 2.3B). Specific mACh receptor binding in lymphocytes was altered by time and temperature. Receptor binding was unaffected following storage at 4°C for up to 2 days, however, binding decreased by more than 40% after longer storage times (Figure 2.3C). Lymphocytes stored at –20°C exhibited decreased specific binding relative to fresh samples. Specific binding in lymphocytes stored at –80°C for less than 7 days were similar to fresh samples; however sample variation appeared to increase over time.

Storage conditions modulated both MAO and ChE activity in mink cortex samples (Figure 2.4A, 4B). Mink cortex samples stored at –80°C for longer than

7 days and at –20°C for longer than 2 days resulted in a 25% decrease in MAO activity. Mink cortex ChE activity also decreased significantly after 2 days of storage at –80°C and –20°C. However, storage time did not alter MAO and ChE as the activity remained stable during the remaining storage period at all storage conditions. Tissue samples stored at 4°C and in the –20°C defrost freezer exhibited no significant change in MAO or ChE activity during the storage period. Both mACh and D2 receptor B_{max} values from mink cortex samples were unaffected by 4°C, –20°C, and –80°C storage for 7 days, while storage in the –20°C defrost freezer decreased B_{max} values (Figure 2.5A, 5B). K_d values for mACh receptors from mink cortex significantly increased following storage at all conditions, while there was no apparent change in K_d values for D2 receptors from samples stored at -20°C and –80°C.

2.4.5 Effects of Freeze/Thaw Cycling on mink tissue

Mink cortex MAO activity decreased by 18 and 21% following the second and third freeze/thaw cycles, respectively. Mink cortex ChE activity was not significantly affected by multiple freeze/thaw cycles, however a decreasing trend in activity was observed with repeated cycles (Table 2.1).

Both D2 and mACh receptor binding characteristics were altered following multiple freeze thaw cycles (Table 2.1). mACh receptor B_{max} increased by 21 and 30%, and K_d values increased by 180 and 120%, following the first and second freeze/thaw cycles, respectively. Similarly, the D2 receptor B_{max} values increased by 46 and 53%, and K_d values increased by 40 and 95% following the first and second freeze/thaw cycle, respectively.

2.5 Discussion

Alterations in neurochemical markers such as MAO and ChE enzyme activity, and D2 and mACh receptor binding characteristics, which result from neurotoxicant exposure, can be used as quantitative measures in the early stages of neurotoxicity. Relatively few studies have examined the relationships

among these neurochemical biomarkers and toxicant exposure in wildlife or human studies at a population level (Costa and Manzo 1995). In order to demonstrate the feasibility of measuring these biomarkers from field samples, the limits of storage and transport conditions were identified. Methods for assays that are suited for processing large batch samples are also described.

The assays described here address several previous methodological limitations (efficiency, cost and sensitivity) that can restrict the application of neurochemical biomarker analysis. Previously described methods for the measurement of neurochemical parameters often involve time-consuming multiple step procedures performed in test tubes (Cagiano et al. 1990; Costa et al. 1990; Chakrabarti et al. 1998). Methods that involve the detection of a fluorescent oxidized product of Amplex Red for the continuous measurement of MAO and ChE activity have been previously evaluated using cow brain tissue and purified enzymes, respectively (Zhou and Panchuk-Voloshina 1997; Zhou et al. 2000). In the present paper, data show that these microplate assays can be used to measure enzyme activity in human platelets, plasma, and mink cortex tissue. These single step assays allow for the analysis of 26 samples in less than 2 hr of lab work. The mACh and D2 receptor-binding assay were also adapted for use in 96-well filter plates and describe methods that allow saturation receptor binding curves (6 concentrations of radioligand) to be measured for 12 samples in less than 5 hours of laboratory work. Correlative studies performed with wild mink cortex receptor binding curves validated the use of a single concentration of ligand, ^{3}H-QNB or ^{3}H-spiperone, to represent B_{max}, but not K_d values. Therefore, using a single radioligand concentration would allow the measurement of 72 samples in less than 5 hours. While these microplate assays exhibited non-specific binding patterns for cortex membranes that were similar or lower than previously reported values using individual filters (Fitzgerald and Costa 1993), our lymphocytes microplate experiments revealed higher non-specific binding values (65-75% of total binding) when compared to previous unpublished data from our laboratory (25-35% of total binding) (Stamler and Chan, unpublished observation). The high throughput and cost-effective nature of these microplate

assays allow for ease in measurement of the neurochemical biomarkers in the laboratory.

Populations in rural and isolated communities are susceptible to exposure to environmental contaminants because of their dependence on traditional food for subsistence (Kuhnlein and Chan 2000). In addition, occupational exposure in industries such as mining and agriculture often occur in areas distant from major centers and laboratory facilities. Therefore, studies performed on isolated or dispersed populations require longer sample storage times under limited storage conditions prior to delivery to the laboratory for analysis. Methods have been developed for ChE measurement in the field, but are not as sensitive and accurate as laboratory based methods (Wilson et al. 1996; Oliveira et al. 2002). The measurement of stored biomarkers can be affected by several conditions including protein denaturation, oxidation, proteolysis, and microbial contamination, which may be dependent upon storage conditions such as temperature, freeze/thaw cycles, and storage time. Data indicated that isolated blood platelets and plasma could be stored at 4°C for up to 4 weeks prior to measurement of MAO and ChE without significantly altering enzyme activity. Therefore minimal laboratory facilities (i.e. centrifuge) are required in the field prior to delivery of samples to laboratory facilities, and biomarkers will remain stable if shipped on ice. Data also indicated that freezing human platelet reduces MAO activity, a possible result of ice crystal formation and protein denaturation (Franks 1985). Similarly, mACh receptor binding on isolated lymphocytes is best performed on fresh, unfrozen cells that have been stored for less than 2 days on ice. These data suggest that use of these biomarkers for epidemiological studies in isolated communities can be justified, provided proper storage conditions are maintained.

Evaluating neurotoxic effects in wildlife tissue by measuring cellular protein biomarkers can be complicated due to unknown and uncontrollable temperature conditions during post-mortem storage. Here studies show that tissue storage at 4°C or in a –20°C defrost freezer did not affect MAO and ChE enzyme activity, relative to time-zero samples. While mink cortex D2 and mACh receptor B_{max}

values were not affected by storage temperature, mACh receptor affinity (K_d) fluctuated and could not be considered a reliable endpoint. It is possible that subtle protein damage from storage and temperature changes may have resulted in decreased ligand affinity (K_d) but not altered membrane receptor density (B_{max}). Wildlife carcasses may undergo multiple freeze/thaw cycles prior to laboratory acquisition, producing additional protein damage. These data suggest that multiple freeze/thaw cycles affect MAO activity and both mACh and D2 receptors binding characteristics, but not ChE activity. Stability of ChE under a range of storage temperatures has been previously reported (Balland et al. 1992; Phillips et al. 2002). In summary, while storage conditions generally do not alter these biomarkers in wildlife samples, some parameters are sensitive to temperature fluctuations, and therefore storage conditions should be monitored.

It has been shown that neurochemical changes precede behavioural changes in multiple animal models, and have been proposed as biomarkers of early nervous system dysfunction (Costa and Manzo 1995; Manzo et al. 1996; Manzo et al. 2001). In this study, data show that several biomarkers of neurotoxicity can be measured from human participants and from wild mink samples if optimal storage conditions are used and storage logs maintained. Based on our findings, future epidemiological studies can begin to relate neurotoxicant exposure with changes in neurochemical parameters in human and wildlife populations.

Table 2.1: Effects of multiple freeze/thaw cycles on receptor binding characteristics and enzyme activity in mink cortex

# Freeze Thaw Cycles	mACh Receptor Binding Characteristics		D2 Receptor Binding Characteristics		Enzyme Activity	
	B_{max}	K_d	B_{max}	K_d	MAO	ChE
0	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]
1	121.2 ± 4.6 [b]	283.1 ± 24.8 [b]	146.5 ± 12.5[a]	141.1 ± 27.2[a]	88.1 ± 10.9[a]	103.7 ± 16.5[a]
2	130.2 ± 4.8 [b]	217.4 ± 19.0 [b]	153.4 ± 7.7 [b]	195.8 ± 42.8[b]	82.2 ± 9.3[b]	89.3 ± 7.1[a]
3	-	-	-	-	79.5 ± 6.3[b]	85.9 ± 7.6[a]

[a,b] Letters denote significant ($p<0.05$) differences within columns

Values expressed as % time-zero ± SD (n=6)

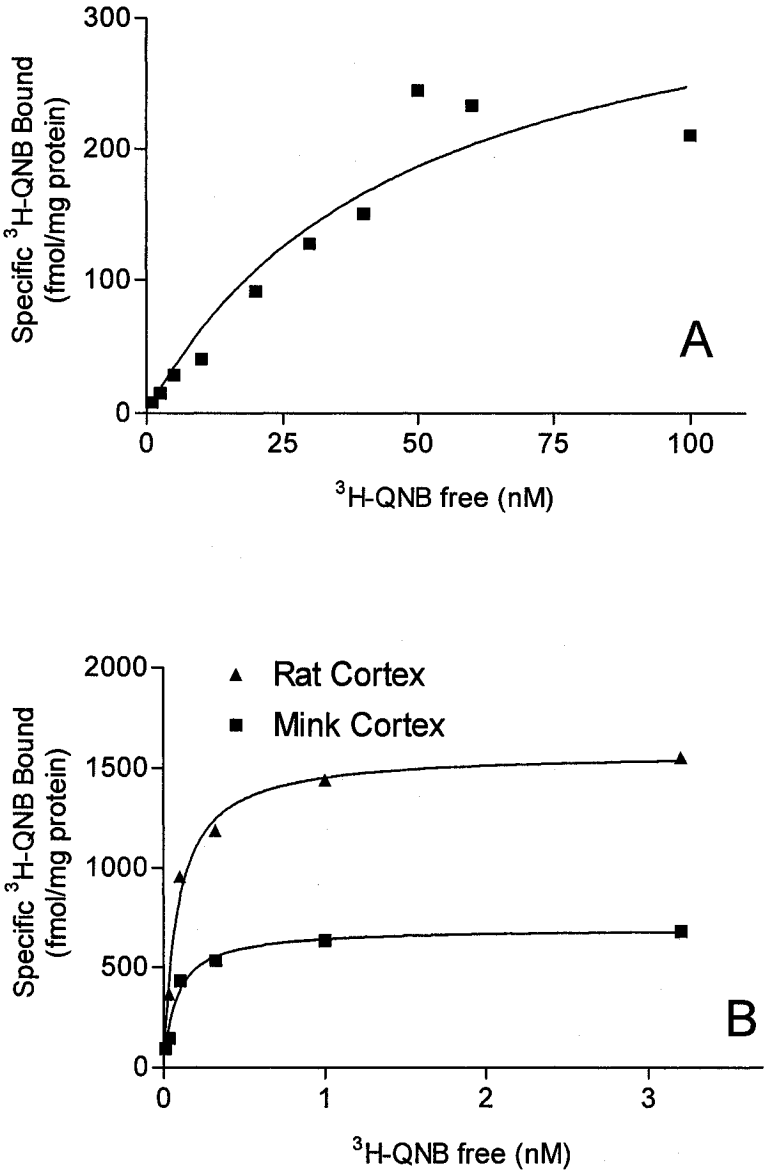

Figure 2.1: Saturation binding curve for specific [^{3}H]-QNB binding to the mACh receptor. (A) Specific binding in human whole cell lymphocytes with 10 concentrations of radioligand (1 – 100nM). Values are means of duplicate determinations from lymphocytes pooled from 6 subjects. (B) Specific binding in mink and rat cortex membrane preparations with 6 concentrations of radioligand (0.01 - 3.2 nM). Values are the means of duplicate determination from a representative experiment. Specific binding was obtained by subtracting non-specific (presence of 100 μM atropine) from total binding.

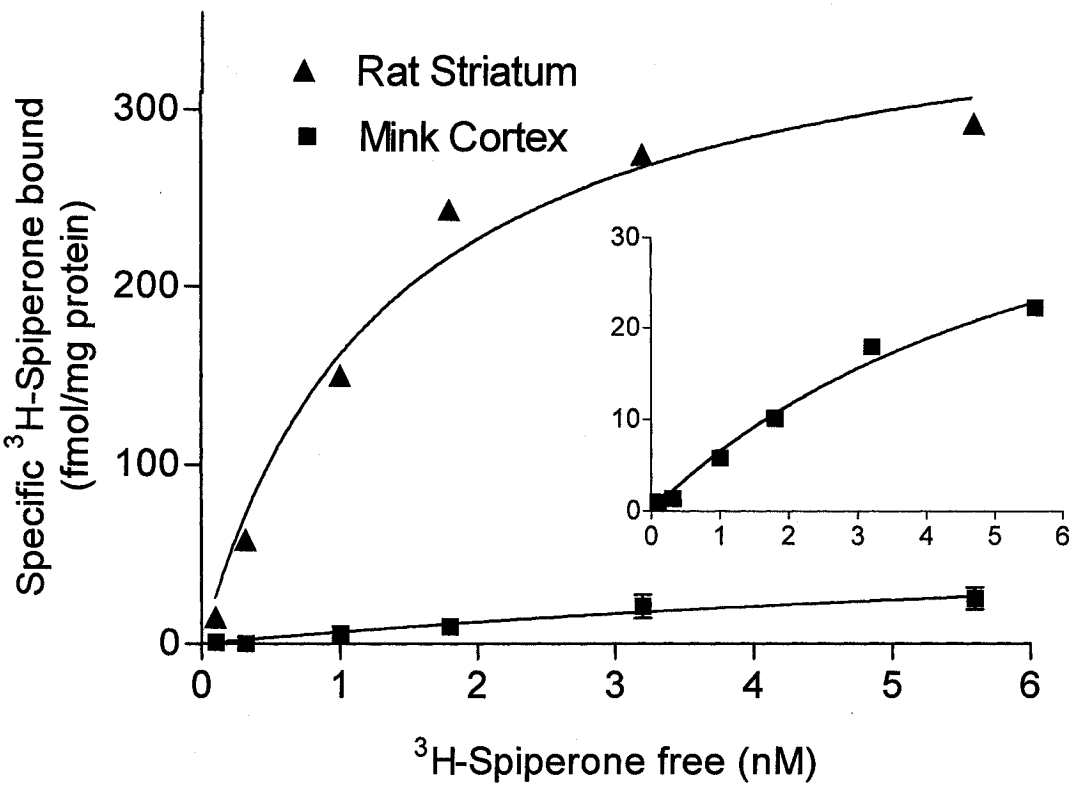

Figure 2.2: Saturation binding curve for specific [^{3}H]-spiperone binding to the D2 receptor in mink cortex and rat striatal membrane preparations with 6 concentrations of radioligand (0.1 – 5.6 nM). Inset, y-axis adjusted to show specific [^{3}H]-spiperone binding to mink cortex membrane preparations. Specific binding was obtained by subtracting non-specific (presence of 100 μM (+)-butaclamol) from total binding. Values are the means of triplicate measurements from a representative experiment.

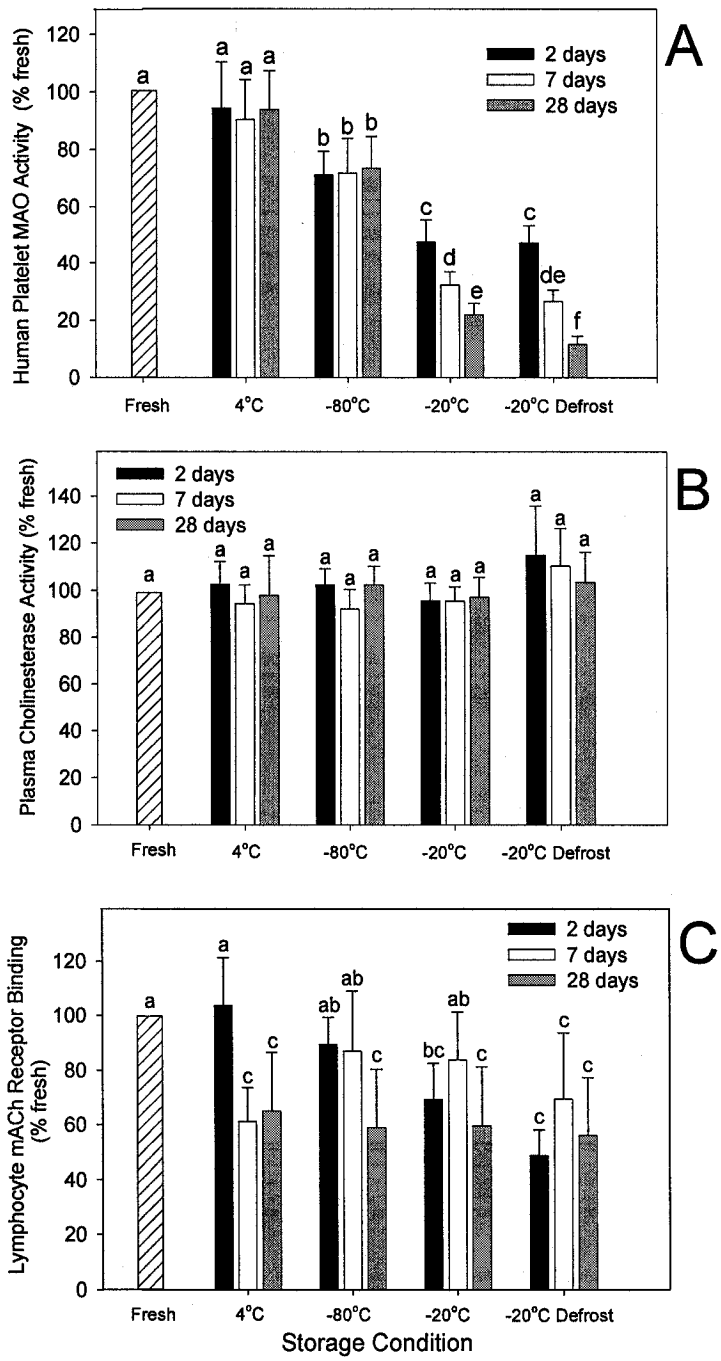

Figure 2.3: Effects of storage time and temperature on (A) MAO, (B) ChE, and (C) mACh receptor function from human blood samples (n=6). Samples were stored at 4°C, -20°C, -80°C or in a –20°C defrost freezer for 2, 7 and 28 days prior to measurement. Each bar represents the mean ± SD as % activity or binding measured in fresh tissue samples. Statistical analysis was performed using a two-way repeated measure ANOVA followed by Tukey's multiple comparison test. Letters indicate significant differences ($p \leq 0.05$).

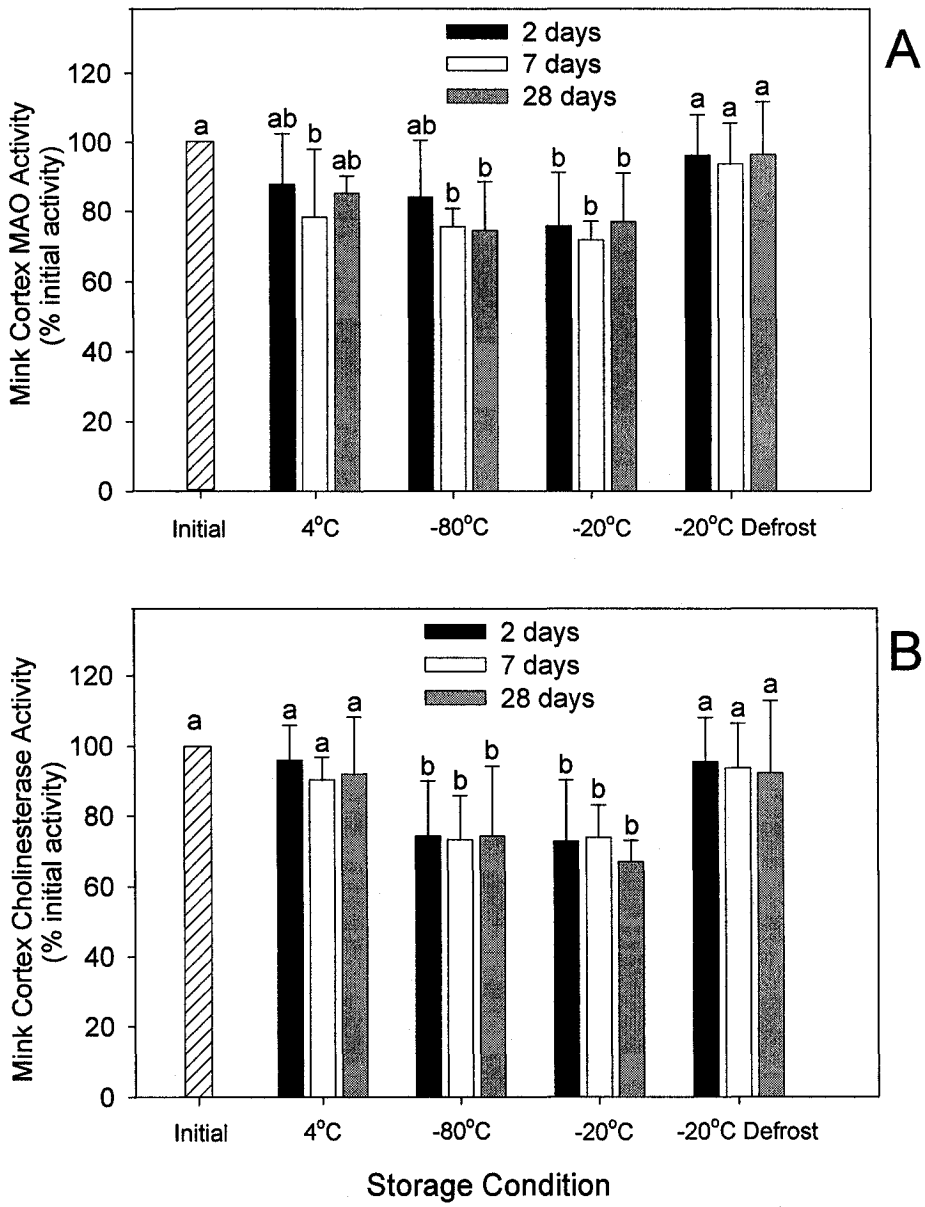

Figure 2.4: Effects of storage time and temperature on (A) MAO, and (B) ChE activity in mink cortex samples (n=6). Samples were stored at 4°C, -20°C, -80°C or in a –20°C defrost freezer for 2, 7 and 28 days prior to measurement. Each bar represents the mean ± SD as % enzyme activity measured at time-zero. Statistical analysis was performed using a two-way repeated measure ANOVA followed by Tukey's multiple comparison test. Letters indicate significant differences ($p \leq 0.05$).

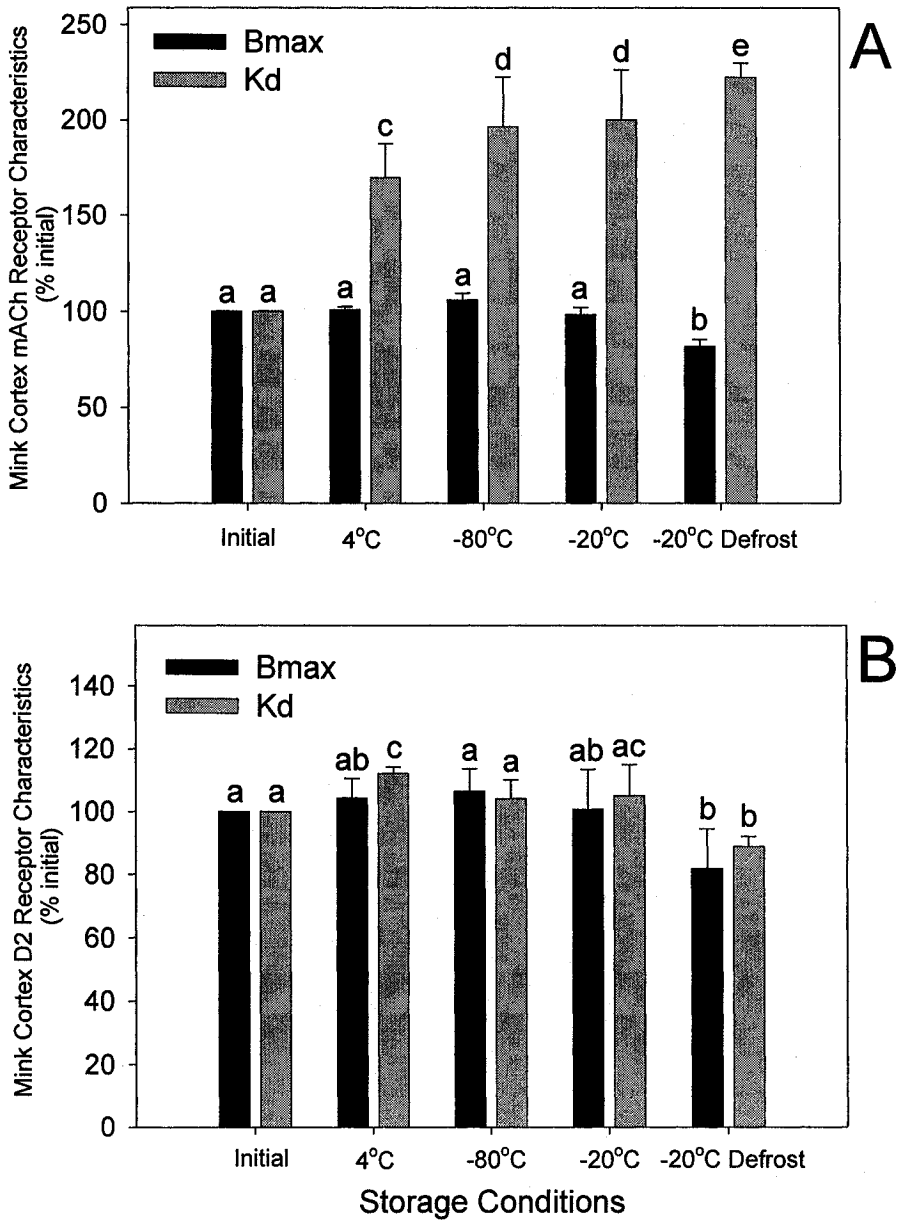

Figure 2.5: Effects of storage time and temperature on (A) mACh and (B) D2 receptor binding characteristics in mink cortex (n=3) membrane preparations. Samples were stored at 4°C, -20°C, -80°C or in a –20°C defrost freezer for 7 days prior to measurement. Each bar represents the mean ± SD as % mACh or D2 receptor B_{max} or K_d measured at time-zero. Statistical analysis was performed using a two-way repeated measure ANOVA followed by Tukey's multiple comparison test. Letters indicate significant differences ($p \leq 0.05$).

Connecting Paragraph

Chapter 2 describes microplate techniques for measuring muscarinic acetylcholine (mACh) receptor. Mercury compounds have been shown to inhibit the function of the mACh receptor, but only in the rat brain tissue. This next chapter examines if this interaction is consistent in other species, specifically humans. Autopsied human brain tissue obtained from the Douglas Hospital Brain Bank was used to compare the interactions of Hg compounds on mACh receptor binding with other relevant species. This study provides additional evidence that mACh receptors can be used as a molecular target of Hg exposure in several species including humans. The wildlife component of this manuscript is the primary focus of N. Basu's PhD thesis.

Chapter 3: An inter-species comparison of mercury inhibition on muscarinic acetylcholine receptor binding in the cerebral cortex and cerebellum‡

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Abstract

Mercury (Hg) is a ubiquitous pollutant that can disrupt neurochemical signalling pathways in mammals. It is well-documented that inorganic Hg ($HgCl_2$) and methyl Hg (MeHg) can inhibit the binding of radioligands to the muscarinic acetylcholine (mACh) receptor in rat brains. However, little is known concerning this relationship in specific anatomical regions of the brain or in other species, including humans. The purpose of this study was to explore the inhibitory effects of $HgCl_2$ and MeHg on [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to the mACh receptor in the cerebellum and cerebral cortex regions from human, rat, mouse, mink, and river otter brain tissues. Saturation binding curves were obtained from each sample to calculate receptor density (B_{max}) and ligand affinity (K_d). Subsequently, samples were exposed to $HgCl_2$ or MeHg to derive IC50 values and inhibition constants (Ki). Results demonstrate that $HgCl_2$ is a more potent inhibitor of mACh receptor binding than MeHg, and the receptors in the cerebellum are more sensitive to Hg-mediated mACh receptor inhibition than those in the cerebral cortex. Species sensitivities, irrespective of Hg type and brain region, can be ranked from most to least sensitive: river otter > rat > mink > mouse > humans. In summary, our data demonstrate that Hg can inhibit the binding [^{3}H]-QNB to the mACh receptor in a range of mammalian species. This comparative study provides data on inter-species differences, and a framework for interpreting results from human, murine, and wildlife studies.

3.1 Introduction

Mercury (Hg) is a hazardous trace metal that is released into the environment from both natural and anthropogenic sources (ATSDR 1999; Wiener et al. 2003). Hg can exist in one of three allotropic forms: elemental Hg (Hg^0), inorganic Hg ($Hg^{+/++}$), and organic Hg. Each form of Hg has distinct chemical properties that can affect its distribution and effects at the level of the cell, organism, and ecosystem. In freshwater ecosystems methyl Hg (MeHg), the predominant type of organic Hg, is assimilated by aquatic organisms and biomagnified through the food web from 10,000 to 100,000 times (ATSDR 1999). As a result, dietary consumption of aquatic animals, such as fish, represents the major route of Hg exposure for human and wildlife populations. This is of particular concern for groups that depend upon fish products as an important source of nutrients, such as remote communities (e.g. inhabitants in the Faroe Islands and Brazilian Amazon), indigenous peoples that rely on subsistence hunting, and obligate fish-eating wildlife (e.g. mink and river otter) (Chan et al. 2003).

Once in the body, MeHg can be transported across the blood-brain-barrier via a carrier-mediated system (Aschner and Aschner 1990), facilitating its neurotoxicity. MeHg can be metabolized to the divalent cation, although the capacity of the brain to do so is limited when compared to other organs. As such, both Hg^{2+} and MeHg are found in mammalian brains, although there is still debate as to which form of Hg is the primary toxic compound (Charleston et al. 1996, Eto, 1997). Histopathological examinations of brain tissues collected from humans (Eto 1997), experimental rodents (Nagashima 1997), and wild animals (Wobeser and Swift 1976) exposed to Hg demonstrated widespread neuronal degradation and associated proliferation of glial cells. While the toxicity of Hg can be classified as non-specific given its high affinity for sulfhydryl groups, it is evident that certain anatomical regions of the brain, such as the granule cells of the cerebellum and calcarine region of the occipital cortex, are particularly at risk (Eto 1997). Due to these structural effects, multiple studies have demonstrated that humans and wildlife exposed to Hg can suffer from a range of adverse

functional neurobehavioural impairments to motor (e.g. ataxia, paresthesia, insomnia, tremors) and sensory (e.g. peripheral vision, deafness, hallucinations) systems (reviewed by (ATSDR 1999).

Given its non-discriminate mode of toxicity, the specific cellular mechanisms underlying Hg's toxic effects on the nervous system are still unclear. Hg can disrupt signalling pathways that facilitate cellular communication throughout the central and peripheral nervous systems. For example, Hg can impair components of the muscarinic cholinergic (mACh) signalling pathway including choline acetyltransferase activity (Kobayashi et al. 1979; Omata et al. 1982), choline uptake (Bondy et al. 1979; Kobayashi et al. 1979), and acetylcholinesterase activity (Tsuzuki 1981). Receptor binding studies have also demonstrated that Hg can directly prevent the binding of radioactive ligands to the mACh receptor in rats (Bondy and Agrawal 1980; Von Burg et al. 1980; Abd-Elfattah and Shamoo 1981; Castoldi et al. 1996). The mACh receptor belongs to a highly conserved class of transmembrane receptors that are coupled to guanine nucleotide binding proteins (G-protein) (Wess 1996). Given the physiological importance of G-protein mediated signalling cascades, disruption of endogenous ligand (i.e. acetylcholine) binding to the mACh receptor, as a result of Hg exposure, can have profound impacts on diverse functions, including memory, locomotion, and thermoregulation (Wess 2004).

While it is established that Hg can inhibit mACh receptor binding in the brain of rats, the extent of this inhibition has not been studied in specific brain regions or in different mammalian species, including humans. As a result, the current study was conducted to characterize the effects of $HgCl_2$ (inorganic Hg) and MeHg (organic Hg) on mACh receptor ligand binding on tissues (cerebral cortex and cerebellum) from human, murine, and wildlife samples. This comparative approach allowed us to test the null hypothesis that relevant toxicological factors (i.e. mercury type, brain region, animal species) do not affect mACh receptor binding.

3.2 Materials and Methods

3.2.1 Chemicals

[^{3}H]-Quinuclidinyl benzilate ([^{3}H]-QNB; 42 Ci/mmol) was obtained from NEN/Perkin Elmer (Boston, MA, USA). Atropine, bovine serum albumin, (+)-butaclamol, ketanserin, and mercuric chloride ($HgCl_2$, 99.6% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl mercury chloride (>95% purity) was obtained from Alfa Aesar (Ward Hill, MA, USA).

3.2.2 Samples

Brain tissues (n=6; age 1 yr; 6 females) from mink (*Mustela vison*) were obtained from a captive colony maintained at the Experimental Fur Farm, Michigan State University (East Lansing, MI, USA). Brain tissues (n=6; age = 1-3 yr; 4 males, 2 females) from wild river otters (*Lontra canadensis*) were collected from licensed trappers in Nova Scotia (Canada). Human brain tissues (n=5; age=52-64 yr; 3 males, 2 females) were obtained from the Douglas Hospital Research Centre Brain Bank (Montreal, QC, Canada). Brain tissues (n=6) from adult Sprague-Dawley rats (age = 5-7 mo; 6 females) and adult mice (age = 3-6 mo; 6 females) were obtained from the Animal Care facilities at McGill University (Montreal, QC, Canada). Ethics approval was obtained from McGill University for all aspects of this study.

3.2.3 Preparation of Cellular Membranes

Brain tissues from each animal were dissected into cerebral cortex and cerebellum regions and stored at -80°C until analysis. To prepare membranes, tissues were homogenized in ice-cold NaK buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4). The homogenate was centrifuged at 32,000 *g* for 15 min at 4 °C, and the resulting pellet was washed twice under the same conditions. The final pellet was re-suspended in NaK buffer and aliquots were immediately frozen in liquid nitrogen and stored at –80 °C until required. The concentration of protein in the membrane preparation was determined with the Bradford assay

(Bradford 1976) using bovine serum albumin as external standard.

3.2.4 Development of [^{3}H]-QNB Saturation Binding Curves

Membrane preparations from brain tissues (20μg) were pre-incubated in NaK buffer for 30 min at 25 °C in triplicate on 96-well 0.22μM GF/B filter plates (Millipore Inc., Boston, MA, USA). Samples were mixed with various concentrations (0.01 to 3.2 nM) of the mACh receptor antagonist, [^{3}H]-QNB, for 60 min at 25 °C with gentle agitation. Receptor bound [^{3}H]-QNB was separated from free [^{3}H]-QNB by vacuum filtration, and the filters were washed three times with NaK buffer. Filters were extracted and allowed to dissolve overnight in liquid scintillation cocktail (ICN Biomedicals, Aurora, OH, USA). Radioactivity retained by the filters was quantified by a liquid scintillation counter (LKB Wallac 1209 Rackbeta) with approximately 65% counting efficiency. Specific binding was defined as the difference in [^{3}H]-QNB bound in the presence and absence of 100 μM atropine.

3.2.5 Hg Inhibition Studies

Membrane preparations from brain tissues (20μg) were incubated with a range of MeHgCl or $HgCl_2$ (0 to 1000μM) for 15min in 96-well 0.22μM GF/B filter plates (Millipore Inc., Boston, MA, USA). Following the incubation period, receptor binding assays were performed on each sample as described previously. Samples were incubated with 0.1nM to 0.3nM [^{3}H]-QNB, reflecting concentrations that closely approximated ligand affinity (K_d) for a given sample.

3.3 Statistical Analysis

Data from receptor binding studies were curve fitted using a non-linear regression program (GraphPad Prism Version 3.02, GraphPad Software Inc., San Diego, CA, USA) to calculate receptor density (B_{max}) and ligand affinity (K_d) according to the following equation:

$$Y = (B_{max})(R)/K_d + R,$$

where Y represents specific binding (fmol/mg protein), R represents the concentration of radioligand, B_{max} represents receptor density (fmol/mg protein), and K_d represents ligand affinity (nM).

For *in vitro* studies, concentrations of $HgCl_2$ and MeHgCl that cause 50% inhibition of control binding (IC50) were calculated (GraphPad Prism, Version 3.02, GraphPad Software Inc., San Diego, CA, USA). To alleviate the influence of radioligand concentration and ligand affinity on IC50 data, inhibition constants (Ki) were derived to describe the affinity of the mACh receptor for Hg. The IC50 data was normalized according to the Cheng-Prusoff equation (Cheng and Prusoff 1973):

$$Ki = IC50/1+((R)/K_d),$$

where Ki represents the inhibition constant, R represents the concentration of radioligand tested, and K_d represents the ligand affinity.

A p-value less than, or equal to, 0.05 was considered statistically significant in all analysis, and data are represented as mean ± SEM. Two-way ANOVAs were used to compare receptor B_{max} and K_d (mammalian species x brain region). Three-way ANOVAs were used to compare inhibition data (mammalian species x brain region x Hg type). When significant differences were detected, Tukey's post hoc tests were applied.

3.4 Results

Binding of [^{3}H]-QNB to mACh receptors exhibited one-site binding in all

species studied. Representative saturation binding curves for cerebral cortex (Fig 3.1A) and cerebellum (Fig 3.1B) are shown. mACh receptor B_{max} was significantly higher in the cerebral cortex compared to the cerebellum across all species ($F=16.452$, $df=4$, $P<0.001$; Table 3.1). Both mouse cerebellum and mouse cortex mACh receptor B_{max} was highest compared to all other species. There were no significant differences in mACh receptor K_d in the cerebellum among the species tested.

IC50 (Table 3.2) and Ki (Table 3.3) data were derived for each sample. Analysis of data by three-way ANOVAs revealed significant differences between the factors (i.e. species, type of Hg, brain region). $HgCl_2$ was more potent in inhibiting mACh receptor binding compared to MeHg (IC50: $F=44.368$, $df=1$, $P<0.001$; Ki: $F= 39.908$, $df=1$, $P<0.001$). While IC50 and Ki values were higher in MeHg exposed samples compared to $HgCl_2$ in both brain regions tested across all species, this difference was only significant in the mink cortex, human cortex, and human cerebellum (Tables 3.2 and 3.3).

Inhibition of mACh receptor binding was more potent in the cerebellum compared to the cerebral cortex (IC50: $F=44.368$, $df=1$, $P<0.05$; Ki: $F= 22.784$, $df=1$, $P<0.001$). While there were no differences in IC50 and Ki between the human cerebellum and cortex exposed to $HgCl_2$, there was a significant difference in MeHg inhibition under the same conditions (Tables 3.2 and 3.3).

There were significant differences in mACh receptor inhibition among the species tested (IC50: $F=113.078$, $df=4$, $P<0.001$; Ki: $F= 93.062$, $df=4$, $P<0.001$). Following exposure to $HgCl_2$ or MeHg, IC50 and Ki values were generally highest in human tissue samples (Table 3.3). Irrespective of Hg type and brain region studied, species sensitivities could be ranked from most to least sensitive as: otter > rat > mink > mouse > humans (Fig 3.2 and 3.3).

3.5 Discussion

The major finding of this study is that $HgCl_2$ and MeHg can inhibit the binding of [^{3}H]-QNB to the mACh receptor in the cerebellum and cerebral cortex regions in several mammalian species. Specifically, $HgCl_2$ is a more potent inhibitor of mACh receptor binding than MeHg, and the receptors in the cerebellum are more sensitive than those in the cerebral cortex. Species sensitivities, irrespective of Hg type and brain region, can be ranked from most to least sensitive as: otter > rat > mink > mouse > humans.

It is still unclear which allotropic form of Hg is the primary neurotoxicant in mammals. Some have suggested that the latency period associated with Hg neurotoxicity is attributable to the accumulation of mercuric ions in the brain as a result of slow de-methylation processes (Charleston et al. 1996). This notion is supported by the current and previous studies that collectively demonstrate $HgCl_2$ is a more potent inhibitor of mACh receptor binding than MeHg (Bondy and Agrawal 1980; Von Burg et al. 1980; Abd-Elfattah and Shamoo 1981; Castoldi et al. 1996). Interestingly, the disparate effects on receptor binding inhibition by inorganic and organic Hg were more prominent in human tissues. Physicochemical differences between $HgCl_2$ and MeHg likely facilitate these observations. First, $HgCl_2$ has two potential binding sites while MeHg has one available binding site, suggesting the divalent cation is more reactive with multiple amino acids (Abd-Elfattah and Shamoo 1981). Second, MeHg is more likely to interact with proteins and functional groups that are embedded within the lipid bilayer since the organic compound is more hydrophobic than $HgCl_2$. Third, inhibition of mACh receptor binding by $HgCl_2$ has previously been described as non-competitive while that of MeHg is competitive (Castoldi et al. 1996). Based on these facts, $HgCl_2$ likely binds two thiol groups located near the extracellular membrane surface in a manner that would mask the mACh receptor binding site, while MeHg may prefer a single thiol group located closer to the receptor binding domain to prevent direct binding of ligands. Future studies are required to test the validity of this hypothesis.

The mACh receptor is divided into five classes (M1 to M5) based on

pharmacological function. The stimulation of M1, M3, and M5 isoforms (coupled to $G_{q/11}$-protein) causes increases in intracellular [Ca^{2+}] by activating phospholipase C, whereas the stimulation of M2 and M4 isoforms (coupled to $G_{i/0}$-protein) decreases cyclic AMP (cAMP) levels by inhibiting adenylyl cyclase activity (Wess 1996; Caulfield and Birdsall 1998). The conservation of this receptor family across mammalian species is evident by examining the amino acid sequences among rat, mice and humans greater than 98% and 95% homologous for the M1 and M2 isoforms, respectively (Caulfield and Birdsall 1998). While we did not discern the effects of Hg on the various mACh receptor isoforms, a previous study demonstrated that MeHgOH and $HgCl_2$ are equipotent inhibitors for the M1 receptor, but $HgCl_2$ is a more potent inhibitor of the M2 receptor (Castoldi et al. 1996). High densities of M2 receptors are located within the cerebellum and occipital cortex of mammals (Wei and Hung 1990; Gu 2003); the two brain areas particularly susceptible to Hg exposure based on evidence from humans (Eto 1997), rodents (Nagashima, 1997), and wildlife (Wobeser and Swift 1976).

Inhibition of mACh receptor binding was more potent in the cerebellum compared to the cerebral cortex, across all species studied. The cerebellar granule cells are more sensitive to Hg exposure relative to other neurons. Mechanistic studies demonstrate this sensitivity may be due to MeHg's direct interaction with M3 receptors causing phopholipase C-mediated rises in intracellular Ca^{2+} and subsequent cell death (Limke et al. 2004). As a result, loss of granule neurons from the cerebellum may be dependent upon interactions between Hg and the components of the cholinergic signalling pathway.

The ranking of species sensitivities to Hg-mediated mACh receptor inhibition were remarkably parallel across both brain regions and types of Hg examined. There may be different mechanistic factors involved across the species since complete inhibition of mACh receptor binding by Hg was not achieved in some tissue samples, especially in the human cerebellum, and this requires further investigation. The in vitro data from mink and river otter are of concern since the concentrations of Hg we tested fall within a range of Hg that

are commonly found in the brains of wild mink and river otters (upwards of 4.8 µg/g Hg, *w.w.*; (Basu et al. 2005). Therefore, naturally occurring levels of Hg may be causing significant effects on neurochemical pathways in fish-eating wildlife and monitoring the health of fish-eating wildlife represents a novel strategy to warn against potential risks associated with fish consumption in human populations that inhabit mutual ecosystems.

All the inhibitory data (i.e. IC50 and Ki values) in the current study were within one order of magnitude of each other across the species tested. This is not surprising given that Hg intoxication causes structural damage to specific anatomical regions of the brain and that neurobehavioural outcomes are highly conserved across different mammals. Neurobehavioural deficits commonly observed in afflicted human populations (e.g. ataxia, impaired gait, tremors) have also been observed in rodents and wild mammals (Nagashima 1997). Such a high degree of conservation in Hg's toxicological effects supports the extrapolation of rodent neurotoxicological data to both humans and wildlife in risk assessment. This approach is valid at the level of whole animal (i.e. neurobehavioural tests) and cell (i.e. disruption of cholinergic signalling pathways).

In summary, we demonstrate that Hg can inhibit the binding of ligands to the mACh receptor in a range of mammalian species. Such comparative studies on inter-species differences are required to gain a better understanding on the significance and relevance of neurochemical biomarkers in human and ecological health assessments.

Table 3.1: Receptor binding characteristics for [^{3}H]-QNB binding to mACh receptors in the cerebral cortex and cerebellum of various mammals.

	mACh receptor B_{max} (fmol/mg)		mACh receptor K_d (nM)	
	Cerebral Cortex	Cerebellum	Cerebral Cortex	Cerebellum
Mouse	2317.8 (122.2)[a]	504.4 (69.6)[y]	0.24 (0.02)[1]	0.08 (0.06)[9]
Rat	1142.7 (41.1)[b,d]	168.0 (10.6)[z]	0.17 (0.01)[1,2,3]	0.10 (0.02)[9]
Human	1460.9 (160.9)[b]	185.0 (14.4)[y,z]	0.23 (0.02)[1,2]	0.09 (0.02)[9]
Mink	801.8 (44.0)[d]	112.8 (31.0)[z]	0.14 (0.02)[2,4]	0.13 (0.05)[9]
Otter	876.1 (140.9)[c,d]	157.7 (35.7)[z]	0.10 (0.01)[3,4]	0.10 (0.02)[9]

Values represent mean (± SEM). Superscript letters/numbers represent significant differences in mACh receptor B_{max} or K_d in a specific brain region among the mammalian species tested.

Table 3.2: IC50 data for [^{3}H]-QNB binding to mACh receptors in the cerebral cortex and cerebellum of various mammals.

	Cerebral Cortex		Cerebellum	
	$HgCl_2$	MeHg	$HgCl_2$	MeHg
Mouse	4.44 (0.26)^{b}	5.87 (0.50)^{x}	3.15 (0.64)^{2}	4.48 (0.56)^{8}
Rat	1.67 (0.08)^{c,d}	3.32 (0.23)^{x,y}	1.70 (0.21)^{2,3}	1.97 (0.12)^{8}
Human	8.13 (1.04)^{a}	17.90 (1.92)^{w}	9.05 (0.51)^{1}	13.00 (3.36)^{9}
Mink	2.87 (0.12)^{c}	5.45 (0.31)^{x}	2.64 (0.26)^{2}	3.53 (0.17)^{8}
Otter	1.41(0.21)^{d}	2.58 (0.36)^{y}	1.16 (0.15)^{3}	1.59 (0.12)^{8}

Values represent mean (± SEM). Superscript letters/numbers represent significant differences in IC50 among mammalian species in a specific brain region following exposure to $HgCl_2$ or MeHg (μM).

Table 3.3: Inhibition constant (Ki) data for [^{3}H]-QNB binding to mACh receptors in the cerebral cortex and cerebellum of various mammals.

	Cerebral Cortex		Cerebellum	
	$HgCl_2$	MeHg	$HgCl_2$	MeHg
Mouse	2.41 (0.14)[b]	3.18 (0.27)[y,z]	1.54 (0.32)[1]	2.01 (0.31)[8,9]
Rat	0.78 (0.05)[d]	1.54 (0.12)[y]	0.82 (0.074)[1]	0.98 (0.10)[7,8]
Human	4.23 (0.52)[a]	9.41 (1.10)[z]	3.94 (0.57)[2]	5.79 (1.66)[9]
Mink	1.63 (0.14)[c]	3.10 (0.29)[y,z]	1.24 (0.15)[1]	1.65 (0.13)[7,8,9]
Otter	0.69 (0.10)[d]	1.27 (0.17)[y]	0.52 (0.06)[1]	0.75 (0.11)[7]

Values represent mean (± SEM). Superscript letters/numbers represent significant differences in Ki among mammalian species in a specific brain region following exposure to $HgCl_2$ or MeHg (μM).

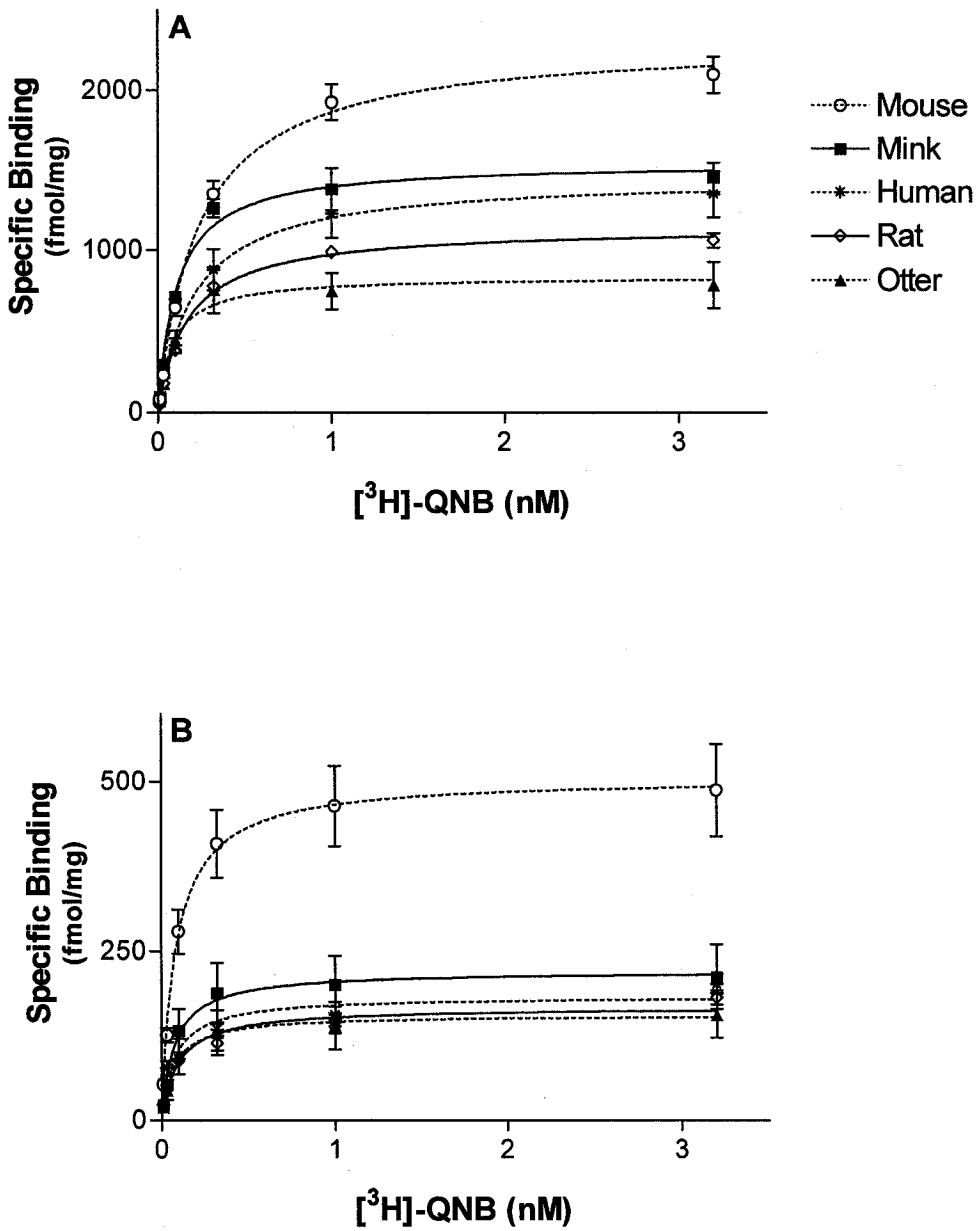

Figure 3.1: Saturation analysis of [^{3}H]-QNB binding to isolated membranes preparations from the A) cerebral cortex, and B) cerebellum of mammalian species. All data points represent mean (± SEM) specific binding from 5-6 samples.

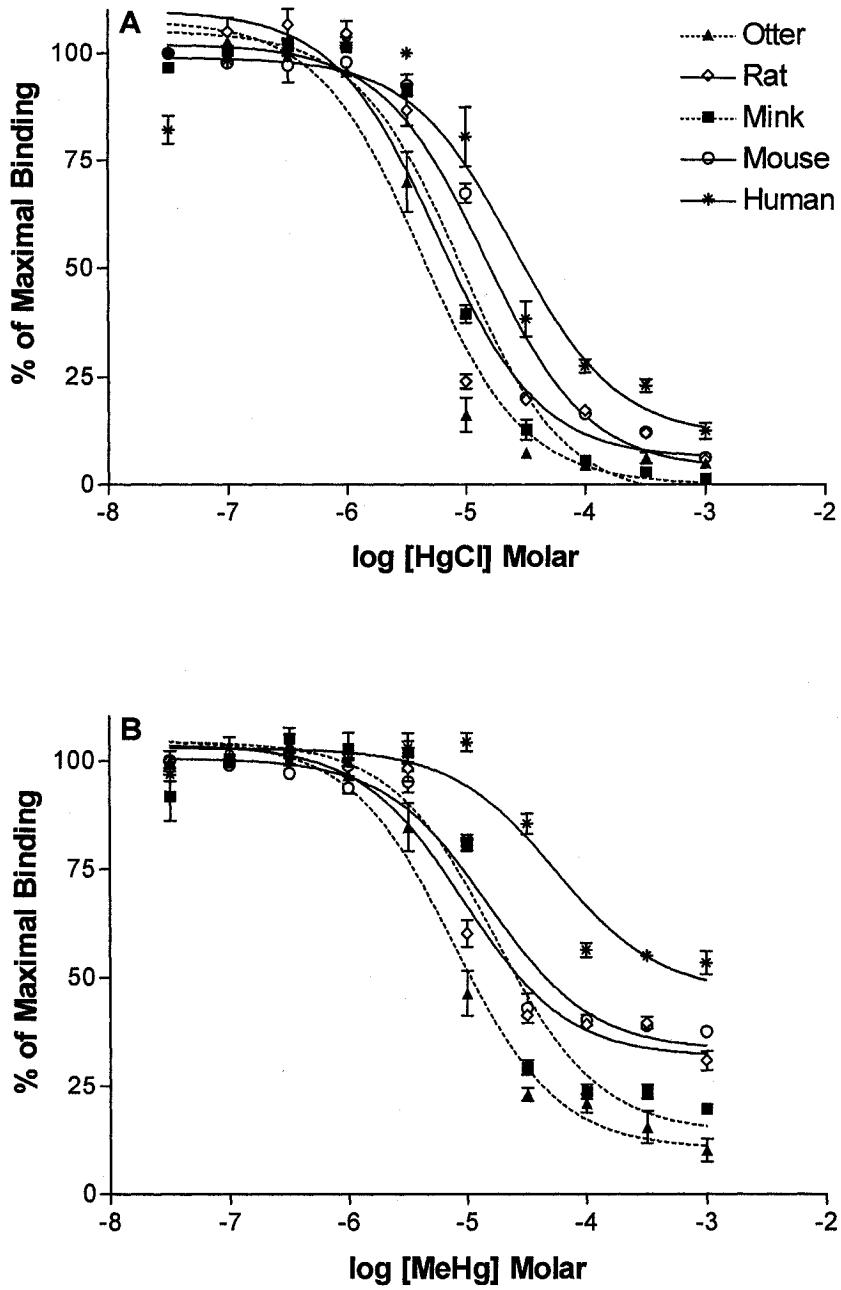

Figure 3.2: Inhibition of [^{3}H]-QNB binding by (A) $HgCl_2$, or (B) MeHg in membrane preparations isolated from the cerebral cortex in mammalian species. Values are compared to membrane preparations not exposed to Hg, and data points represent mean (± SEM) specific binding from 5-6 samples.

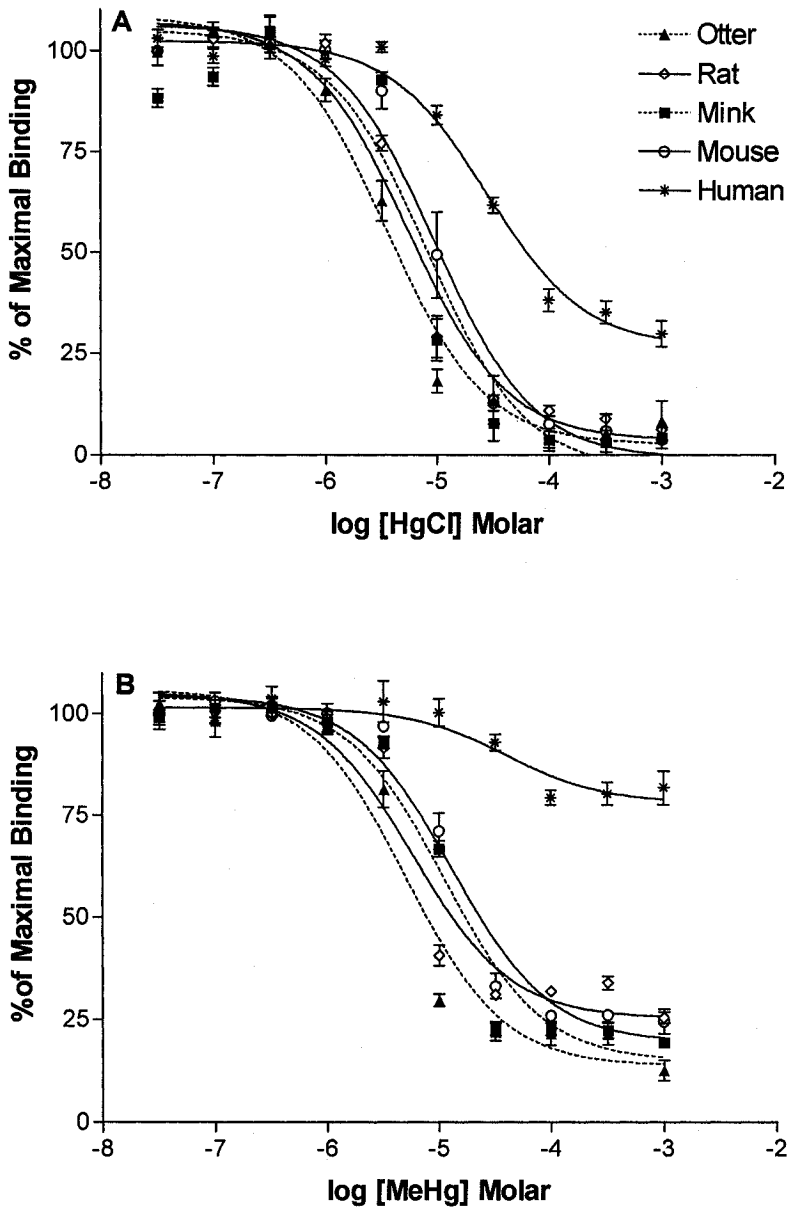

Figure 3.3: Inhibition of [^{3}H]-QNB binding by (A) $HgCl_2$ or (B) MeHg in membrane preparations isolated from the cerebellum in mammalian species. Values are compared to membrane preparations not exposed to Hg, and data points represent mean (± SEM) specific binding from 5-6 samples.

Connecting Paragraph

Chapter 3 examined the effects of Hg compounds on muscarinic acetylcholine receptor in several different animal species. This next chapter examines the effects of MeHg exposure on another potential biochemical marker of CNS function, monoamine oxidase (MAO). The developing brain is particularly sensitive to the neurotoxic effects of MeHg. MAO regulates neurotransmitter concentrations and has been shown to play an important role in early brain development. Therefore, the effects of low-level prenatal MeHg exposure on MAO activity in various brain regions of rat offspring were evaluated. mACh receptor binding was also assessed in the offspring brain, however, no significance was observed and therefore this data is included as Appendix A.

Chapter 4: Effects of gestational exposure to methylmercury on monoamine oxidase activity in rats

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4.1 Abstract

Monoamine oxidase (MAO; EC 1.4.3.4) regulates biogenic amines in the brain and is involved in brain development. The purpose of this study was to determine the effects of low-level gestational exposure to methylmercury (MeHg) on MAO activity in rats. Embryos (gestation day 13.5) were removed from pregnant Sprague-Dawley rats and cultured for 12 h in media containing 0, 100 or 750 µg/L of MeHg. MAO activity in whole embryos cultured in 750 µg/L of MeHg was reduced by 15% ($p=0.005$) when compared to controls. To determine the effects of low-level gestational MeHg exposure on offspring MAO, female rats were treated by gavage with saline or MeHg at dose levels of 0.5 or 1.0 mg MeHg/kg body weight/d for four weeks prior to mating and throughout pregnancy. MAO activity was measured in the cerebral cortex, striatum, hippocampus, cerebellum and brain-stem of female and male offspring (postnatal day 41). Female offspring MAO activity was significantly reduced in both exposure groups when compared to controls, with specific effects observed in the brain-stem of the high dose group. No significant differences in MAO activity were observed in male offspring brain. In conclusion, this data demonstrated that a low gestational exposure to MeHg reduced MAO activity in the developing embryo and in adult female rat offspring. Changes in MAO activities may serve as an indicator for neurotoxicity following developmental exposure to MeHg and should be further investigated.

4.2 Introduction

Mercury (Hg) is a neurotoxic persistent pollutant that is widespread in the environment. The dietary intake of the organic form of Hg, methylmercury (MeHg), in contaminated fish and aquatic mammals is the main route of human exposure (ATSDR 1999). High dose accidental poisonings in Minamata, Japan and Iraq indicated that the developing brain is particularly sensitive to the neurotoxic effects of MeHg (Bakir et al. 1973; Harada 1995). Infants exposed to high concentrations of MeHg during gestation, had symptoms similar to congenital cerebral palsy and brain pathology showed severely damaged

neurons throughout the cortex (Takeuchi and Eto 1999). More recently, epidemiological studies from fish-eating communities suggest that subtle variations in child motor and cognitive behaviour were related to low-level prenatal MeHg exposure (Grandjean et al. 1997). However, in order to fully evaluate the health risks and impacts of low-level MeHg exposure, it is useful to understand potential mechanisms of toxicity (Castoldi et al. 2003). While the neurotoxic targets and mechanisms of high dose exposure have been well established in animal models (ATSDR 1999), there has been a need to develop specific and functional methodologies to identify possible subtle neurotoxic effects of low-level gestational exposure to MeHg.

Monoamine neurotransmitters, including serotonin, dopamine, and noradrenaline, are involved in neurofunctional behaviours such as fight-or-flight response, emotion, motor activity, learning and memory (Whitaker-Azmitia et al. 1994; Yeomans and Frankland 1995; Koch 1999; Holschneider et al. 2001; Dare et al. 2003; Brown and Silva 2004). Disruptions of these neuronal signalling pathways have been proposed as a mechanism for MeHg neurotoxicity in prenatal and adult rodent models (Lindstrom et al. 1991; Lakshmana et al. 1993; Faro et al. 2003). Administration of Hg compounds increase concentrations of noradrenalin and serotonin in various regions of the brain (Lindstrom et al. 1991; Lakshmana et al. 1993), and also increase striatal dopamine release (Faro et al. 1997). Brain monoamine neurotransmitters are metabolized and degraded by a flavin-enzyme, monoamine oxidase (EC 1.4.3.4, MAO) located in the outer mitochondrial membrane of astrocytes and neuronal cells (Shih 2004). Two isoenzymes have been identified, MAO-A and MAO-B, which differ in the specificities for inhibitors, substrates and expression in tissue (Shih 2004). Pharmacological and genetic studies have shown that MAO is important for serotonergic neuronal development and neurobehaviour (Whitaker-Azmitia et al. 1994; Cases et al. 1996). Initial structure/function studies by Gomes et al (Gomes et al. 1976) suggested that mercuric compounds interact with functionally critical cysteine residues in MAO, resulting in non-competitive inhibition and permanent damage to the enzyme. In a more recent study, repeated exposure to MeHg

resulted in MAO activity reduction in various brain regions of adult Sprague Dawley rats (Chakrabarti et al. 1998). This literature suggests that MAO may be a useful neurochemical marker for the effects of MeHg on the monoaminergic nervous system.

The objectives of this study were to 1) evaluate effects of MeHg on MAO in rat embryos cultured in vitro, and 2) evaluate effects of low-level prenatal MeHg exposure on MAO in brain regions of adult male and female rat offspring.

4.3 Methods

4.3.1 Animals

The protocol was approved in advance by the Institutional Animal Care and Use Committee (IACUC), and the care and use of animals was conducted in accordance with guidelines of the USA National Research Council and the Canadian Council on Animal Care. Adult Sprague-Dawley rats (Charles River Canada, Canada), approximately 6.5 weeks of age, were acclimated for one week prior to study. All animals were maintained at constant temperature (21°C), 50% humidity, photoperiod of 12-h light/dark and had *ad libitum* access to a commercial available diet (PMI Rodent Chow, 5002) and water.

4.3.2 Embryo Culture

Adult female rats were placed in a cage for mating with adult male rats overnight. Embryos were obtained from pregnant rats and cultured based on a previously described method (Blair et al. 1996) with modifications. Briefly, embryonic gestational day (GD) 0 was assumed to be the mid-point of the dark-cycle during which copulation occurred (12:00 midnight) as confirmed by the presence of sperm in the vaginal smear. On GD 13.5, female rats were euthanized by CO_2 asphyxiation. The uterus was immediately excised and harvested into a Petri dish containing sterile Hanks' balanced salt solution (HBSS). The uterus was cut along the antimesometrial edge and each conceptus was explanted. The remnants of the decidua, trophoblast and Reichart's

membrane were removed from the yolk sac. Embryos were extruded into the medium, still attached to the opened yolk sac and placenta through the vitelline and umbilical vessels. Two embryos were transferred to each culture bottles containing 10 mL of 15% rat serum (heat-inactivated), and 85% Dulbecco's Modified Eagle's Medium with either 0, 100 or 750 µg/L of methyl mercury chloride (Alfa Aesar, U.S.A.). Bottles were gassed with 95% O_2 and 5% CO_2 for 2 min, incubated at 37°C, rotated at 20 cycles/min, and regassed at a 6 hours gassing interval. Following 12 hours incubation, embryos were transferred into cryo-vials, and stored at -80°C until biochemical analysis.

4.3.3 MeHg In Vivo Dosing Experiment

Fifteen female rats were randomly assigned to control, low dose (0.5 mg of MeHg/kg body weight/day), or high dose (1.0 mg of MeHg/kg body weight/day) groups. Methylmercury chloride was dissolved using a sonicator in nitrogen purged deionized water to achieve a final dose volume of 5 ml/kg body weight. Control animals received nitrogen purged water alone. The females were dosed orally by gavage each day for 28 days, and were then placed for mating with untreated adult males (same strain and source) until confirmation of mating. The presence of sperm in the vaginal smears indicated copulation (first day of pregnancy). The females were treated with either MeHg or saline until delivery of the offspring (approximately 20 days following successful mating). Dams were observed daily and the food consumption and body weights were measured weekly. After birth the general condition of the offspring were evaluated and body weights were recorded periodically. Each litter was culled to give a total of 4 males and 4 females. When necessary, the selected pups were identified by injection of India ink into their paws four days after birth. The offspring were weaned on postnatal day (PND) 21.

The maternal rats were euthanized approximately 21 days after weaning and the offspring were euthanized on PND 41. The method of euthanasia was carbon dioxide asphyxiation followed by exsanguinations from the abdominal aorta. The brain regions (cortex, striatum, cerebellum, brain-stem and

hippocampus) were immediately dissected on ice (Chakrabarti et al, 1998), from one female and one male offspring from each exposure group. The whole brain was also removed for mercury measurement from one male and one female offspring from each exposure group. Tissue was frozen at -80°C until MAO or total mercury analysis was performed.

4.3.4 MAO Analysis

Whole embryos were sonicated in 1mL of NaK buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) for 30 sec. MAO activity in homogenates was measured based on a microplate method described by Zhou *et al* (Zhou and Panchuk-Voloshina 1997) and modified by Stamler *et al* (Stamler et al. 2005). Embryo samples (50 µg of protein) were diluted with NaK buffer and added to a 96-well plate. Reaction was initiated with the addition of reaction buffer (100 µM Amplex Red, 2 U/mL horseradish peroxidase, and 4 mM tyramine). Fluorescence was measured every 5 min between 30-90 min at 540/590 nm (excitation/emission) using a fluorometric plate reader (Wallac Victor 2, Perkin Elmer, Boston, MA) at 23°C. The concentration of resorufin was determined from a standard curve (0.5-5.0 µM resorufin), and specific activities of samples were expressed as pmol of resorufin per min per mg of protein. NaK buffer was used as a sample blank and 2.5 µM H_2O_2 was used as a positive control.

MAO activity was measured in the five brain regions as previously described by Krajl (Krajl 1965) with modifications described by Chakrabarti et al (Chakrabarti et al. 1998). Briefly, samples were homogenized in 10 volumes of 50 mM phosphate buffer (pH 7.8) and mixed thoroughly in the presence of 2% Triton X-100 (v/v). The homogenate was added to 2.5 mL of phosphate buffer and 0.3 mL of 0.9% NaCl solution and pre-incubated for 10 min at 37°C. A single concentration of kynauramine dihydrobromide (22 µM) was added to the reaction mixture and incubated for 30 min at 37°C. The reaction was stopped by the addition of 0.2 mL of 5 M perchloric acid followed by centrifugation at 1500 x g for 10 min. An aliquot of the supernatant was diluted with 1 M NaOH solution and the fluorescence of the product, 4-hydroxylquinoline, was measured with a RF-

551 Shimadzu spectrofluorometric detector (excitation/emission; 318/380nm). The concentration of product was determined from a standard curve of 4-hydroxyquinoline and expressed as nmol product formed per 30 min per mg of protein.

4.3.5 Hg Analysis

Concentrations of total mercury in the kidney, liver and whole brain of the offspring were measured as described by Neugebauer *et al* (Neugebauer et al. 2000). Approximately 1.5 g of tissue was digested for 16 h in concentrated nitric acid and then heated for 5 h at 105°C. Digests were cooled and diluted with distilled water to give a 12% (v/v) nitric acid solution. Mercury concentrations were measured using a Z-8200 Polarized Zeeman cold vapour Atomic Absorption Spectrophotometer (Hitachi, Tokyo Japan). Certified reference material (DORM-2; dog fish muscle, National Research Council, Ottawa, ON) was analyzed for each batch of sample for quality control. Detection limit was 7.0 ng mercury/g of tissue. Samples with Hg below the limit of detection were assigned of a value that was half of the detection limit (3.5 ng/g).

4.4 Statistics

Analyses were performed using SAS software (SAS Institute, 2002) and data was considered significant when p-value was less than or equal to 0.05. Means values and standard error of each dose group were calculated for numerical data. Only one female and one male offspring were taken as a representative of the litter to avoid over-inflation of statistical power (Holson and Pearce 1992). The effects of MeHg dose on offspring body weight, offspring food intake and MAO activity in embryos cultured in vitro were analyzed using an analysis of variance (ANOVA) and multiple comparisons were performed using the Tukey post-hoc test. Analysis of offspring MAO in different dose-groups and brain-regions were based on a two-way ANOVA and multiple comparisons were performed using the Tukey post-hoc test.

4.5 Results

4.5.1 Embryo Culture

MAO activity in whole embryos (Figure 4.1) was altered following incubation with MeHg ($F(2, 35) = 7.43$; $p=0.002$). Embryos cultured in the presence of 750 µg/L of MeHg exhibited significantly reduced MAO activity (15%) when compared to control embryos ($p=0.005$).

4.5.2 In Vivo Experiment

No significant differences were observed in maternal body weight gained and food consumed during the pre-mating, gestation, lactation and post-lactation period in the exposure and the control groups. All offspring survived following birth. A significant effect of MeHg on total body weight gain was observed in male offspring ($F(2,19) = 5.05$, $p=0.017$) as those in the high dose group gained less weight when compared to controls (Table 4.1). MeHg exposure did not affect weight gain in female offspring.

4.5.3 MAO Activity

Brain MAO activity in female offspring was affected by MeHg dose group ($F(2,74) = 8.20$; $p < 0.001$) but not by brain region ($F(4,74) = 1.29$; $p = 0.282$). There was no statistical interaction between brain region and dose group ($F(8,74) = 0.72$; $p = 0.673$). Brain MAO activity was significantly reduced in the offspring of the high dose ($p<0.001$) and low dose group ($p=0.044$) when compared to the controls. Analysis of enzyme activity in each brain region showed that MAO was significantly decreased in the brain-stem of the high dose female offspring ($p=0.011$) (Figure 4.2). MAO activity in male offspring was not affected by MeHg dose group ($F(4,74) = 1.97$; $p = 0.149$) or by brain region ($F(4,74) = 1.34$; $p = 0.266$), although enzyme activity was lower in the striatum of the high dose male offspring when compared to controls.

4.5.4 Hg Analysis

Whole brain total Hg concentrations in female and male offspring at PND 41 were affected by the MeHg dose group (Table 4.2). There was a significant difference in brain Hg concentrations among the male offspring; control < low dose group < high dose groups. While, female offspring in the MeHg dosed groups have elevated brain Hg compared to controls, there was no significant difference between the low and high dose groups.

4.6 Discussion

This study is the first to demonstrate that MeHg can reduce MAO activity in whole rat embryos cultured in vitro, as well as in female rat offspring brain tissue exposed during gestation. These studies suggest that MAO may be a marker of long-term monoaminergic nervous system damage as a result of low-level prenatal MeHg exposure.

The choice of MeHg dose in the embryo culture model was based on reported elevated concentrations in human cord blood in Nunavik, Quebec (Muckle et al. 2001; Després et al. 2005) and Minamata, Japan (Dalgard et al. 1994). No effect on MAO activity was observed at a dose level of 100 µg/L, which is higher than the average concentrations in the cord blood collected from Faroe Island where neurological effects were reported (5). However, the lack of sensitivity may be due to the fact that rats are more resistant to MeHg toxicity (per kg/body weight) than humans (Rice 1996). Recently, a human study conducted with fish consumers from Lac St-Pierre, (QC, Canada) showed that Hg exposure (<20 µg/L blood-Hg) is related to reduced MAO activity in circulating platelets (Stamler et al. 2004). Therefore, the decrease in MAO activities during gestation may still be a significant factor for observed neurological symptoms in human populations. It has been estimated that gestational exposure to mercury during the poisoning in Minamata may have been as high as 750 µg/L umbilical cord blood (Dalgard et al. 1994). We showed that this concentration reduced MAO activity by 15% in whole rat embryos cultured for 12 hours. In humans, this

concentration of cord blood-Hg resulted in severe neuronal damage and behavioural abnormalities in the children, including hyperkinesis, abnormal reflexes, and ataxia (Harada 1995).

Previous studies show that a repeated oral administration of MeHg (5.0 mg of MeHg/kg body weight/d for 7 consecutive days) inhibited MAO activity in several brain regions immediately following administration of the final dose (Chakrabarti et al. 1998). In the present study, female offspring had reduced MAO activity in the brain, with specific effects being seen in the brainstem of the high dose group. These effects were observed when Hg concentrations in whole brains were below 60 ng/g. It is important to note that biochemical changes were detected 6 weeks after the final dose of Hg, indicating the effects on MAO may be long-term or permanent. Future studies are required to evaluate these temporal changes in MAO activity following Hg exposure.

Structure and function evaluations of MAO have demonstrated that several cysteine residues are required for proper enzymatic function (Hubalek et al. 2003). Mercuric compounds have a high affinity for thiol groups (Clarkson 1997) and it is likely that Hg binds to cysteine residues, resulting in permanent disrupted structure and decreased MAO function as suggested by Gomes *et al* (Gomes et al. 1976). Previous i*n vitro* studies using rat brain synaptosome preparations reported that MeHg can inhibit MAO activity (Chakrabarti et al. 1998). While MeHg exposure may alter MAO gene expression and protein levels, these in vitro studies suggest that MeHg exposure directly inhibited enzymatic function.

The specific disruption of MAO function during development has been associated with specific behavioural impairments in animal models (Whitaker-Azmitia et al. 1994; Shih 2004). The administration of specific inhibitors for both MAO-A and MAO-B (clorgyline and deprenyl, respectively) to pregnant rats throughout gestation, resulted in a 50% inhibition of brain MAO, increased open-field activity and deficits in learning and memory function in the offspring (Whitaker-Azmitia et al. 1994). These biochemical and behavioural changes were associated with a reduction in serotonergic neuron innervations in the cortex (Whitaker-Azmitia et al. 1994). Behavioural and pathological evaluation of

MAO deficient mice suggested an association with novelty seeking, increased aggression and impaired cortical somatosensory neuron barrel formation (Cases et al. 1996; Holschneider et al. 2001; Rebsam et al. 2002; Shih 2004). These studies suggest that impairment of MAO activity during gestation may have profound effects on brain development and on the monoaminergic system.

In conclusion, a low-level gestational exposure to MeHg inhibited MAO function in female rat offspring and in embryos cultured in vitro. This study suggests that MAO may serve as a biochemical marker of monoaminergic nervous system dysfunction related to low-level MeHg exposure. Recent developmental studies in embryos indicate that MAO may play an important role in neuronal growth, migration and consequently offspring neurobehaviour (Rebsam et al. 2002). Inhibited cortical MAO increases serotonin concentrations, over-activates the serotonin receptor (5-HT1b), thereby inhibits thalamocortical axon growth (Rebsam et al. 2002). Therefore, future mechanistic studies should evaluate the interaction between MeHg exposure, MAO inhibition and impaired neuronal development.

Table 4.1: Effects of MeHg on overall body weight gain in offspring

MeHg Dose Group (# litters)	Body Weight Gain (g)
Males	
Control (5)	184.0 ± 3.5
Low dose (5)	185 ± 3.3
High dose (5)	170 ± 4.4 *
Females	
Control (5)	143.4 ± 2.9
Low dose (5)	146.0 ± 2.6
High dose (5)	134.6 ± 3.1

Mean ± SEM of the body weight gain of each litter (n) between PND 4 and 41.
* $P < 0.05$ (vs controls; Dunnet's Test)

Table 4.2: Total mercury concentrations in rat offspring whole brain tissue.

MeHg Dose Group (# litters)	Male (ng/g)	Females (ng/g)
Control (5)	5.3 ± 1.8^{a}	9.2 ± 3.5^{a}
Low (5)	20.2 ± 1.6^{b}	34.6 ± 3.2^{b}
High (5)	58.2 ± 3.6^{c}	45.5 ± 5.1^{b}

Values represent the means ± SEM. Means with different letters represent significant differences within columns ($p<0.05$)

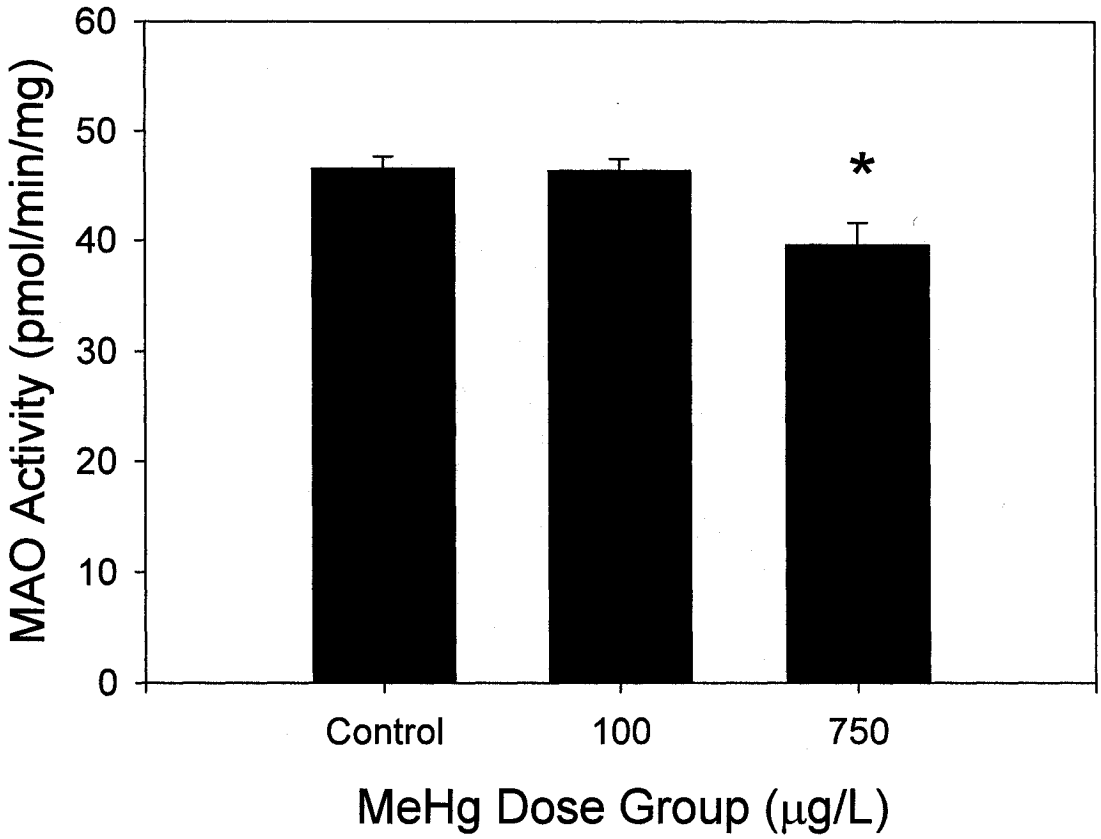

Figure 4.1: Effects of MeHg exposure on MAO activity in whole rat embryos cultured in vitro. Untreated embryos (GD 13.5) were cultured for 12h in the presence of 0, 100 or 750 µg/L of MeHg. Each bar represents the mean MAO activity ± SEM in whole rat embryos (n=12). *$p<0.05$ compared to control group.

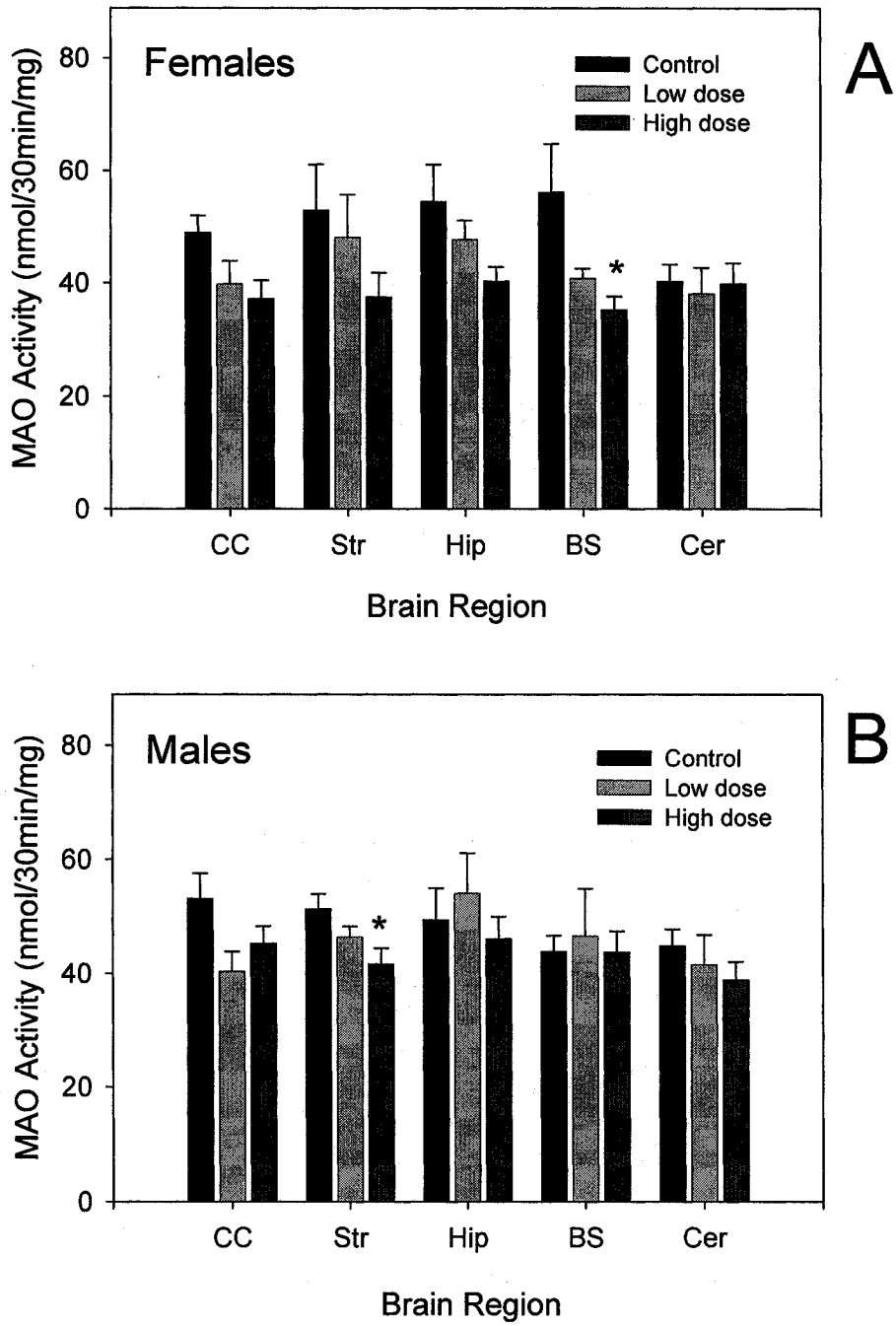

Figure 4.2: Effects of MeHg exposure on MAO activity in different brain regions of male and female offspring (PND 41). Each bar represents the mean MAO activity ± SEM in male rats (n=5) and female rats (n=5) in the cerebral cortex (CC), striatum (Str), hippocampus (Hip), brain-stem (BS), and the cerebellum (Cer). *$p<0.05$ compared to control group.

Connecting Paragraph

Chapter 5 is a natural progression of the previous three chapters. The second chapter describes methodological considerations for the measurement of the proposed biomarkers in a human population and suggests that blood tissue can be adequately stored until laboratory analysis. The previous two chapters evaluated the interaction of Hg compounds with two proposed biomarkers of neurotoxicity, monoamine oxidase (MAO) and the muscarinic acetylcholine (mACh) receptor. The mACh receptor, ChE and MAO can be measured in accessible non-neuronal tissue and may reflect CNS dysfunction. The associations between Hg exposure and these biomarkers have never been examined in human populations. Therefore Chapter 4 brings these ideas together to evaluate the association between MeHg exposure and peripheral biochemical markers in a fish-eating community from Lac St-Pierre, Quebec.

Chapter 5: Mercury exposure and peripheral biochemical markers in fish consumers from the Lac St-Pierre Region, Quebec, Canada

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5.1 Abstract

Humans are exposed to methylmercury (MeHg) primarily through dietary consumption of predatory fish and marine mammals. MeHg can damage the nervous system by disrupting neurochemical parameters. Analogues of these parameters, including monoamine oxidase (MAO), cholinesterase (ChE), and the muscarinic acetylcholine (mACh) receptor, are measurable in non-neuronal peripheral blood tissue and may reflect central nervous system function. The objective of this study was to evaluate the association between these peripheral biochemical markers and MeHg exposure in 130 fish-eating adults from Lac St-Pierre, Quebec, Canada. Blood mercury (Hg) concentrations were used as a marker of MeHg exposure and ranged from 0.2 to 17.0 µg/L. We observed no relationship between blood-Hg and the mACh receptor on lymphocytes or ChE activity in serum. However, a significant negative association (r=-0.193, p=0.029) was observed between blood-Hg and MAO activity. Multiple linear regression analysis demonstrated that blood-Hg (β = -4.9, P = 0.007) and heavy smoking (β = -8.5, P = 0.001) were associated with reduced platelet-MAO activity. In addition, mean MAO activity in participants above the 75th percentile for blood-Hg (3.4 µg/L) was significantly lower than mean value below the 25th percentile (23.5 ± 8.2 vs. 30.6 ± 14.0 nmol/min/20µg; P = 0.035). Our results suggest that MAO in blood platelets may be a useful tool to assess biochemical effects of MeHg exposure in human populations. These functional changes in platelet-MAO may reflect enzymatic changes in neuronal tissue and should be further investigated as a risk assessment strategy.

5.2 Introduction

Methylmercury (MeHg) is a widespread neurotoxic pollutant (ATSDR 1999). The dietary intake of contaminated fish and marine mammals are the main route of exposure to MeHg in human populations (Van Oostdam et al. 1999). MeHg is absorbed efficiently by the gastrointestinal tract and is able to cross the blood brain-barrier (NRC 2000). Once in the brain, mercury (Hg) compounds can disrupt protein function by interacting with sulfhydryl residues,

eventually leading to cellular death and neuronal loss (Castoldi et al. 2001). Accidental poisoning episodes in Japan and Iraq have shown that exposure to high-levels of MeHg causes neurological abnormalities including ataxia, paresthesia, tremors, and visual and auditory impairments (Bakir et al. 1973; Harada 1995). Additionally, studies conducted in the Brazilian Amazon have suggested an association between MeHg and diminished motor and visual function at blood-Hg concentrations below 200µg/L (Mergler 2002), a guideline level associated with a low-risk of neurological damage (WHO 1990). There is increasing interest to detect early signs of neurotoxicity at low-levels of MeHg exposure (NRC 2000). The detection of MeHg related biochemical disruption has been proposed as a strategy to better understand these potential neurotoxic risks (Castoldi et al. 2001).

Biochemical changes in the nervous system generally occur prior to permanent damage and therefore could be used to predict future neurotoxic outcomes (Costa and Manzo 1995; Manzo et al. 1996). MeHg exposure disrupts the function and transmission of the cholinergic (Hastings et al. 1975; Kobayashi et al. 1980; Coccini et al. 2000) and monoaminergic nervous systems (Oudar et al. 1989; Lindstrom et al. 1991; Faro et al. 1997). Key protein components of these systems, including monoamine oxidase (MAO, EC 1.4.3.4) (Tsuzuki 1981; Chakrabarti et al. 1998), muscarinic acetylcholine (mACh) receptor (Coccini et al. 2000; Basu et al. 2005), and cholinesterase (ChE, EC 3.1.1.7) (Hastings et al. 1975) are targeted by Hg compounds and may serve as biochemical markers of neurotoxicity. Our laboratory has recently demonstrated the negative effects of MeHg on mACh receptors in vitro (Basu et al. 2005) and in brain tissues of fish-eating wildlife such as mink (Basu et al. 2005) and river-otter (Basu et al. 2005). These neurochemical changes may reflect early stages of MeHg neurotoxicity. However, due to the complexity and inaccessibility of nervous tissue, monitoring these biochemical markers is not feasible in humans.

Blood platelets and lymphocytes are unique non-neuronal cells as they utilize similar cellular machinery as monoaminergic and cholinergic neurons, respectively (Manzo et al. 1996; Reed et al. 2000; Tayebati et al. 2002).

Specifically MAO, ChE and mACh receptors are present in peripheral blood cells (Costa et al. 1988; Chen et al. 1993) and are similarly targeted by neurotoxic compounds (Costa et al. 1990; Chakrabarti et al. 1998; Coccini et al. 2000). Chakrabarti et al reported that repeated oral exposure to MeHg in rats resulted in a parallel reduction of MAO activity in cortex, striatum and platelet (Chakrabarti et al. 1998). In a similarly designed study, MeHg exposure resulted in an immediate increase in lymphocyte mACh receptor density, which was followed by a similar increase of mACh receptor density in the cerebellum and hippocampus (Coccini et al. 2000). These animal studies suggest that monitoring peripheral biomarkers in non-neuronal tissue may offer a method to screen for relevant neurochemical perturbation following MeHg exposure. Additionally, human studies have demonstrated that platelet-MAO is reduced by heavy tobacco smoking (Whitfield et al. 2000) and by industrial exposure to styrene and manganese (Manzo et al. 1996). PET (positron emission tomography) imaging studies have confirmed that MAO activity is also reduced in brain tissue of heavy smokers (Fowler et al. 1996), suggesting that platelet-MAO may serve as a surrogate marker for other inaccessible tissue. Relationships between these potential peripheral biomarkers and MeHg exposure have not been reported in human populations. The identification of biochemical markers that may indicate the effects of MeHg exposure may serve as a strategy to evaluate health risks in human populations.

Lac St-Pierre is a widening of the St-Lawrence River system located between Montreal and Quebec City (Quebec, Canada), where residents are involved in commercial and sport fishing during the summer and winter seasons. Reports of MeHg concentrations in fish from the St-Lawrence River suggest that several species are above recommended guideline levels (Chan et al. 2000), and consumption of these fish may result in neurobehavioural toxicity (Mergler et al. 1998). Therefore, the objective of this study was to investigate the association between MeHg exposure and potential peripheral biomarkers (i.e. platelet-MAO, lymphocyte mACh receptor and serum ChE) in fish-eating adults living along the St-Lawrence River.

5.3 Materials and methods

5.3.1 Population and Sampling

The study population habited the Lac St-Pierre region in the municipality of Sorel – Tracy (Quebec, Canada). A research agreement was drafted and signed by community representatives and researchers. The participants were recruited through the Lac St-Pierre Fisher's Association, radio stations and local newspapers. This cross-sectional study was carried out from February to April 2003. Participants were excluded if they did not report eating fish from Lac St-Pierre. For this study, 130 participants, 18 years and older were selected. Informed consent was obtained by the participants and coded questionnaires were completed to determine socio-demographic and lifestyle information. For the purpose of this study, information regarding age, gender, alcohol intake, smoking habits, garden pesticide use (y/n), body mass index (kg/m^2), medical and recreational drug use, industrial exposure to chemicals (y/n) and neurological diseases were collected as potential confounding variables. Ethical approval to conduct this study was granted by the McGill University and the University of Quebec at Montreal ethical review committee.

Blood samples (30 ml) were drawn into vacutainer tubes by a registered nurse. Peripheral lymphocytes were isolated from whole blood by density gradient centrifugation using Ficoll-Paque Plus reagent (Amersham Biosciences, Brown Deer, WI, USA) (Boyum 1968). Isolated lymphocytes were washed twice, and suspended in phosphate buffer (50 mM, pH 7.4). Platelet-rich-plasma was centrifuged at 3000 *g* for 25 min at 4°C, to pellet platelets. Platelets were washed twice and suspended in phosphate buffer. Whole blood, collected in tubes without anticoagulant, was centrifuged at 2000 g for 10 min to obtain serum. The concentration of protein in lymphocytes and platelets preparations were determined by the Bradford method (Bradford 1976) using bovine serum albumin as the standard. Lymphocytes were counted with a hemacytometer. Platelets and serum were stored at –80°C until biochemical assays were performed. The storage condition and stability of the samples have been tested and optimized (Stamler et al. 2005).

5.3.2 Biomarker Analysis

MAO activity analysis was based on the method described by Krajl (Krajl 1965). Platelets (20 μg protein) were mixed in a Triton X-100 (0.5%) solution, and then diluted in 3 ml of phosphate buffer saline (50 mM, pH 7.8). Reaction was initiated by the addition of a concentration range of the MAO substrate, kynauramine dihydrobromide (1.5 – 50 μM), followed by a 30 min incubation at 37°C. Following the incubation, the reaction was stopped by the addition of 5 M perchloric acid, and centrifuged at 2000 x *g* for 10 min. The supernatant was diluted with 1 M NaOH and the fluorescence was read at 318nm excitation and 380nm emission on a spectrofluorometer. Product concentration was calculated based on the standard curve of 4-hydroxyquinoline (0 – 30 nM) and results were expressed in nmol of product formed per min per 20 μg of platelet protein. Intra assay variation was less than 5% and inter assay variation was less than 10%.

ChE activity in serum was measured based on the commercially available kit developed by Molecular Probes Inc (Eugene, OR, USA) (Zhou et al. 1997; Zhou et al. 2000). Human serum was diluted with phosphate buffer and added to a 96-well plate. Reaction was initiated with the addition of reaction buffer (100 μM 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red), 1 U/mL horseradish peroxidase, 0.1 U/ml choline oxidase and 50 μM acetylcholine). Fluorescence was measured every 5 min between 30-90 min at 540 excitation and 590 emission with a fluorometric plate reader (Wallac Victor 2, Perkin Elmer, Boston, MA). All samples were measured in triplicate. The concentration of resorufin was determined from a standard curve (0.5 - 5.0 μM), and specific activities of samples were expressed as nmol of product per min per mL of serum.

The mACh receptor-binding assay (Costa et al. 1988) was performed on freshly isolated human lymphocytes in duplicate. Approximately 20 μg of cell protein was pre-incubated in 3ml of phosphate buffer (+/- 100 μM atropine) for 30 min at 25°C. Samples were incubated with 10nM [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB; NEN/Perkin Elmer, Boston, MA, USA), a mACh receptor specific

radio-ligand, for 60 min at 25°C. The incubation was terminated by filtration through 0.22 μM GF/C glass filters (Millipore Inc., Boston, MA, USA) and washed three times with cold phosphate buffer. Radioactivity retained by the filters was quantified by a liquid scintillation counter (LKB Wallac 1209 Rackbeta). Specific binding was defined as the difference in [^{3}H]-QNB bound in the presence and absence of atropine and expressed as fmol per number of cells.

Iron status was determined from serum ferritin levels, which were measured in duplicate using the ACTIVE® Ferritin Enzyme-Linked Immunosorbent Kit (Diagnostic Systems Laboratories, Inc. Webster, Texas, USA). Ferritin concentration was expressed as ng per ml of serum. Iron deficiency was defined as serum ferritin levels below 12 ng/ml.

5.3.3 Blood Metal Analysis

Whole blood samples (7 ml) were drawn into heparinized tubes and stored at -20°C prior to analysis. Blood total-Hg concentrations were determined using cold-vapour atomic-absorption spectrometry. The detection limits were 0.2μg/L for blood Hg analysis. Certified reference material was analyzed for quality control purposes. Selenium (Se), cadmium (Cd) manganese (Mn) and lead (Pb) blood concentrations were determined using inductively coupled plasma-mass spectrometry technique. All metal analyses were carried out at the Centre de Toxicologie du Québec, Canada.

5.4 Statistical Analysis

Data from MAO saturation kinetics were curve fitted using GraphPad Prism (Version 3.02, GraphPad Software Inc., San Diego, CA) to calculate maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m). References to MAO activity refer to MAO_{Vmax}. All other statistical analyses were conducted using SPSS statistical software (SPSS Version 11.5.0, San Rafael, CA). A *P*-value less than or equal to 0.05 was considered statistically significant and all values were reported as means ± standard deviation unless otherwise noted.

Data was analyzed for normality using the Kolmogorov-Smirnov test. Serum ferritin, and blood total Hg, Cd, Mn, and Pb data were log-transformed for statistical analysis. MAO_{Km} was not normally distributed. The relationship between biomarkers (MAO, mACh receptor, and ChE) and independent variables were evaluated using an independent Student's *T*-test, one-way ANOVA for multiple comparisons or Pearson correlation. Spearman correlations, Mann-Whitney-U Tests or ANOVA performed on ranks, were used to evaluate the relationship between MAO_{Km} and independent variables. A forward step-wise multiple-linear regression analysis was performed to determine the predictors of biomarker function in the population. Age, gender, heavy smoking (y/n), alcohol intake, garden pesticide use (y/n), serum ferritin (ng/L), industrial exposure to chemicals (y/n), recreational drug use (y/n), cardiac drug use (y/n) and blood selenium and metal concentrations were considered as independent variables.

Additionally, the population was divided into quartile groups based on blood-Hg levels, and mean MAO values were compared using a General Linear Model Univariate procedure adjusting for heavy smokers followed by a Bonferroni post-hoc analysis.

5.5 Results

5.5.1 Study Population Description and Exposure Measurements

The age of the participants ranged from 18-73 y with a mean of 49 ± 13 and 52 (40%) were women. Twenty two percent described themselves as current smokers, of which 20 individuals reported smoking more than 14 cigarettes per day (heavy smokers). Mean BMI (SD) in the population was 26.5(5.7). Thirty percent of the population abstained from the use of alcohol, and 12% consumed greater than 420 grams of ethanol/week (high alcohol consumption). None of the participants reported having neurological diseases such as Parkinson's, Alzheimer's disease or Schizophrenia, and none were taking MAO inhibiting drugs. Median (range) serum ferritin levels was 169.2 (7.4 -

1000) ng/ml and iron deficiency was observed in 4 participants. Three individuals reported working with agricultural pesticides, while 37 reported using garden style pesticides. The mean, median and range of blood metal levels are summarized in Table 5.1. Mean (SD) blood-Hg concentration was 2.4 ± 2.9 µg/L and values ranged from 0.2 to 17.0 µg/L.

5.5.2 Biomarkers in Study Population

Blood tissue MAO, mACh receptor and ChE levels in the study population and in subjects grouped by gender, smoking habits, and alcohol consumption are listed in Table 5.2. Relationships between continuous variables (age, BMI, ferritin levels, alcohol, and cigarette intake) are listed in Table 5.3. One man out of the total 130 participants was unable to donate blood for this study and two blood samples had insufficient platelets for MAO analysis. Mean mACh receptor binding on lymphocytes was greater in men when compared to women (P = 0.006). MAO was not significantly affected by smoking status (y/n); however heavy smokers (≥15 cigarettes/d) displayed lower MAO activity than moderate smokers (1-14 cigarettes/d) and non-smokers combined (P = 0.001). No difference in MAO was observed between non-smokers and light-smokers (P > 0.05). There was a positive relationship between plasma ChE and both age (P = 0.019) and BMI (P = 0.001), as shown in Table 5.3. Of the drugs reported being used by more than 5% of the study group, only cardiac medication (n = 17) resulted in a significant increase in serum ChE (P = 0.016). There was no observed interaction between alcohol intake (abstainers, drinkers, and heavy drinkers (>420 g/week)) and the measured biomarkers. Serum ferritin levels were positively correlated with mACh receptor binding (P = 0.022) and negatively correlated (P < 0.01) with MAO activity. Blood-Cd levels were strongly correlated with the number of cigarettes per day (Spearman, r = 0.724, P < 0.0001) and heavy smokers had over four times greater mean blood-Cd concentrations when compared to the remaining population (3.8 ± 1.7 versus 0.9 ± 1.0 µg/L, P < 0.001).

5.5.3 Association Between Biomarkers and Contaminant Exposure Levels

Simple correlation analysis between blood metal concentrations and biochemical markers indicated that MAO negatively correlated with blood total-Hg ($P = 0.029$), Cd ($P = 0.008$), and Se ($P = 0.045$) levels (Table 5.3). No significant correlations were observed between the remaining biomarkers (mACh receptor, ChE or MAO_{Km}) and blood metal concentrations. To further investigate the relationship between platelet-MAO and blood-Hg, a forward stepwise multi-regression analysis that considered the previously mentioned independent variables was performed. In the regression model ($r^2 = 0.12$, $P < 0.001$), both heavy smoking and blood-Hg explained 7.4 and 5.4% of the MAO variation in the population, respectively (Table 5.4). To account for the potential effects of Fe deficiency, removal of the 4 iron deficient individuals from the multiple regression analysis did not change the model. Predictors of serum ChE and lymphocyte mACh receptors were also evaluated (Table 5.4). Gender, garden pesticide use and blood Mn levels are predictors of lymphocyte mACh receptor binding levels in this population, while gender, BMI and pesticide use were predictors of serum ChE activity.

MAO activity values, adjusted for the effects of heavy smoking, were stratified in blood-Hg quartile groups (Figure 5.1). Blood-Hg levels in the lowest quartile group ranged from 0.2 – 0.5 µg/L while those in the highest group ranged from 3.4 – 17 µg/L. Mean MAO from participants in the upper Hg quartile group was significantly ($P = 0.035$) less than the mean values from the lowest quartile group. Further analysis showed that participants with blood-Hg concentrations above 3.4 µg/L exhibited lower mean MAO activity when compared to the remaining population (23.6 ± 7.5, n=29 versus 29.1 ± 10.9 nmol/min/20µg, n=98, $P = 0.012$).

5.6 Discussion

This study explores the association between MeHg exposure and

peripheral biochemical markers in a fish-eating community. The key finding in this study is that blood-Hg was significantly associated with reduced platelet-MAO activity, but not with serum ChE activity or lymphocyte mACh receptor binding. A 19 - 24% reduction in platelet-MAO activity was associated with blood-Hg concentrations above 3.4µg/L. It has been proposed that platelet-MAO may reflect nervous system function (Costa and Manzo 1995; Manzo et al. 2001), and therefore these variations in biomarker activity may indicate early signs of MeHg related neurotoxicity.

Platelets are secretory cells that play an important role in hemostasis, thrombosis, vascular remodeling and repair (Reed et al. 2000). These cells are considered to be a peripheral model of serotonergic nerve endings, as they utilize similar transmitters, enzymes, receptors and transporters for cellular communication (Reed et al. 2000; Polgar et al. 2002). MAO exists as two isoenzymes, MAO-A and MAO-B (Shih 2004), however platelets only exhibit MAO-B activity (Chen et al. 1993). MAO is involved in the oxidative deamination of amine neurotransmitters, including serotonin, dopamine and noradrenaline (Shih 2004). Reduction of this enzyme can lead to rapid changes in neurotransmitters concentrations and other cellular functions which can result in behavioural effects (Murphy and Kalin 1980). The reduction of brain MAO activity following MeHg exposure has been previously observed in animals (Tsuzuki 1981; Chakrabarti et al. 1998). Similar to these animal experiments, this study only assessed MAO activity and therefore it is difficult to conclude if the observed relationship with Hg is a result of reduced MAO protein or a reduction in specific activity. In vitro studies using rat brain synaptosomes and isolated platelets show that MAO is dose dependently inhibited through incubation with MeHg (Chakrabarti et al. 1998). Additionally, early studies examining the structure of MAO, proposed that mercuric compounds bind to essential cysteine residues (Wu et al. 1993), reducing activity and permanently damaging enzyme function (Gomes et al. 1969; Gomes et al. 1976). While secondary effects of Hg exposure on MAO can't be ruled out, these in vitro studies provide a mechanistic explanation of reduced MAO function. Given evidence that platelet-MAO may act

as a surrogate marker for brain MAO activity (Fowler et al. 1996; Chakrabarti et al. 1998; Whitfield et al. 2000), it is possible to suggest that Hg related effects on platelet-MAO would also be observed in brain tissue of the Lac St-Pierre cohort.

Platelet-MAO activity is influenced by a range of environmental and genetic factors. While genetic analysis was not evaluated in this study, several polymorphisms have been shown to influence MAO activity in humans (Damberg et al. 2001). Environmental factors, including tobacco smoking (Whitfield et al. 2000; Berlin and Anthenelli 2001), gender (Whitfield et al. 2000), iron deficiency (Youdim et al. 1975) and alcohol intake (Rommelspacher et al. 1994) have also been shown to affect platelet-MAO activity. Of these variables examined in the present study, only heavy smoking significantly contributed to reduced platelet-MAO. Whitfield et al has shown that moderate and heavy smokers (>10 cigarettes per day) have a reduction in platelet-MAO when compared to non smokers, former smokers, and light smokers (i.e. <10 cigarettes per day) (Whitfield et al. 2000). Cigarettes are the major contributor to Cd levels in the blood (dell'Omo et al. 1999), and therefore MAO was also negatively associated with blood-Cd. Cd levels were strongly related to the reported number of cigarettes smoked per day, and were approximately 4-fold higher in heavy smokers compared to moderate and non-smokers. While it has been shown that various constituents of tobacco smoke inhibit MAO activity in vitro (Castagnoli et al. 2002), the direct inhibition of MAO by Cd cannot be ruled out (Leung et al. 1992). Because, tobacco is a strong modulator of MAO activity, it is important to control for the effects of smoking when measuring platelet-MAO activity in population studies.

Neither of the non-neuronal cholinergic markers was associated with blood-Hg concentrations. ChE activity was positively correlated with BMI, which has previously been observed in several other studies (Kutty and Payne, 1994, Lawrence and Melnick, 1961). ChE is synthesized by the liver and secreted bound to lipoproteins (Kutty and Payne, 1994). This relationship may be explained by the fact that participants with higher BMI may have increased Very-low-density-lipoprotein secretion as a result of hyperlidemia (Cucuianu et al.,

1996). Additionally, after correcting for confounding factors, the use of pesticides (i.e. insecticides, fungicides, and herbicides) was positively associated with an increase in both cholinergic markers. The specific types of pesticides were not recorded, however only anti-cholinergic pesticides would be anticipated to alter cholinergic function (Wilson et al., 1997). It is important to note that while acute exposure to anti-cholinergic pesticides are known to decrease ChE activity in blood, chronic exposure has been shown to increase enzymatic activity. Overall, the understanding of variables that predict variations in mACh receptor binding and ChE activity in a population will assist in the development and planning of future investigations with these biomarkers in humans.

Certain species of fish from the St. Lawrence River system have been shown to have levels of MeHg above recommended guidelines (i.e. Walleye, Lake Trout), and that consumption of these fish are the major determinant of human MeHg-exposure in this area (Mahaffey and Mergler 1998; Chan et al. 2000). Although blood concentrations of Hg were relatively low in this study group, associated biochemical variations in platelet-MAO can be observed at doses that are below the lowest observable effect levels reported in the literature (ATSDR 1999). The physiological and clinical consequences of reduced MAO activity are not clear, and must be further evaluated. These results suggest that MAO in blood platelets may be a useful tool to detect early effects of MeHg exposure in human populations.

Table 5.1: Blood metal concentrations (µg/L) in the Lac St-Pierre study group

Blood Metal	Mean ± SD	Median	Range
Hg	2.4 ± 2.9	1.25	0.2 – 17
Cd	1.4 ± 1.53	0.80	0.1 – 7.5
Se	210 ± 30.3	209	127 – 296
Mn	8.8 ± 2.7	8.4	3.5 – 20
Pb	31.3 ± 19.7	27.0	8.9 – 135

Table 5.2: The distribution among various independent variables of mean blood MAO, MAO_{Km}, ChE and mACh receptor levels (± SD) in the study population

	n	MAO (nmol/min/20µg)	MAO_{Km} (µM)	ChE (nmol/min/ml)	mACh receptor (fmol/cell x 10^6)
Total	129	27.8 (10.6) ‡	19.5 (7.5) ‡	340 (80)	77.4 (32.1)
Gender					
Women	52	29.9 (9.8) a ‡	19.1 (8.2) a ‡	324 (80) a	67.9 (35.5) a
Men	77	26.4 (10.9) a	19.7 (7.0) a	348 (80) a	83.7 (28.0) b
Smoking Status					
Non smokers	100	28.7 (10.4) a‡	19.5 (7.8) a ‡	340 (80) a	77.5 (33.2) a
1-14 cig./d	9	32.1 (12.6) a	19.4 (6.4) a	306 (94) a	84.5 (26.9) a
≥ 15 cig./d	20	21.3 (7.8) b	19.1 (6.3) a	354 (72) a	72.9 (29.6) a
Heavy Smokers					
<15 cig./d	109	29.0 (10.6) a ‡	19.5 (7.7) a ‡	336 (82) a	78.2 (32.6) a
≥15 cig./d	20	21.3 (7.8) b	19.1 (6.3) a	354 (72) a	72.9 (29.6) a
Alcohol Consumption					
Abstainers	29	27.8 (7.5) a	18.5 (6.1) a	352 (82) a	81.9 (32.7) a
1–420 g/week	85	28.0 (11.8) a †	19.6 (7.8) a †	334 (80) a	76.1 (33.0) a
> 420 g/week	10	27.2 (9.7) a †	21.8 (9.3) a †	352 (78) a	71.4 (21.3) a
Pesticide Use					
No	92	27.8 (11.0) a	19.9 (8.8) a	332 (76) a	74.9 (28.3) a
Yes	37	27.8 (9.4) a	18.2 (6.3) a	361 (89) a	84.8 (41.1) a
Cardiac Drugs					
No	112	28.2 (10.2) a	19.8 (7.8) a	322 (78) a	77.0 (32.0) a
Yes	17	25.2 (13.4) a	17.8 (3.8) a	382 (86) b	80.0 (30.0) a

Letters represent ($P < 0.05$) significant differences
‡ Two samples were not included due to insufficient protein
† One sample was not included due to insufficient protein

Table 5.3: Correlation analysis among biomarkers of neurotoxicity and blood metal concentrations and other parameters

	Peripheral Biomarkers			
Variables	**MAO**	**MAO_{Km} [b]**	**mAChR**	**ChE**
Age (y)	-0.008	-0.002	0.147	0.208*
BMI (kg/m^2)	-0.036	-0.067	0.068	0.291**
Cigarettes (no./d)[b]	-0.199*	0.012	0.024	0.002
Alcohol(g/week)[b]	-0.050	0.080	-0.011	-0.022
Ferritin (ng/L) [a]	-0.177*	0.003	0.198*	0.209*
Blood Metals				
Hg (µg/L) [a]	-0.193*	-0.035	0.004	0.029
Cd (µg/L) [a]	-0.235**	-0.032	-0.008	0.052
Se (µg/L)	-0.179*	0.057	-0.073	0.093
Mn (µg/L) [a]	0.048	0.009	0.157	0.064
Pb (µg/L) [a]	-0.132	0.141	-0.132	0.151

[a] Log transformed data
[b] Spearman Correlations
*$P < 0.05$, ** $P < 0.001$

Table 5.4: Predictors of peripheral biochemical markers from multiple linear regression analysis[b].

Predictors	β-Coefficient (±SE)	P-Value
Platelet-MAO activity (nmol/min/20µg)		
Heavy Smoker	-8.31 ± 2.46	0.001
Blood-Hg[a]	-4.52 ± 1.74	0.011
Total Model: Adjusted R^2 = 0.105, P < 0.001		
Lymphocyte mACh receptor (fmol/cell x 10^6)		
Blood-Mn[a]	48.9 ± 21.5	0.024
Gender (♀=1,♂=0)	-18.2 ± 5.5	0.001
Pesticide (n=0, y=1)	12.6 ± 6.3	0.048
Total Model: Adjusted R^2 = 0.105, P = 0.001		
Serum ChE activity (nmol/min/ml)		
BMI (kg/m^2)	4.45 ± 1.26	0.0001
Gender (♀=1,♂=0)	-30.0 ± 13.6	0.029
Pesticide (n=0, y=1)	33.6 ± 15.5	0.032
Total Model: Adjusted R^2 = 0.125, P < 0.001		

[a] Values were log transformed
[b] β-Coefficient were determined by stepwise multiple linear regression. Inclusion criteria: $P < 0.05$.

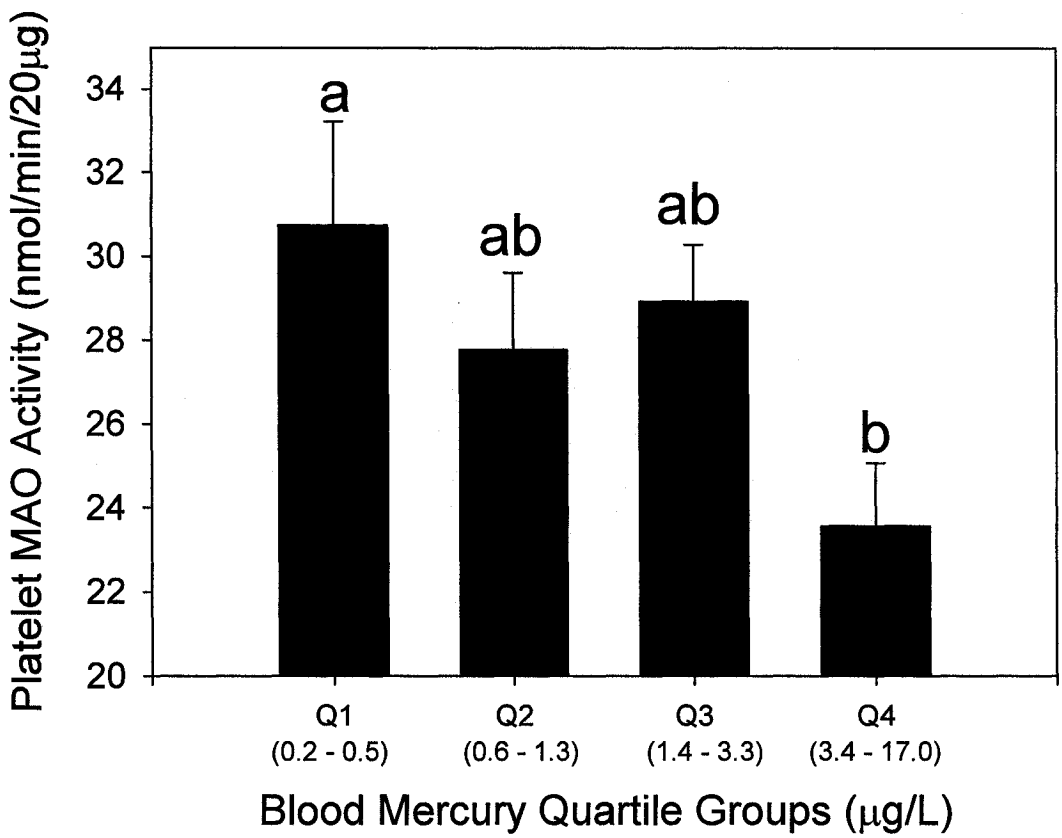

Figure 5.1: Mean (± SE) platelet-MAO for subjects in blood-Hg quartile groups (Qs). MAO was adjusted for the effects of heavy smoking. There were 30-32 subjects per quartile. Bars with different letters are significantly different, $P < 0.05$.

SUMMARY AND CONCLUSION

Humans are exposed to MeHg through dietary consumption of predatory fish and aquatic mammals (ATSDR 1999). While the symptoms associated with high dose Hg poisoning can be clinically observed (Bakir et al. 1973; Harada 1995), neurotoxic effects associated with chronic low dose exposure are more difficult to identify (Mergler 2002). Currently the most sensitive method for evaluating early stages of neurotoxicity in humans is through neurobehaviour tests designed to evaluate cognitive, sensory and motor function. Detection of biochemical changes may offer a sensitive method that may allow us to understand early stages of neurotoxicity in humans (Manzo et al. 1996). Currently, there are no useful biochemical markers of MeHg neurotoxicity for human populations. Following a review of the literature, three biomarkers were proposed for their potential use in the detection of the early neurotoxic effects of MeHg exposure (i.e. mACh receptors, ChE and MAO). These biomarkers are present in blood tissue, offering an ethical method to detect CNS dysfunction in human populations. The goal of this thesis was to develop and explore the application of these biomarkers in human populations exposed to MeHg.

The first step was to evaluate the feasibility of measuring the peripheral biomarkers in human blood from rural communities. Due to the potential large population sizes, screening tools were adapted for use in microplates (Chapter 2). Controlled storage studies suggest that platelets and plasma can be stored on ice without significant loss of biomarker function (MAO and ChE, respectively). While plasma ChE activity remains stable when frozen, platelet-MAO activity is more stable when stored at -80°C when compared to -20°C. The mACh receptor assay performed with isolated lymphocytes must be performed immediately, limiting the application to epidemiological studies. These findings address feasibility concerns for the application of the potential peripheral biochemical markers in epidemiological studies.

Hg compounds are known to disrupt mACh receptor binding in rat models (Castoldi et al. 1996). In order to examine the application of this biomarker for the detection of Hg toxicity in human populations, an in vitro examination was

performed to identify the interactions of Hg compounds with brain tissue from various animal species, including humans (Chapter 3). This study confirms that the inhibitory effect of Hg on mACh receptor radioligand binding is consistent in the animal species examined (rats, mouse, otter, mink and humans). However, analyses of IC50 values indicate that the sensitivity to Hg varies in different species, possibly due to isoform expression and genetic differences. Of the species examined, human brain mACh receptor binding was the least sensitive. These data conclude that the human form of the mACh receptor is inhibited by Hg compounds, and suggest that examination of lymphocyte mACh receptor binding may be a relevant target for MeHg in human epidemiological studies.

Repeated oral exposure to MeHg has been shown to inhibit MAO activity in several brain regions in adult Sprague Dawley rats (Chakrabarti et al. 1998). While the physiological consequence of reduced MAO function in adults is unclear, impairment of MAO during brain development is known to cause moderate to severe behavioural abnormalities (Whitaker-Azmitia et al. 1994; Shih and Thompson 1999; Shih 2004). Chapter 4 demonstrated low-level maternal exposure to MeHg before and throughout gestation reduced MAO activity in the brain of adult female offspring. Additionally, whole rat embryos cultured in the presence of low concentrations of MeHg have significantly reduced MAO activity. These findings also suggest MAO may act as an indicator of long-term neurotoxicity following gestational exposure to MeHg.

Some species of fish from the St-Lawrence River have been shown to have elevated levels of MeHg (Mahaffey and Mergler 1998; Mergler et al. 1998). The region of the St-Lawrence River located near Lac St-Pierre is home to commercial and recreational fishers. In this study, the association between Hg exposure levels and peripheral biomarker function in fish-eating adults were evaluated (Chapter 5). Exposure in this community was generally low and none of the participants were within the Health Canada's increasing risk range for blood-Hg (20-200 µg/L). Platelet-MAO activity was inversely associated with blood-Hg concentrations suggesting a biochemical effect of exposure. Reduced MAO activity is associated with blood-Hg levels above the EPA's guideline level

(5.8 µg/L). No association was observed between blood-Hg and lymphocyte mACh receptor or serum ChE activity. Heavy smoking was also associated with decreased MAO activity in the population. Given that platelet-MAO activity was affected by Hg exposure, MAO may serve as a marker of the early neurotoxic effects of MeHg exposure in human populations.

While animal studies suggest that the cholinergic nervous system is involved in mechanism of MeHg neurotoxicity, no effects were observed between Hg exposure and lymphocyte mACh receptor binding or ChE activity in the Lac St-Pierre community. The *in vitro* data from Chapter 3, suggest that the human form of the mACh receptor may be less sensitive to Hg inhibition than the rodent receptor. While not directly comparable, the in vitro concentrations of Hg required to inhibit human mACh receptor binding in human brain membrane preparations were much greater than blood concentrations observed in Lac St-Pierre community.

It is known that Hg compounds are capable of disrupting protein function in the brain, resulting in secondary effects related to altered cellular function and organism behaviour. Because protein disruption represents the general mode of Hg toxicity in the brain, detection of any biochemical perturbation related to neuronal transmission may serve as an indicator of neurotoxicity. Under this definition, because MAO is found in catecholaminergic neurons in the brain, enzymatic changes are relevant marker of CNS dysfunction. These Hg related biochemical changes will therefore assist in the understanding of the continuum of Hg toxicity progression and may represent a hallmark of the earliest stages CNS disease states.

Future studies are required to explore the interaction between Hg compounds and MAO. In vitro studies suggested that Hg can bind to essential cysteine residues, which may result in permanent damage to the enzyme (Gomes et al. 1969). It is still unclear how organic and inorganic Hg compounds differ in their ability to inhibit MAO activity. Additional studies should investigate other 5-HT related markers, including receptors and transporters as biochemical targets of Hg compounds. The developing embryo is particularly sensitive to

changes in MAO activity, as high levels of serotonin during brain growth inhibit neuronal migration and synaptogenesis (Shih and Thompson 1999). Given that MeHg neurotoxicity is particularly sensitive to the developing brain, future studies should evaluate how MAO inhibition and potential increases in serotonin levels may be involved in neurotoxicity.

The epidemiological studies should also be repeated in other communities to confirm these findings and evaluate the effects of other confounding neurotoxic pollutants. The Lac St Pierre community was exposed to low-levels of Hg, and it would be important to understand the interaction of Hg at higher exposure levels to complete the platelet-MAO dose/response curve. If future studies consistently show an interaction between platelet-MAO and Hg exposure, then case-control studies (high fish consumers versus non-fish consumers) designed to evaluate MAO levels through brain imaging techniques are warranted to validate MAO as a surrogate biomarker of neurotoxicity. Longitudinal studies would also help explain and identify variations in biomarker activity in order to establish a platelet-MAO baseline level.

The development of indicators of neurotoxicity has not progressed as quickly as in other medical research areas. While some biomarkers are available for the detection of Hg toxicity, they generally identify the irreversible stages of Hg neurotoxicity. The understanding of the molecular mechanism of Hg neurotoxicity will help identify a continuum of effects observed in following low-level exposure. Sensitive protein targets altered by Hg compounds may provide new opportunities for biomarker evaluations. This thesis provides a basic framework of results that suggest that platelet-MAO may be an early target of Hg toxicity. Platelet-MAO is the first biochemical marker that has shown to correlate with Hg exposure in a human population. The stability and ease of measurement of this biomarker offers a unique opportunity to explore Hg related biochemical interactions in epidemiological studies. If the robustness of the use of platelet-MAO as a biomarker can be shown in other populations, it will provide an important tool for future environmental health investigations.

Limitations

There are several limitations to the findings in this thesis. The major impacts of these findings are based on the assumption that reduced platelet-MAO activity reflects brain MAO-activity and Hg related toxicity. Despite years of laboratory research, the specific effects of Hg compounds on the nervous system leading to behavioural and developmental impairments, has not been clearly identified. General mechanisms of Hg neurotoxicity include oxidative stress, mitochondrial dysfunction, calcium signalling, and membrane disruption, may all contribute to cellular damage and eventual cell death (Castoldi et al. 2001). Therefore, the conclusion that reduced platelet-MAO is an indication of neurotoxicity is limited due to the insufficient understanding of Hg neurotoxicity. The assumption that reduced platelet-MAO mirrors brain-MAO activity may also be dependent on the neurotoxic compound, or the state of the disease causing neuronal damage. Therefore, it is possible that reduced platelet-MAO activity may not relate to altered physiological change in the nervous system, and may be only used as a biomarker of exposure.

The literature does not identify any clear toxicity resulting from the direct effects of reduced platelet-MAO activity in the adult brain. Human studies have shown that platelet-MAO activity reflects brain MAO function in heavy smokers (Berlin et al. 1995; Fowler et al. 1996), however few studies have identified any neurological consequence of subtle and chronic enzyme reduction (Shih 2004). There is some evidence that MAO and serotonin levels in platelets may be related to neurological diseases, including Parkinson's, Alzheimer's and Schizophrenia (Bond et al. 1979; Jossan et al. 1991; Jarman et al. 1993; Fitzgerald et al. 1996; Zhou et al. 2001), however this evidence is inconsistent (Konings et al. 1995; Simpson et al. 1999). While it is hypothesized that the reduction in brain-stem MAO in the female offspring may represent direct MAO inhibition or reduced monoaminergic neurons due to Hg exposure, these changes may only represent brain homeostasis and not actual Hg related neurotoxicity. Based on the lack of understanding of the consequences of reduced MAO

activity, it is difficult to clearly identify the risks of Hg exposure in this population from biomarker measurement.

Peripheral biochemical markers, including platelet-MAO, may vary widely in the population and therefore the specificity of MAO for detection of Hg related effects are limited. In the Lac St-Pierre study, only 10% of the variation in platelet-MAO activity could be explained by the independent variables. Therefore, the use of this marker for identification of neurotoxicity on an individual-level is extremely limited. Platelet-MAO is more useful in large epidemiological studies, where the effects of smoking, alcohol and other relevant confounders can be controlled using multiple regression approaches. At the present time, platelet-MAO should only be used to complement current strategies of risk assessment in a population. Another limitation of the Lac St Pierre study is the limited sample size. While 130 participants were recruited for this study, a larger sample size would have increased the power of the study and allowed for opportunities to stratify the population in order to examine relationships in women and men individually, with regards to MAO activity.

Animal studies indicate a clinical significance of reduced brain MAO activity during critical periods of gestation. This is a result of increased serotonin levels and subsequent over activation of the 5-HT receptors, leading to decreased neuronal growth and development in the offspring. The rodent gestational study (Chapter 4) showed reduced MAO activity in the brain-stem region of the adult female offspring, while no effect was observed in the male offspring. Ideally, the MAO activity should have been measured shortly after birth in order to evaluate the effects during this critical period of development. This study was designed to simultaneously evaluate offspring behavioural changes, and therefore MAO was only measured on PND 41. Information regarding temporal changes in MAO activity in the brain would greatly increase the understanding of this biochemical effect.

These animal studies were also limited by the power of the experimental design as a result of low sample size. Sample size for this study was based on the previous MeHg dosing study performed in adult rats (Chakrabarti et al. 1998).

In the study, the MAO inhibition in the adult rodent brains by MeHg exposure was more robust than in this gestational study. Therefore if the power of this study been increased, specific gender related effects could have been confirmed.

Finally, the rodents in this study had access to regular rodent chow diets, which may contain additional contaminants. It has been recently shown that rodent chow contains significant levels of MeHg and even organochlorines from the fish-meal components (Weis et al, 1995). It is a concern that these diets used in this study may contain sufficient background contaminant levels to influence the results of this animal study. Chow diets that do not include fish-meal would have been more appropriate for this gestational study. Future animal studies should be particularly aware of potential contaminants that may influence toxicity and subsequent biochemical endpoints.

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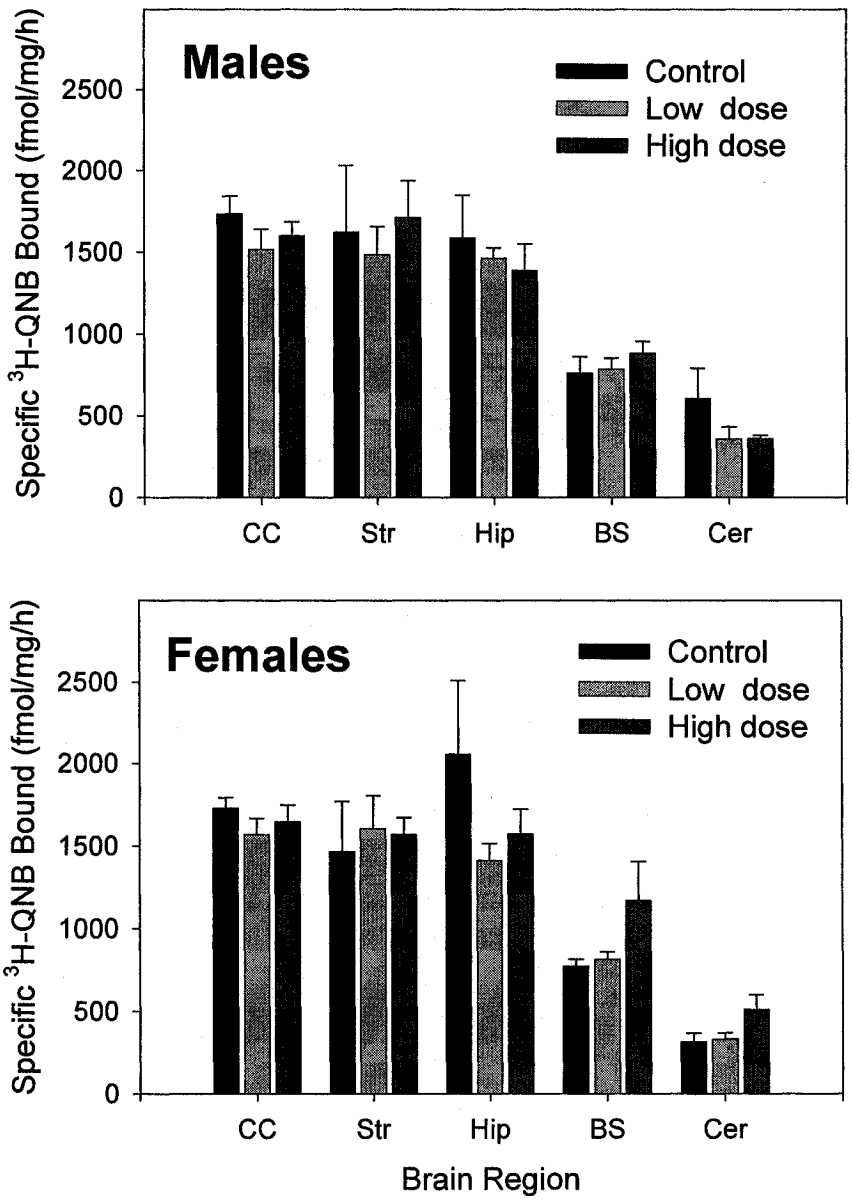
APPENDICES



Appendix A. Effects of MeHg exposure on mACh receptor binding in different brain regions of male and female offspring (PND 41). Each bar represents the mean ^{3}H-QNB bound ± SEM in male rats (n=5) and female rats (n=5) in the cerebral cortex (CC), striatum (Str), hippocampus (Hip), brain-stem (BS), and the cerebellum (Cer) of the dose groups. Brain region had an effect in females ($F(2,74)=29.0$, $p<0.001$) and in males ($F(2,74)=32.2$, $p<0.001$). No dose effect was observed.

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

McGill University
Animal Use Protocol – Research

Protocol #: 3929
Investigator #: 960
Approval End Date: MARCH 31, 2004
Facility Committee: AGR

Title: Dietary Efftcts of toxicity of environmental contaminants
(must match the title of the funding source application)

☐ New Application ☒ Renewal of Protocol # 3929 ☐ Pilot Category *(see section 11)*: B

1. Investigator Data:

Principal Investigator: Laurie Chan **Phone #:** 398-7765

Department: Dietetics and Human Nutrition **Fax#:** 398-1020

Address: CINE, MacDonald Campus, McGill University, 21111 Lakeshore Rd, Ste-Anne-de-Bellevue, Quebec H9X 3V9 **Email:** chan@macdonald.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Laurie Chan	**Work #:**	398-7765	**Emergency #:**	457-7388
Name:	Donna Leggee	**Work #:**	398-7588	**Emergency #:**	694-8649

3. Funding Source:

External ☒
Source (s): NSERC operating
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: Apr 2002 to Jun 2006

Internal ☐
Source (s): _____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: _____

For Office Use Only:

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): _____ or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): _____ or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: _____ **Date:** March 8 2003

Approved by:

Chair, Facility Animal Care Committee:		**Date:** 16/4/03
University Veterinarian:		**Date:** April 16 2003
Chair, Ethics Subcommittee (as per UACC policy):		**Date:**
Approved Animal Use	**Beginning:** April 1, 2003	**Ending:** MARCH 31, 2004

☐ This protocol has been approved with the modifications noted in Section 13.

Certificate of Ethical Acceptability for Research Involving Humans.

Project Title: An ecosystem approach to mercury and human health

Applicant's Name: Dr. Laurie Chan

Supervisor (if applicable):

Type of review: Full.

Decision: The applicant made extensive modifications to the original proposal (as requested by the REB). These were deemed satisfactory and on this basis

APPROVAL HAS BEEN GRANTED for

1)-with UQAM on the design and the administration of the food questionnaire and

2)-measuring of biomarkers.

Edmund S. Idziak
Chair
Research Ethics Board
Faculty of Agricultural and Environmental Sciences
December 19, 2001

REB #: 820-0603

McGill University
Environmental Safety

THIS IS TO CERTIFY THAT

Chris Stamler

HAS SUCCESSFULLY COMPLETED A BASIC

LABORATORY COURSE IN

RADIATION SAFETY

December 2001

Radiation Safety Officer
J. Vincelli

Manager, Environmental Safety
W. Wood, ROH

McGill University Internal Radioisotope Permit

20050125

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Permit Holder & Position	Building (Office)	Building (Lab)	Laboratory Classification		Date Issued
LAURIE CHAN, ASSOCIATE PROFESSOR	CINE BLDG. MACDONALD CAMPUS	CINE BLDG. MACDONALD CAMPUS	BASIC	**PLEASE POST**	2004/09/01
Department	Room Number(s) / Telephone	Room Number(s) / Telephone			Expiry Date
DIETETICS AND HUMAN NUTRITIAN	398-7765	100 398-7588			2005/08/31

PERSON(S) APPROVED TO WORK WITH RADIOISOTOPES

Name	Train.	Cond(s)	Class(es)	Radioisotope(s)
LAURIE CHAN, ASSOC. PROFESSOR	N	4	5	Ni-63
CHRISTOPHER STAMLER, GRADUATE STUDENT	Y	4	4	H-3
JIUN NI LIU, GRADUATE STUDENT	N	4	5	Ni-63
JOANNE POIRIER, GRADUATE STUDENT	N	4	5	Ni-63
DONNA LEGGEE, RESEARCH ASSISTANT	Y	4	5	Ni-63
NILADRI BASU, GRADUATE STUDENT	Y	4	4	H-3
KIMBERLY BULL, GRADUATE STUDENT	Y	4	4	H-3
ELLEN LYE, GRADUATE STUDENT	N	4	4	H-3

GENERAL LICENCE CONDITIONS (OPEN AND/OR SEALED SOURCES)

- The permit must be posted with the CNSC safety poster in the permit holder's premises.
- Radioisotope handling shall be in accordance with the McGill Radiation Safety Policy Manual.
- The permit holder must ensure that all persons mandated to work with radioisotopes be properly trained in radiation safety prior to start of work.
- Radioactive work areas must be clearly identified with radiation warning signs.
- Smoking, eating, drinking, storage of foods or drink and the application of cosmetics and contact lenses are prohibited in areas where radioisotopes are used.
- All procedures involving radioactive materials should be carried out on spill trays or on benches lined with disposable absorbent material.
- Procedures that might produce airborne radioactive contamination should be carried out in a functioning fume hood.
- When hand or clothing contamination is possible, protective gloves and clothing must be worn.
- After handling radioactive material and especially before leaving the laboratory, personnel must ensure that all parts of their cloths are not contaminated.
- Purchase and disposal of radioisotopes must be kept electronically or documented in a log book.
- For disposal of radioactive waste, consult the McGill Radiation Safety Policy Manual and/or McGill WMP.
- Wipe tests must be performed and records be kept in a log book.
- The permit must reflect the exact conditions under which radioactive material is used. If changes must be made, contact the RSO at 398-1538.
- The device(s) containing the sealed source(s) must have a radiation symbol and an identification label bearing the name and telephone number of the permit holder.
- Leak tests must be performed on sealed sources equal to or greater than 50 MBq (1.35mCi).
- Extremity dosimeter (i.e. ring or wrist badges) must be worn if 50 MBq or more of P-32, Sr-89, Sr-90 & Y-90 are used.
- Workers using I-125 or I-131 on open bench (5 MBq), in a fume hood (50 MBq) or a vented glove box

Approved Unsealed Radioisotope(s) and Location(s)

Isotope	Possession Limit	Stored	Handled
H-3	< 40 MBq (1.1 mCi)	MS2-002	MS2-002, MS1-125

Approved Sealed Radioisotope(s) and Location(s)

Permanently Housed Source(s)				*Accessible Source(s)*			
Isotope	Activity	Stored	Handled	Isotope	Activity	Stored	Handled
Ni-63	15 mCi	108	108				

Personnel Conditions

1 .Must attend thyroid bioassays within 5 days of use if 50 MBq (1.35 mCi) of I-125 are manipulated in a fume hood.
2 .Must wear a whole-body film badge, if gamma, x-ray or high energy beta emitters are used.
3 .Must wear an extremity TLD dosimeter, if more than 50 MBz (1.35 mCi) of P-32, Sr-89, Sr-90 or Y-90 are used.
4 .Classified as Radiation User.
5 .Classified as Nuclear Energy Worker (NEW).
6 .Does not work with any radioisotopes but may be indirectly exposed.

Workload Classes

1 .Work load < 10MBq (270 uCi) of unsealed radioisotopes in open areas.
2 .Work load < 10MBq (270 uCi) of unsealed radioisotopes in a fume hood.
3 .Work load > 10MBq (270 uCi) of unsealed radioisotopes in open areas.
4 .Work load > 10 MBq (270 uCi) of unsealed radioisotopes in a fume hood.
5 .Work with sealed sources.
6 .Individual does not work with radioactive sources but normal working conditions involve presence in a room where radioactive material is used or stored.

Joseph Vinceiti
RSO & Occupational Hygienist
McGill Environmental Health & Safety

Date

For: Dr. Ian Butler, Chairperson
McGill University Laboratory Safety Committee
Associate Vice-Principal (Research)

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

project should be commenced without prior approval of an application to use biohazardous materials. Submit this plication to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved plication.

PRINCIPAL INVESTIGATOR: Laurie Chan

ADDRESS: CINE building, Macdonald Campus

TELEPHONE: 7765

FAX NUMBER: 1020

DEPARTMENT: Dietetics and Human Nutrition

E-MAIL: Laurie.Chan@mcgill.ca

PROJECT TITLE: Dietary Effects of toxicity of environmental contaminants

2. FUNDING SOURCE: MRC ☐ NSERC ☑ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify)

Grant No.: 154270-02 Beginning date: May 2002 End date March 2006

3. Indicate if this is

☑ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: SIGNATURE date: 16 (day) April (month) 2002 (year)

Chairperson, Biohazards Committee: SIGNATURE date: 18 (day) 04 (month) 02 (year)

Approved period: beginning 18 (day) 04 (month) 02 (year) ending 31 (day) 03 (month) 06 (year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

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REPUBLICATION PERMISSION AGREEMENT AND INVOICE # J1727

June 2, 2005

Christopher Stamler
CINE, McGill University
21,111 Lakeshore Road
Ste-Anne-de-Bellevue
Canada H9X 3V9

Selection: **Biochemical Markers of Neurotoxicity in Wildlife and Human Populations: Considerations for Method Development**

From: **Journal of Toxicology and Environmental Health, 2005**

Within the following publication: **Ph.D. Thesis**

Publisher: **McGill University**

Pub date: **2005**

Format(s): Print:☒ CD-ROM:☐ E-Book:☐ Web Site:☐ Elec Storage: ☐

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Christopher Stamler — June 2/05

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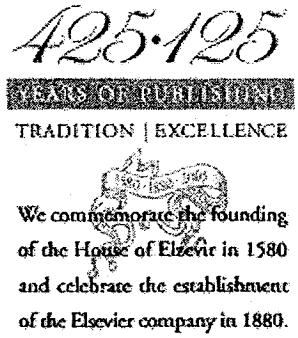

20 May 2005

Our ref: HG/smc/May 2005.jl124

Mr Christopher Stamler
Chris.stamler@mail.mcgill.ca
Dear Mr Stamler

Chapter 6: TOXICOLOGY AND APPLIED PHARMACOLOGY, 2005, Basu et al, 'An inter-species comparison of ...', (in press)

As per your letter dated 26 April 2005, we hereby grant you permission to reprint the aforementioned material at no charge **in your thesis** subject to the following conditions:

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Helen Gainford, Rights Manager

May 15th, 2005

To Whom It May Concern:

This letter is to confirm that the co-authors (Peter Beyrouty, Jiun-Ni Liu, Dr. Stan Kubow and Dr. H.M. Chan) agree that the Ph.D. candidate (Christopher Stamler) may include the following manuscript in his thesis. The manuscript entitled, *"Methylmercury reduces monoamine oxidase activity in rat embryos and offspring"* was a collaborative research project.

May 15/05

Christopher Stamler

I, the co-author agree that the candidate, Christopher Stamler, include this manuscript in his thesis.

* See attached e-mail pg 142

Peter Beyrouty _____ date

Jiun-Ni Liu _____ May 31, 2005 date

Dr. Stan Kubow _____ July 25, 2005

Dr. Hing Man Chan _____ May 25, 2005 date

Date: Fri, 10 Jun 2005 13:47:55 -0700 (PDT)
From: peter bey <peter_c_bey@yahoo.com>
To: Chris Stamler <chris.stamler@MAIL.MCGILL.CA>
Subject: Re: Thesis Chapter 4: agreement

2 unnamed text/html 4.35 KB

Sure Chris, go ahead and include MAO data.

Chris Stamler <chris.stamler@mail.mcgill.ca> wrote:

Hi Peter,

As we discussed over the phone, I will be submitting my Ph.D thesis as a "manuscript based thesis". McGill now requires e-mail confirmation that you (as a co-author) are okay that I include our co-authored work as a chapter in my thesis.

So therefore this letter is to confirm that the co-authors (Peter Beyrouty, Jiun-Ni Liu, Dr. Stan Kubow and Dr. Hing Man Chan) agree that the Ph.D. candidate (Christopher Stamler) may include the following manuscript in his thesis. The third manuscript (Chapter 4), entitled *"Methylmercury reduces monoamine oxidase activity in rat embryos and offspring"* will be included as Chapter 4 in my Ph.D. thesis.

Please reply to this e-mail, indicating that you agree that I include our co-authored manuscript in my thesis.

Thanks in advance for your cooperation.

Christopher Stamler

May 15th, 2005

To Whom It May Concern:

This letter is to confirm that the co-authors (Nadia Abdelouahab, Claire Vanier and Donna Mergler) agree that the PhD candidate (Christopher Stamler) include the following manuscript in his thesis.

The manuscript entitled, "Mercury exposure and peripheral biochemical markers in fish consumers from the Lac St-Pierre Region, Quebec, Canada" is co-authored by C. Stamler, N. Abdelouahab, C. Vanier, D. Mergler and H.M. Chan.

May 15/05

Christopher Stamler

I, the co-author agree that the candidate, Christopher Stamler, can include this manuscript in his thesis.

Nadia Abdelouahab Date

Claire Vanier 26/05/05 Date

Dr. Donna Mergler 26/05/05 Date

May 25. 2005

Dr. Hing Man Chan Date