EFFECTS OF MERCURY ON NEUROCHEMICAL RECEPTOR-BINDING CHARACTERISTICS IN WILD MINK

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(Received 28 January 2004; Accepted 30 November 2004)

Abstract—Piscivorous wildlife, such as mink (Mustela vison), routinely are exposed to mercury (Hg) in their natural environment at levels that may cause adverse behavioral outcomes. The purpose of this study was to determine if a correlation exists between neurochemical receptors and concentrations of Hg in the brains of wild mink. Specifically, receptor-binding assays were conducted to characterize the muscarinic cholinergic (mACh) and dopaminergic-2 (D2) systems in brain tissues collected from mink trapped in the Yukon Territory, Ontario, and Nova Scotia (Canada), and values were correlated with total Hg and methyl Hg (MeHg) concentrations in the brains. A significant correlation was found between Hg (total Hg and MeHg) and mACh receptor density (r = 0.546; r = 0.596, respectively) or ligand affinity (r = 0.413; r = 0.474, respectively). A significant negative correlation was found between total Hg and D2 receptor density (r = −0.340) or ligand affinity (r = −0.346). These correlations suggest that environmentally relevant concentrations of Hg may alter neurochemical function in wild mink, and that neurochemical receptor-binding characteristics can be used as a novel biomarker to assess Hg’s effects on wildlife. Given the importance of the muscarinic cholinergic and dopaminergic pathways in animal behavior, further studies are required to explore the physiological and ecological significance of these findings.

Keywords—Wildlife toxicology Neurotoxicology Biomarkers Mercury Mink

INTRODUCTION

Mercury (Hg) is a ubiquitous neurotoxicant and naturally occurring element that exists in multiple allotropic forms [1,2]. Methylmercury (MeHg), the primary organic species of Hg, can readily traverse biological membranes and biomagnify through the aquatic food chain. Consequently, MeHg is a potential risk to high trophic-level piscivorous wildlife, such as mink (Mustela vison) [3]. Historical declines of some wild populations of mink are thought to be associated with Hg exposure [4,5]. Additionally, controlled feeding experiments have demonstrated that ranch mink ingesting as little as 1 µg/g dietary MeHg display clinical signs of toxicity, such as reproductive impairment [6], behavioral changes [7,8], and lethality [7,8]. The effects of Hg on wildlife and ecosystem health is a growing concern because concentrations of Hg measured in the brains of wild mink (range: 0.11–13.4 µg/g wet wt total Hg) [9–11] generally are within one order of magnitude of concentrations that may cause ill effects (i.e., greater than 5 µg/g wet wt total Hg) [7,12], and global concentrations continue to rise due to industrial activities and long-range atmospheric transport [13–16].

To understand the physiological and ecological risks associated with Hg exposure, feeding trials have been conducted in a variety of avian and mammalian wildlife species [12,17–19]. The endpoints tested in a majority of these studies included bioindicators of health that may be classified as irreversible, such as ataxia and brain lesions. Although these data increase our basic understanding of Hg toxicity, there is a need to develop specific biomarkers to predict the adverse risks associated with chronic exposure to low concentrations of Hg by individuals and populations. Assuming that molecular events at the cellular level precede functional impairments at the organ level, monitoring biochemical changes in the nervous system represents a unique tool to predict possible neurobehavioral outcomes associated with Hg exposure in organisms [20–22].

The ability of wildlife to survive in the environment requires a functional neurological signaling pathway whereby the animal can receive, process, and store information. Two major pathways in neurotransmission are the cholinergic and dopaminergic systems that play critical roles in cognition, somatosensory, and motor function [23,24]. Rodent studies have shown that Hg can disrupt multiple aspects of these pathways including the synthesis, storage, or release of neurotransmitters [25–29], receptor-binding events [30–32], and re-uptake or clearance mechanisms [25,26,28]. Furthermore, studies on fish have shown that exposure of animals to pesticides and heavy metals altered components of the cholinergic [33] and dopaminergic [34] system, which were related to behavioral outcomes. Collectively, these findings suggest that prolonged disruptions in neurotransmission ultimately may alter animal be-
behavior and lend support to the idea that neurochemical changes may be used as biomarkers to study the neurotoxic effects of Hg and other toxicants on wildlife.

Recently, we have demonstrated the versatility and discussed the applications of receptor-binding characteristics to study neurochemistry in epidemiological studies of humans and wildlife [35]. The purpose of the present study is to investigate if there is a correlation between neurochemistry and brain Hg concentrations in wildlife. Specifically, muscarinic cholinergic (mACh) and dopaminergic (D2) receptor-binding characteristics were measured in whole brains of mink collected from different geographical regions of Canada. Receptor data were correlated with concentrations of brain Hg (total and MeHg) to test the following null hypothesis: There is no association between Hg exposure and neurochemical function in wild animals.

METHODS

Chemicals

Radioligands, [3H]-quinuclidinyl benzilate ([3H]-QNB; 42 Ci/mmol) and [3H]-spiperone (15.7 Ci/mmol), were obtained from NEN/Perkin-Elmer (Boston, MA, USA). Atropine, bovine serum albumin, (+)-butaclamol, ketanserin, and polyethylenimine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample collection

Animals were collected from licensed trappers during the 2002 to 2003 trapping season from the Yukon Territory (Watson Lake), Southern Ontario (Peterborough and Parry Sound areas), and Nova Scotia, Canada. The gender of each animal was noted and all carcasses were stored at −20°C until processed. The lower jaw was removed to age each animal using cementum annuli readings (Matson’s Laboratory, Milltown, MA, USA).

Hg analysis

Concentrations of total Hg and MeHg were quantified as described by Scheuhammer et al. [36]. To quantify total Hg, approximately 0.35 g of freeze-dried brain tissue was digested overnight in concentrated nitric acid, heated for 5 h at 105°C, and diluted eightfold with distilled water. For MeHg analysis, acidic sodium bromide was used to extract the MeHg from a tissue sample into toluene. The Hg complex then was reverse-extracted into the aqueous phase as a thiosulfate conjugate, and this sample was digested and stored in a mixture of strong acids. Concentrations of total Hg and MeHg were measured in the digests using cold vapor atomic absorption spectrophotometry (Hitachi Atomic Absorption Spectrophotometer model Z8200, Tokyo, Japan) at a wavelength of 253.7 nm. Certified reference materials (Dogfish Muscle Certified Reference Material for Trace Metals [DORM-2]), Analytical Chemistry Unit, National Research Council, Ottawa, ON, Canada) and sample blanks were included in all analyses for quality control purposes, and all data are expressed as dry weight concentrations, unless otherwise indicated.

Preparation of brain membranes

Brains were excised from each animal and stored at −80°C until membranes were prepared. Frozen tissues were homogenized for 30 s in ice-cold NaK buffer (50 mM NaH2PO4, 5 mM KCl, 120 mM NaCl, pH 7.4). The homogenate was centrifuged at 16,500 g for 15 min at 4°C, and the resulting pellet was washed twice under the same conditions. The final pellet was resuspended in NaK buffer and aliquots were frozen immediately in liquid nitrogen and stored at −80°C until required. The concentration of protein in the membrane preparation was determined with the Bradford assay [37] using bovine serum albumin as the standard.

mACh receptor-binding assay

One hundred micrograms of membrane preparation were preincubated in NaK buffer for 30 min at 25°C in duplicate. Samples then were mixed with various concentrations (0.01–3.2 nM) of [3H]-QNB, a mACh receptor-specific radioligand, for 60 min at 25°C under constant agitation. The incubation was terminated by rapid vacuum filtration through 1.2-μM glass fiber filters (Millipore, Boston, MA, USA). The filters were washed three times with 3 ml of ice-cold NaK buffer and placed into glass vials. The filters were allowed to dissolve overnight in 5-ml liquid scintillation cocktail (ICN Biomedicals, Aurora, OH, USA). Radioactivity retained by the filters was quantified by a liquid scintillation counter (LKB Wallac 1209 Rackbeta, Turku, Finland) with approximately 68% counting efficiency. Specific binding was defined as the difference in [3H]-QNB bound in the presence and absence of 100 μM atropine, and the total volume in each tube was 1 ml. To reduce nonspecific binding of the radioligand to the filters, filters were soaked for 30 min in 0.1% (weight/volume) polyethyleneimine before use.

D2 receptor-binding assay

The receptor-binding assay for the D2 receptor was modified for a 96-well microplate filter system. Twenty micrograms of membrane preparation were preincubated in Tris buffer (50 mM Tris, 5 mM KCl, 2 mM MgCl2, pH 7.4) for 30 min at 25°C in triplicate. Samples then were mixed with various concentrations (0.1–5.6 nM) of [3H]-spiperone, a D2-specific radioligand, for 90 min at 25°C under constant agitation. The incubation was terminated by rapid vacuum filtration through 1.0-μM glass fiber filters (Millipore). Filters were washed three times with 200 μl Tris buffer and placed into glass scintillation vials. The filters were allowed to dissolve overnight in 5-ml liquid scintillation cocktail. Radioactivity retained by the filters was determined as described earlier. Specific binding was defined as the difference in [3H]-spiperone bound in the presence and absence of 100 μM atropine, and the total volume in each tube was 1 ml. To reduce nonspecific binding of the radioligand to the filters, filters were soaked for 30 min in 0.5% (weight/volume) polyethyleneimine before use, and 50 μM ketanserin (5-HT2 receptor antagonist) was added to each well to prevent binding of [3H]-spiperone to 5-HT2 receptors.

Statistical analysis

The critical significance value for all statistical analyses was set at α = 0.05. All data are represented as mean ± standard deviation. Data from all receptor-binding studies were curve-fitted using GraphPad Prism (Ver 3.02, GraphPad Software, San Diego, CA, USA) to calculate receptor density (Bmax) and ligand affinity (Kd). To minimize the sum of squares, an F-test determined that mACh binding was best fit with a rectangular hyperbolic equation and D2 binding was best fit with a three-parameter logistic equation.

Mercury (total and MeHg) data were log-transformed for statistical analysis (SigmaStat Ver 2.03, SPSS, San Rafael, CA,
**RESULTS**

**Hg analysis**

Concentrations of total Hg were measured in two independent laboratories at McGill University (Montreal, QC, Canada) and Trent University (Peterborough, ON, Canada). The mean difference in values was 6.6 ± 4.8% and the cold-vapor atomic absorption spectrophotometry detection limit was 1 μg/L Hg. Mean recovery of Dogfish Muscle Certified Reference Material for Trace Metals (DORM-2) standard reference material was 94.0 ± 4.9% and 93.4 ± 4.3% of the expected value for total Hg and MeHg, respectively. Concentrations of total Hg in the mink brain ranged between 0.27 and 18.84 μg/g and concentrations of MeHg ranged between 0.26 and 13.52 μg/g (Table 1). Concentrations of Hg (total and MeHg) were significantly (p < 0.001) higher in Nova Scotia samples compared to those collected from Ontario and the Yukon Territory (Table 1). Methylmercury was measured only at McGill University and accounted for 88.8 ± 15.4% of the total Hg (Table 1). The relationship between total Hg and MeHg was significant (r = 0.966, p < 0.0001, n = 44). Mean moisture content of brain tissue was 74.6 ± 1.2%. No effect of age or gender was found on the concentrations of brain Hg.

**Receptor-binding characteristics**

Analysis of all mACh receptor-binding data revealed a mean receptor density and ligand affinity of 721.5 ± 227.2 fmol/mg protein and 0.11 ± 0.02 nM, respectively, with highest values recorded in the samples from Nova Scotia (Table 1). Non-specific binding, as determined by incubation of samples with atropine was less than 5% of total binding at 1 nM [³H]-QNB. Analysis of all D2 receptor-binding data revealed a mean receptor density and ligand affinity of 112.2 ± 32.8 fmol/mg/protein and 1.64 ± 0.33 nM, respectively (Table 2).

Although significantly higher D2 receptor density was measured in samples from the Yukon Territory, there were no significant differences in D2 ligand affinity among mink collected from different regions. Non-specific binding, as determined by incubation of samples with (+)-butaclamol, was 50 to 55% of total binding at 1.8 nM [³H]-spiperone. No effects of age or gender were found on mACh and D2 receptor-binding characteristics.

**Correlation of Hg with receptor-binding characteristics**

A significant positive correlation was found between total Hg and mACh receptor density (r = 0.546, p < 0.0001, n = 47; Fig. 1A) and ligand affinity (r = 0.413, p < 0.05, n = 47; Fig. 2A). Similar to total Hg, a significant positive correlation was found between brain MeHg and mACh receptor density (r = 0.596, p < 0.0001, n = 43; Fig. 1B) and ligand affinity (r = 0.474; p < 0.001, n = 43; Fig. 2B).

Contrary to the mACh receptor data, a significant negative correlation was found between total Hg and D2 receptor density (r = −0.340, p < 0.05, n = 48; Fig. 3A) and ligand affinity (r = −0.346, p < 0.05, n = 48; Fig. 4A). Correlation of MeHg with D2 receptor density (Fig. 3B) and ligand affinity (Fig. 4B) also was negative, but this association was not statistically significant (p > 0.05).

**DISCUSSION**

Multiple studies have demonstrated that piscivorous wildlife are exposed to Hg via their natural diet, but little is known regarding the physiological and ecological consequences of this long-term exposure. The major finding of this study is that significant differences in mACh and D2 receptor-binding characteristics (receptor density and ligand affinity) can be correlated to concentrations of Hg (total and MeHg) in the brains of wild mink collected across Canada (Figs. 1–4), thus rejecting our null hypothesis. Given the importance of the cholinergic and dopaminergic systems in cognitive processes and motor function, prolonged alterations in receptor properties may precede, and even be used to predict, adverse changes in neurobehavior and animal health.

Concentrations of brain Hg (total and MeHg) measured in this study correspond well with previously published values for mink collected in Ontario [11,38] and Yukon Territory [39]. This is the first report to publish brain Hg concentrations in mink collected from Nova Scotia (Table 1). Significantly higher concentrations of brain Hg (total and MeHg) were measured across the study regions. Analysis of covariance was conducted to examine the relationship between Hg and receptor-binding characteristics among the study regions. Analysis of covariance was conducted to evaluate if any effects were related to age or gender of mink.

**Table 1.** Mean (±standard deviation) values of Hg levels in mink brains collected across Canada, 2002 to 2003. Values are expressed as a dry weight concentration assuming brain moisture content of 74.6 ± 1.2%.

<table>
<thead>
<tr>
<th>Sampling region</th>
<th>Total Hg (μg/g dry wt)</th>
<th>MeHg (μg/g dry wt)</th>
<th>MeHg (% of total Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nova Scotia</td>
<td>5.7 ± 5.2</td>
<td>4.9 ± 4.1</td>
<td>90.0 ± 14.9</td>
</tr>
<tr>
<td>Ontario</td>
<td>1.4 ± 0.6</td>
<td>1.2 ± 0.7</td>
<td>86.8 ± 19.7</td>
</tr>
<tr>
<td>Yukon Territory</td>
<td>1.2 ± 0.8</td>
<td>1.1 ± 0.8</td>
<td>86.8 ± 14.1</td>
</tr>
</tbody>
</table>

* Significant (p < 0.05) differences among groups.

**Table 2.** Mean (±standard deviation) values of neurochemical receptor-binding characteristics (Bmax = receptor density; Kd = ligand affinity) in brain membrane preparations from mink collected across Canada, 2002 to 2003.

<table>
<thead>
<tr>
<th>Sampling region</th>
<th>Muscarinic cholinergic receptor-binding characteristics</th>
<th>Dopamine-2 receptor-binding characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bmax (fmol/mg protein)</td>
<td>Kd (nM)</td>
</tr>
<tr>
<td>Nova Scotia</td>
<td>1,269.9 ± 378.1Aa</td>
<td>0.12 ± 0.02A</td>
</tr>
<tr>
<td>Ontario</td>
<td>598.9 ± 203.4</td>
<td>0.11 ± 0.02AB</td>
</tr>
<tr>
<td>Yukon Territory</td>
<td>550.6 ± 88.8</td>
<td>0.09 ± 0.01B</td>
</tr>
</tbody>
</table>

*A, B = significant (p < 0.05) differences among groups.*
Fig. 1. Relationship between cholinergic muscarinic acetylcholine (mACh) receptor density (Bmax) and levels of total Hg (A) and MeHg (B) in brains of wild mink (Mustela vison) collected from Nova Scotia (V), Ontario (O), and the Yukon Territory (□) in Canada during 2002 to 2003; d.w. = dry weight.

Fig. 2. Relationship between cholinergic muscarinic acetylcholine (mACh) receptor ligand affinity (Kd) and levels of total Hg (A) and MeHg (B) in brains of wild mink (Mustela vison) collected from Nova Scotia (V), Ontario (O), and the Yukon Territory (□) in Canada during 2002 to 2003; d.w. = dry weight.

in mink from Nova Scotia, relative to the Yukon Territory and Ontario, which can be attributed to both the geochemistry of this region [40] and its proximity to industrial point sources that emit Hg [3,41]. Wildlife inhabiting low-alkaline regions, such as Nova Scotia, generally have higher Hg tissue burdens because methylation of inorganic Hg is enhanced under acidic conditions [15]. Mean concentrations of brain MeHg, as a percentage of total Hg, were consistent across the study regions (Table 1) and support previous observations that mink have a limited capacity to de-methylate Hg compared to other piscivorous wildlife [11,42].

Historical reports of Hg poisoning [5,9] and controlled feeding studies [6–8,43] have demonstrated that mink are sensitive to chronic Hg exposure [44]. Given that average concentrations of Hg in North American mink are within one order of magnitude of concentrations measured in severely poisoned mink [3], and MeHg concentrations in many aquatic ecosystems may exceed the U.S. Environmental Protection Agency’s (U.S. EPA) derived mammalian wildlife criteria for mink (57 pg MeHg/L) [19], there is a need to explore the subtle effects associated with Hg exposure in the natural environment. After reviewing the available literature, the U.S. EPA [3] recommended a lowest-observable-adverse-effects level of 1.1 μg/g dietary MeHg. This criterion largely was derived from observations that mink fed this ration had brain concentrations of 7.1 to 9.3 μg/g wet weight Hg and resulting neuronal lesions [7]. Others [12] have suggested that brain concentrations of 5 μg/g wet weight MeHg (~19.7 μg/g dry weight MeHg, assuming moisture content of brain to equal 74.6%), may be low enough to cause subtle neurological effects. However, our data demonstrate that significant neurochemical changes exist in wild mink (Figs. 1–4), and levels of Hg (total and MeHg) measured in the brains of these animals were below the concentrations proposed by the U.S. EPA [3] and Wolfe et al. [12] that may cause adverse effects.

Despite several decades of research on the ecotoxicological effects of Hg, the only acceptable biomarker for Hg is to quantify exposure by measuring concentrations of Hg in blood, fur, or organs. Although this type of information is necessary for risk assessment, it does not provide much information about the cellular changes that precede functional impairment. A major limitation in wildlife studies is the rapid postmortem degradation of cellular components, such as enzymes and genes, which may be used as possible biomarkers of Hg effect. We have demonstrated recently the versatility of neurochemical receptors in field-based wildlife studies because receptor-binding characteristics were minimally affected by tissue storage temperatures and multiple freeze thaw cycles [35]. These findings, in addition to the results from the current study, lend support to the idea that neurochemical receptor-binding char-
characteristics are a novel biomarker to assess Hg’s neurotoxic effects in wildlife.

It is well-established that Hg can alter neurobehavior in wildlife [12,17,19], but little is known about the mechanisms that mediate these physiological changes. The proper transmission of signals between the animal’s external environment and its nervous system is necessary for survival. Mercury is a nonspecific cytotoxic compound [45,46] and rodent studies have shown that organic and inorganic Hg can impair various aspects of neurotransmission. For example, laboratory rats [47] and mice [48] exposed to MeHg had decreased concentrations of brain acetylcholine, the primary agonist of the mACh receptor. Reduced acetylcholine levels, as a result of MeHg exposure, are supported by mechanistic studies demonstrating that MeHg can suppress the activity of choline acetyltransferase [25,26], inhibit the voltage-gated entry of acetylcholine into pre- and postsynaptic nerve endings [49,50], and impair the binding of [3H]-QNB to the mACh receptor [31]. Because Hg may reduce the cellular pool of acetylcholine, up-regulation of the mACh receptor in Hg-exposed mink (Figs. 1 and 2) may represent an adaptive response by these animals to ensure that cholinergic neurotransmission occurs within a normal physiological range. However, the duration an animal can sustain changes in neurochemistry needs to be studied because behavioral changes (e.g., impaired ability to hunt, breed, migrate) may become evident once this latency period is exceeded.

In contrast to the muscarinic cholinergic receptor data, a negative correlation was calculated between Hg and D2 receptor-binding characteristics (Figs. 3 and 4). Mercury has been demonstrated to affect the dopaminergic system in laboratory animals by causing a net increase in cellular levels of dopamine as a result of decreased monoamine oxidase activity [26,51], increased tyrosine hydroxylase activity [25], and spontaneous release of dopamine from neurons [27,29]. In the field, larval mummichogs (Fundulus heteroclitus) residing in Hg-contaminated waters had higher levels of dopamine, relative to controls [34]. Knowing that Hg can increase cellular dopamine levels, down-regulation of D2 receptors in high Hg-exposed mink may represent an adaptive mechanism to prevent the hyperstimulation of the dopaminergic system by the animal.

In conclusion, significant Hg-related changes in neurochemistry are measurable in wild mink collected across Canada, and these changes occur at concentrations of brain Hg below values known to cause adverse clinical effects. Because mink are exposed routinely to potentially harmful concentrations of Hg in their natural environment and a functional neurological signaling pathway is essential for animal behavior...
and survival, further studies are required to resolve the physiological and ecological significance of these neurochemical changes to individuals and populations. These data also demonstrate that receptor-binding characteristics represent a novel tool to assess the ecotoxicological risks of Hg.

Acknowledgement—This work was supported by operating grants from the Collaborative Mercury Research Network, Natural Sciences and Engineering Research Council of Canada to H.M. Chan and A.M. Scheuhammer. Niladri Basu was funded by a Natural Sciences and Engineering Research Council and Hydro-Quebec postgraduate fellowship. The authors are grateful to the Ontario Fur Managers Federation, Nova Scotia Trappers Association, and the Yukon Territory trappers for providing samples, and to D. Legge, C. Stamler, M.J. Boudreau, H. Broadbent, L. Tivoli, and K. Marcel Loua for technical assistance and critical discussion.

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