MERCURY BUT NOT ORGANOCHLORINES INHIBITS MUSCARINIC CHOLINERGIC RECEPTOR BINDING IN THE CEREBRUM OF RINGED SEALS (PHOCA HISPIDA)

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Elevated concentrations of organochlorines and mercury (Hg) have been reported in marine mammals on a global scale. While risk assessments are generally based on quantifying body burdens of toxicants, much less is known about associated adverse health effects and their underlying mechanisms. The purpose of this study was to characterize the inhibitory effects of methylmercury (MeHg⁺), mercuric chloride (Hg²⁺), p,p’-DDT, Aroclor 1254, chlordane, dieldrin, lindane, and toxaphene on [³H]quinuclidinyl benzilate ([³H]-QNB) binding to the muscarinic cholinergic (mACh) receptor in cellular membranes isolated from the cerebrum of ringed seals (Phoca hispida). [³H]-QNB binding to the mACh receptor was saturable with a mean receptor density (Bmax) of 826.9 ± 68.4 fmol/mg and ligand affinity (Kd) of 0.31 ± 0.04 nM. MeHg⁺ and Hg²⁺ were the only neurotoxicants that inhibited radioligand binding by greater than 50%. Hg²⁺ was significantly more potent at inhibiting mACh receptor binding than MeHg⁺ when the IC50 data were compared (IC50 = 1.92 ± 0.06 µM versus 2.75 ± 0.22 µM), but when the data were normalized to derive inhibition constants (Ki) there was no statistical difference in inhibition (Hg²⁺ = 1.38 ± 0.07 µM; MeHg⁺ = 1.26 ± 0.12 µM). Toxaphene also inhibited mACh receptor binding by 22.4%, but this was only significant at the highest concentration tested (320 µM). Overall, these data suggest that Hg, and not organochlorines, inhibits ligand binding to the mACh receptor. These mechanistic findings may be used to support and develop specific biomarkers of Hg exposure and neurotoxicity in sensitive ecological species.

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INTRODUCTION

Ringed seals (*Phoca hispida*) are key marine mammal species that inhabit aquatic ecosystems in northern circumpolar regions (Smith, 1987). Due to their high trophic status, piscivorous diet, and long life span, they can bioaccumulate relatively high concentrations of mercury (Hg) and persistent organic pollutants, such as organochlorines (Wagemann & Muir, 1984; Muir et al., 1999; Muir & Norstrom, 2000; Troisi et al., 2001; Nyman et al., 2002). Accordingly, such exposures have been associated with adverse health outcomes in this species (Hutchinson & Simmonds, 1994; Colborn & Smolen, 1996), including reproductive impairment (Helle et al., 1976; Helle, 1980; Reijnders, 1986), pathological lesions (Helle et al., 1976; Bergman et al., 2001), skeletal deformities (Zakharov & Yablokov, 1989), and biochemical changes (Brouwer et al., 1989; Nyman et al., 2001, 2003). While most research efforts have been directed toward quantifying tissue residues or assessing endocrine and/or reproductive effects, little is known regarding possible neurological effects associated with chronic exposure to environmental toxicants. This is of particular concern since most of the pollutants that ringed seals bioaccumulate are potent neurotoxicants (National Research Council, 1992; Tilson & Kodavanti, 1998; Castoldi et al., 2001).

Given the inherent complexities in assessing wildlife behavior, there is heightened interest in evaluating neurological function by characterizing the underlying neurochemistry (Manzo et al., 2001; Stamler et al., 2005). The cholinergic system is a major neurotransmission pathway with involvement in motor, behavioral, cognitive, sensory, and autonomic functions (Wess, 1996, 2004). Rodent studies demonstrated that components of the cholinergic pathway are susceptible to environmental toxicants. For example, enzymes involved in the synthesis (choline acetyltransferase) and degradation (acetylcholinesterase) of acetylcholine, the primary neurotransmitter in cholinergic signaling, have been inhibited by Hg and organochlorines (Hastings et al., 1975; Tsuzuki, 1981; Chaturvedi, 1993; Lakshmana et al., 1993; Sahoo et al., 1999). Synaptic transmission can also be impaired by pollutants at the level of the cholinergic receptor, as previous studies have shown that methylmercury (MeHg⁺) and mercuric chloride (Hg²⁺) can prevent ligand binding to the muscarinic cholinergic (mACH) receptor in a range of species (Von Burg et al., 1980; Castoldi et al., 1996; Basu et al., 2005a). The mACH receptor is a cell surface receptor that is present in relatively high concentrations throughout the mammalian central nervous system (Wess, 1996). Because they are coupled to G-proteins, mACH receptors mediate a variety of important physiological processes, and alterations in receptor biochemistry have been related to neurobehavioral deficits (e.g., motor coordination and thermoregulation) and morbidity (e.g., Alzheimer’s disease, dementia) (Wess, 1996, 2004).

The derivation of toxicant-specific, exposure-response data for sensitive species, such as marine mammals, is desirable but challenging. Most risk assessments are based on tissue residue data combined with adverse health
effects that are largely extrapolated from laboratory rodent studies. This approach relies heavily on uncertainty factors and is limited by interspecies differences. For example, cetaceans have a reduced ability to metabolize organochlorines relative to terrestrial mammals (Boon et al., 1997).

Accordingly, better model systems are required to understand the mechanisms that underlie toxic outcomes in at-risk ecological species. Biomedical laboratories often characterize cellular pathways by isolating cells or tissues from laboratory animals (Tiffany-Castiglioni et al., 1999), but such in vitro strategies are underutilized in the field of environmental toxicology. To determine whether environmental pollutants of concern to marine mammals have the potential to impair cholinergic neurotransmission, this study was undertaken to test the hypothesis that MeHg\(^+\), Hg\(^{2+}\), Arochlor 1254, \(p,p'\)-DDT, chlordane, dieldrin, lindane, and toxaphene can inhibit mACh receptor binding in cellular membranes isolated from the cerebrum of ringed seals.

**MATERIALS AND METHODS**

**Chemicals**

Atropine and mercuric chloride (Hg\(^{2+}\), 99.6% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Methylmercury chloride (MeHg\(^+\), >95% purity) was obtained from Alfa Aesar (Ward Hill, MA). DDT (\(p,p'\)-dichlorodiphenyltrichloroethane), dieldrin, \(\gamma\)-chlordane, lindane (\(\gamma\)-hexachlorohexane), Arochlor 1254, and toxaphene were purchased from Radian Chemicals (>99% purity; Austin, TX). Organochlorine and mercury stock solutions were prepared in dimethyl sulfoxide (DMSO) and distilled water, respectively, at concentrations below their solubility limits (Budaveri et al., 1996). \([^3H]Quinuclidinyl benzilate ([^3H]-QNB; 42 Ci/mmol) was obtained from NEN/Perkin Elmer (Boston).

**Animals**

Eight feral ringed seals (Phoca hispida) were obtained near Kuujjuak (Northern Quebec, Canada) by local Inuit hunters during the winter season spanning 1998 and 1999 as previously detailed (Muir et al., 1999). Carcasses were shipped frozen to the Makivik Research Center (Kuujjuaq, Quebec, Canada), where the cerebrum was removed and stored at −80°C until analysis. Chemical residues have been measured in these animals under the Northern Contaminants Program (Muir et al., 1999).

**Characterization of the mACh Receptor**

Cellular membranes for receptor binding studies were prepared by homogenizing individual cerebrums for 30 s in cold Na/K buffer (50 mM NaH\(_2\)PO\(_4\), 5 mM KCl, 120 mM NaCl, pH 7.4) followed by centrifugation at 32,500 \(\times\) g for 15 min at 4°C. The resulting pellet was washed twice under the same conditions to remove endogenous neurotransmitters and contaminants,
and the final pellet was resuspended in Na/K buffer and immediately stored at −80°C until required (Basu et al., 2005a; Stamler et al., 2005). To derive saturation binding curves, membrane protein (20 μg) was incubated with various concentrations (0.01 to 3.2 nM) of the mACh receptor antagonist [3H]-QNB for 60 min at room temperature in 96-well 1 μM GF/B filter plates (Millipore, Inc., Boston). Binding was terminated by the application of vacuum filtration to the microplate to separate receptor-bound [3H]-QNB from free [3H]-QNB. Filters were then rinsed three times with Na/K buffer, removed and allowed to soak overnight in liquid scintillation cocktail (ICN Biomedical, Aurora, OH). Radioactivity retained by the filters was quantified by a liquid scintillation counter (LKB Wallac 1209 Rackbeta, Turku, Finland) with approximately 66% counting efficiency. Specific binding was defined as the difference in [3H]-QNB bound in the presence and absence of 100 μM atropine.

**Inhibition Studies**

The eight chemicals were screened for their ability to inhibit mACh receptor binding (Table 1; range tested: 0 to 320 μM). Membrane preparations (20 μg) were pre-incubated with neurotoxicants for 15 min prior to the receptor binding assay as earlier described (Basu et al., 2005a). Samples were then incubated with 0.3 nM [3H]-QNB, reflecting a radioligand concentration that closely approximated the ligand affinity ($K_d$) for these samples, and binding assays were completed as described earlier.

For chemicals that inhibited the binding of [3H]-QNB to the mACh receptor by greater than 50%, subsequent assays were completed to characterize the nature of this inhibition as either competitive or non-competitive. Saturation binding curves were developed for each sample in the presence or absence of neurotoxicants (IC$_{50}$ concentrations) to determine the effects on $K_d$ and receptor density ($B_{max}$).

**TABLE 1.** Effects of Environmental Neurotoxicants on the Binding of [3H]Quinuclidinyl Benzilate ([3H]-QNB) to the Muscarinic Cholinergic (mACh) Receptor in Cellular Membranes Isolated From the Cerebrum ($n = 8$) of Wild Ringed Seals (*Phoca hispida*)

<table>
<thead>
<tr>
<th>Environmental neurotoxicant</th>
<th>mACh receptor binding (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg$^{2+}$</td>
<td>2.89 ± 0.63$^a$</td>
</tr>
<tr>
<td>MeHg$^+$</td>
<td>21.52 ± 0.65$^a$</td>
</tr>
<tr>
<td>DDT</td>
<td>94.13 ± 6.40</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>91.92 ± 4.94</td>
</tr>
<tr>
<td>Chlordane</td>
<td>105.01 ± 3.90</td>
</tr>
<tr>
<td>Lindane</td>
<td>103.26 ± 7.50</td>
</tr>
<tr>
<td>Arochlo 1254</td>
<td>102.96 ± 5.27</td>
</tr>
<tr>
<td>Toxaphene</td>
<td>77.58 ± 2.84$^a$</td>
</tr>
</tbody>
</table>

*Note.* Data represent mACh receptor binding in the presence of the highest concentration of neurotoxicant tested (320 μM) as a percent of binding in nonexposed samples.

*Statistical differences ($p < .05$) between exposed and nonexposed samples.
Statistical Analysis

Data from receptor binding studies were curve fitted using nonlinear regression (GraphPad Prism Version 3.02, GraphPad Software, Inc., San Diego, CA) to calculate $B_{max}$ and $K_d$ according to the following equation:

\[
\text{Specific binding} = \frac{B_{max}R}{K_d + R}
\]

where $R$ represents the concentration of radioligand ([3H]-QNB), $B_{max}$ represents receptor density (fmol/mg protein), and $K_d$ represents ligand affinity (nM).

For inhibition studies, concentrations of chemicals that inhibited maximal [3H]-QNB binding by 50% (IC\textsubscript{50}) were determined (GraphPad Prism, Version 3.02, GraphPad Software, Inc., San Diego, CA). Inhibition constants ($K_i$) were derived by normalizing the IC\textsubscript{50} data with respect to $K_d$ (Cheng & Prusoff, 1973):

\[
K_i = \frac{\text{IC}_{50}}{[1 + (R/K_d)]}
\]

The critical level of significance for all statistical analyses was set at $\alpha = .05$, and all data are represented as mean ± SEM. Parametric methods ($T$ tests or analyses of variance [ANOVAs]) were used to compare the inhibitory data among neurotoxicants tested (SigmaStat Version 2.03, SPSS, Inc., San Rafael, CA).

RESULTS

Binding of [3H]-QNB to the mACh receptor in cellular membranes isolated from the cerebrum of ringed seals was saturable (Figure 1). Saturation binding data was best fit using a one-site binding model, and yielded a mean mACh $B_{max}$ of 826.9 ± 68.4 fmol/mg and $K_d$ of 0.31 ± 0.04 nM. Nonspecific binding was less than 4% of total binding at 3.2 nM [3H]-QNB.

There was no inhibitory effect on mACh receptor binding by the solvent carrier (1% DMSO) alone. Of the 8 chemicals screened, MeHg\textsuperscript{+} and Hg\textsuperscript{2+} were the only ones to inhibit the binding of [3H]-QNB to the mACh receptor by greater than 50% (Table 1 and Figure 2). Hg\textsuperscript{2+} (IC\textsubscript{50} = 1.92 ± 0.06 μM) was significantly more potent at inhibiting specific [3H]-QNB binding than MeHg\textsuperscript{+} (IC\textsubscript{50} = 2.75 ± 0.22 μM) when comparing IC\textsubscript{50} values. However, this difference in inhibition was not statistically significant when the data were normalized to derive $K_i$ values (Hg\textsuperscript{2+} = 1.38 ± 0.07 μM; MeHg\textsuperscript{+} = 1.26 ± 0.12 μM). Toxaphene at the highest concentration tested (320 μM) produced a 22.4% inhibition of [3H]-QNB binding compared to nonexposed tissues (Table 1).

To characterize the nature of Hg-mediated inhibition of [3H]-QNB binding, saturation binding curves were developed in the presence and absence of Hg\textsuperscript{2+} and MeHg\textsuperscript{+} (Figure 3). Both Hg\textsuperscript{2+} and MeHg\textsuperscript{+} significantly increased
FIGURE 1. Saturation analysis of [³H]quinuclidinyl benzilate ([³H]-QNB) binding to the muscarinic cholinergic (mACh) receptor in cellular membranes isolated from the cerebrum of wild ringed seals (*Phoca hispida*). All data points represent mean (± SEM) specific binding from eight samples.

![Graph of specific binding vs. [³H]-QNB concentration](image)

FIGURE 2. Inhibition of [³H]quinuclidinyl benzilate ([³H]-QNB) binding to the muscarinic cholinergic (mACh) receptor by Hg²⁺ and MeHg⁺ in cellular membranes isolated from the cerebrum of wild ringed seals (*Phoca hispida*). Values are compared to membrane preparations not exposed to Hg, and data points represent mean (± SEM) specific binding from six to eight samples.

![Graph of % of maximal binding vs. log [Hg] molar concentration](image)
mACh receptor $K_d$ by 703.1 ± 105.1% and 426.6 ± 72.7%, respectively, compared to nonexposed samples. Neither chemical had any effect on mACh $B_{max}$ suggesting a competitive mode of inhibition.

**DISCUSSION**

Data regarding contaminant burdens in the brains of marine mammals are limited, but excessive concentrations (i.e., >100 ppm) of Hg and organochlorines have been reported in the liver and blubber, respectively, of ringed seals (Wagemann & Muir, 1984; Nyman et al., 2002). To address the possible neurotoxicological consequences associated with these high accumulations, the present study was completed to characterize the effects of environmental pollutants on mACh receptor binding in cellular membranes isolated from the cerebrum of ringed seals. To our knowledge this is the first study to characterize the mACh receptor in the brain tissue of a marine mammal. mACh $B_{max}$ values were similar to those in the cerebral cortex of mink and river otters (Basu et al., 2005a) and whole brains of white-footed mice (Jett et al., 1993), but $K_d$ values were higher. Comparable values in $B_{max}$ among mammalian wildlife are not surprising, as the mACh receptor is highly conserved in nature. For example, there is greater than 95% homology in the amino acid sequence for the mACh receptor among rats, mice, and humans (Caulfield & Birdsall, 1998), and this minimizes uncertainty when comparing biological data for this receptor across species.
Numerous studies have documented that specific binding to the mACh receptor can be impaired by Hg (Von Burg et al., 1980; Castoldi et al., 1996; Basu et al., 2005a). Inhibitory values were generally within one order of magnitude among all species (i.e., 1 to 10 μM), suggesting that Hg-induced inhibition of mACh receptor binding is governed by similar mechanisms. However, differences were evident, as river otters were almost eight times more sensitive than humans (K_i values in cerebral cortex: 0.69 vs. 4.23 μM), and the cerebellum was more sensitive than the cerebral cortex (Basu et al., 2005a). Inhibitory values from the current study were closer to the data obtained from river otters than humans. Inhibition of mACh receptor binding is likely due to the interaction of Hg with thiol residues located within or near the ligand binding domain (Abd-Elfattah & Shamoo, 1981), preventing mACh receptor binding in a competitive manner (Figure 3). The physiological consequence of this impairment is evident, as Hg-mediated inhibition of mACh receptor binding can trigger cell death by activating inositol 1,3,4-triphosphate receptors and producing increased release of Ca^{2+} from the smooth endoplasmic reticulum in granule cells cultured from the rat cerebellum (Limke et al., 2004).

Effects of Hg on the mACh receptor in vivo are not as definitive. Coccini et al. (2000) reported that exposure of rats (adult female Sprague-Dawley, 200–250 g) to MeHg+ (0.5 mg MeHgOH/kg body weight/d for 16 d) resulted in increased mACh B_{max} in the cerebellum and no changes in the cerebral cortex, but Rajanna et al. (1997) observed that mACh B_{max} increased in the cerebral cortex and decreased in the cerebellum of rats (adult male Sprague-Dawley, 175–200 g) following exposure to Hg^{2+} (1 mg HgCl_2/kg body weight/d for 7 d). Field studies on wild mink (Basu et al., 2005b) and river otters (Basu et al., 2005c) demonstrated that variations in mACh B_{max} were related to Hg exposure. While the correlation between concentrations of brain Hg and mACh B_{max} was negative in the cerebral cortex of river otters, this association was positive in the whole brains of mink. The behavioral and physiological consequences of such alterations in mACh receptor levels are unknown. However, studies on mACh receptor knockout mice have proven this receptor class is essential in multiple physiologies relevant for wildlife health, including thermoregulation, locomotion, and feed intake (Bymaster et al., 2003; Wess, 2004). Given that ringed seals bioaccumulate greater concentrations of Hg than river otters or mink, field studies in this species are required to determine whether environmental exposures are related to alterations in neurochemistry (i.e., mACh B_{max}), as previously observed in other mammalian piscivores (Basu et al., 2005b, 2005c). However, it should be noted that ringed seals can better demethylate MeHg^+ to the proximate species (Wagemann et al., 2000) than mink (Basu et al., 2005b) or river otters (Basu et al., 2005c) can, and a majority of the mercuric ion exists as an inert selenocomplex in the tissues of ringed seals (Wagemann et al., 2000). These factors will undoubtedly influence the neurotoxicological effects of Hg in vivo and require consideration.

Weight of evidence suggests that exposure of marine mammals to persistent organochlorines impairs genetic, immune, endocrine, and reproductive
systems (Hutchinson & Simmonds, 1994; Colborn & Smolen, 1996; Troisi et al., 2001). Despite these observations, very little is known regarding the possible neurological effects of organochlorines on marine mammals, and this is a major knowledge gap, considering that most toxicants accumulated by these species have potent neurotoxic characteristics (National Research Council, 1992; Tilson & Kodavanti, 1998; Castoldi et al., 2001). Data from the current study suggested that the organochlorines tested did not exert neurotoxic effects by direct inhibition of mACh receptor binding in the cerebrum of ringed seals (Table 1), and there are no published reports to contradict this finding. In vivo experiments on rats documented that mACh $B_{\text{max}}$ (Fonseca et al., 1986) and choline acetyltransferase activity (Donahue et al., 2004) are affected by organochlorines. However, these measured cholinergic changes from whole-animal bioassays are likely to be secondary responses resulting from the interaction of organochlorines with other neurochemical pathways, such as dopaminergic signaling or calcium homeostasis (Tilson & Kodavanti, 1998), because there is a lack of in vitro evidence linking organochlorine exposure with inhibition of cholinergic function (Table 1; Fonseca et al., 1986). Further research is required to test this hypothesis and characterize the relationships among persistent environmental pollutants and the major neurological systems in marine mammals.

In conclusion, the results from the current study suggest that binding of $[^3H]$-QNB to the mACh receptor in the cerebrum of ringed seals is inhibited by Hg in vitro, and not by organochlorines. Such mechanistic findings at the neurochemical level may help to differentiate and clarify possible neurobehavioural effects associated with chronic exposure of mammalian wildlife to different types of pollutants in nature. Furthermore, the data demonstrate that the mACh receptor may possibly be used as a specific bioindicator for Hg exposure and neurotoxic effects in sensitive marine mammal species, as previously observed in mink (Basu et al., 2005b) and river otters (Basu et al., 2005c), but this will have to be validated in the field by means of cross-sectional studies.

REFERENCES
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Smith, T. G. 1987. *The ringed seal, Phoca hispida, of the Canadian Western Arctic*. Department of Fisheries and Oceans, Ottawa, Canada.


