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ARTICLE

Biomarkers of the cholinergic and dopaminergic signaling pathways in Arctic beluga whales (*Delphinapterus leucas*): relationship to methylmercury and selenium[1]

Sonja K. Ostertag and Hing Man Chan

Abstract: There are increasing concerns about potential neurotoxicity of chronic methylmercury (MeHg) exposure in Arctic wildlife and human populations. The relationships between mercury (Hg), MeHg, inorganic Hg (iHg), and selenium (Se) exposure, and neurochemical and molecular biomarkers in beluga whales (*Delphinapterus leucas* (Pallas, 1776)) were characterized. Samples were collected in 2008 (n = 20) and 2010 (n = 15) in the Canadian Arctic from hunter-harvested beluga whales. Total Hg concentrations in the temporal cortex and cerebellum were 21.0 ± 22.6 mg kg^{-1} dry weight (dw) and 14.9 ± 18.9 mg kg^{-1} dw, respectively. Monoamine oxidase (MAO) activity was predicted by MeHg and the molar ratio of total Hg to Se (Hg:Se, $p < 0.05$); MAO-A mRNA transcription levels were predicted by iHg and Se ($p < 0.05$). Muscarinic AChR binding was predicted by MeHg ($p < 0.05$) and Hg:Se molar ratio ($p < 0.05$), and mRNA transcription levels of mAChR m1 was predicted by the Hg:Se molar ratio ($p < 0.05$). These results suggest that the cholinergic and dopaminergic signaling pathway in Eastern Beaufort Sea beluga whales may be sensitive to MeHg exposure. The response of beluga whales to MeHg exposure at a physiological and population level remains to be elucidated.

Key words: mercury, biomarker, neurochemical, neurotoxicity, Inuit.

Résumé : Il existe des préoccupations grandissantes concernant la neurotoxicité possible engendrée par l'exposition chronique des populations fauniques et humaines en Arctique au méthylmercure (MeHg). Les relations entre l'exposition au mercure (Hg), au MeHg, au Hg inorganique (iHg) et au sélénium (Se) et les biomarqueurs neurochimiques et moléculaires chez les bélugas (*Delphinapterus leucas* (Pallas, 1776)) ont été caractérisées. Des échantillons ont été prélevés en 2008 (n = 20) et 2010 (n = 15) dans l'Arctique canadien sur des bélugas capturés par des chasseurs. Les concentrations totales de Hg dans le cortex temporal et le cervelet étaient de 21,0 ± 22,6 mg kg^{-1} poids sec (dw) et de 14,9 ± 18,9 mg kg^{-1} dw, respectivement. L'activité monoamine-oxydase (MAO) a été prédite par le MeHg et le rapport molaire du total d'Hg au Se (Hg:Se, p < 0,05); les niveaux de transcription MAO-A mRNA ont été prédits par iHg et Se ($p < 0.05$). La liaison muscarinique AChR a été prédite par MeHg ($p < 0,05$) et le rapport molaire Hg:Se ($p < 0,05$) et les niveaux de transcription mRNA de mAChR m1 ont été prévus par le rapport molaire Hg:Se ($p < 0,05$). Ces résultats suggèrent que la voie de signalisation cholinergique et dopaminergique chez les bélugas

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de l'est de la mer de Beaufort puisse être sensible à l'exposition au MeHg. La réponse des bélugas à l'exposition au MeHg sur le plan physiologique et le niveau de la population reste à être élucidée. [Traduit par la Rédaction]

Mots-clés : mercure, biomarqueur, neurochimique, neurotoxicité, Inuit.

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Introduction

There are increasing concerns about the impacts of chronic methylmercury (MeHg) exposure for Arctic wildlife and human populations (Dietz et al. 2013). Mercury levels in Arctic biota are an order of magnitude higher today than in the preindustrial period and approximately 74%–94% of mercury (Hg) in biota is estimated to originate from anthropogenic Hg emissions (Dietz et al. 2009). Although the Arctic lacks point sources of Hg emissions, up to 300 t of Hg are transported annually to the Arctic from southern latitudes (AMAP 2003; Skov et al. 2004; Outridge et al. 2008). Methylmercury biomagnifies in aquatic ecosystems, and there is a millionfold increase in MeHg concentration from seawater to top predators (Clarkson and Magos 2006).

In the Arctic, environmental contaminants of primary concern for the health of beluga whales (*Delphinapterus leucas* (Pallas, 1776)) have been identified as organic pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and heavy metals (Fisk et al. 2005). From 1981 to 2012, mean total mercury (THg) concentrations in livers of Eastern Beaufort Sea (EBS) beluga whales were 18.84 ± 1.1 μg g^{-1} wet weight (ww) for 16–35 yo whales and significantly greater (53.38 ± 3.9 μg g^{-1} ww) for 36 to 55 yo whales (Braune et al. 2015). In general, Arctic belugas had lower THg concentrations in liver (0.04–182 μg g^{-1} dry weight, dw) than belugas from the St Lawrence Estuary (1.42–756 μg g^{-1} dw; Béland et al. 1993), a southern region with a history of contamination from industrial activity. Modern EBS beluga whales have THg concentrations approximately 4–17 times higher than preindustrial period (Outridge et al. 2002; Outridge et al. 2009).

The risk of MeHg exposure for Arctic marine mammals is poorly understood and challenging to assess based on laboratory feeding trials (Krey et al. 2014). In humans, the central and peripheral nervous systems are highly sensitive to MeHg and elevated exposure has been associated with adverse neurological outcomes including ataxia, constriction of the visual field, and damage to the auditory region of the temporal lobe (Clarkson 1997; Ekino et al. 2007). Methylmercury is transported across the intestinal mucosa and is able to cross the blood brain barrier in mammals (Aschner and Aschner 1990), and the presence of inorganic mercury (iHg) in the brain is likely due to in-situ demethylation of MeHg (Clarkson and Magos 2006). The brain is expected to be more sensitive to MeHg than iHg, based on brain pathology and symptoms associated with MeHg poisoning (Magos et al. 1985). Total brain Hg in EBS beluga whales ranged from 0.06 to 22.9 mg kg^{-1} ww in the temporal lobe (Ostertag et al. 2013), which was within the range associated with toxicity in previous studies. For example, animal feeding trials suggested that brain Hg concentrations exceeding 10 mg kg^{-1} ww could be lethal, and concentrations exceeding 6.75 mg kg^{-1} ww were associated with clinical symptoms (as reviewed by Krey et al. 2015). Neurochemical variation was associated with THg concentrations ranging from 0.27 to 18.84 mg kg^{-1} dw (MeHg: 0.26–13.52 mg kg^{-1} dw) in wild mink (*Mustela vison*, Basu et al. 2005*a*) and 0.09 to 14.31 mg kg^{-1} dw (iHg: 0.00–10.65 mg kg^{-1} dw; MeHg: 0.08 and 8.54 mg kg^{-1} dw) in wild river otters (*Lontra canadensis*, Basu et al. 2005*b*). The demethylation of MeHg followed by the binding of iHg species to Se, to form HgSe or stable HgSe–protein complexes has been suggested as one pathway of MeHg detoxification (Khan and Wang 2009). There was

evidence that MeHg was demethylated and potentially detoxified by Se in the brains of EBS beluga whales (Lemes et al. 2011; Ostertag et al. 2013).

Biochemical changes may be an indicator of early-stage effects before the manifestation of disease (Manzo et al. 1996). Messenger RNA (mRNA) transcription levels may provide insight into effects on organisms, prior to effects on the whole organism; however, mRNA abundance is not necessarily related to protein function (Feder and Walser 2005). Cholinergic signaling pathways have been linked to essential physiological processes including learning, memory, stress response, and modulation of sensory information; the disturbance of dopaminergic neurotransmission is related to psychiatric symptoms in humans (Reis et al. 2009). The muscarinic acetylcholine receptor (mAChR) from the cholinergic signaling pathway and monoamine oxidase B (MAO-B) from the dopaminergic signaling pathway are known to be sensitive to MeHg exposure (Chakrabarti et al. 1998; Coccini et al. 2000; Basu et al. 2005*a*, 2006*a*, 2006*b*, 2007*a*; Beyrouty et al. 2006; Stamler et al. 2006; Coccini et al. 2007). The disruption of either of these signaling pathways due to increased THg accumulation in the Arctic could adversely affect beluga whales in the wild.

The objective of this study was to characterize the relationships between neurochemical and molecular biomarkers for the dopaminergic and cholinergic signaling pathways, and concentrations of THg, Se, MeHg, iHg, and the Hg:Se molar ratio in the temporal lobe and cerebellar cortex of EBS beluga whales. Total MAO activity and MAO-A mRNA expression were selected as biomarkers of the dopaminergic signaling pathway; and mAChR binding levels and mAChR m1 mRNA expression were selected as biomarkers of the cholinergic signaling pathway, based on previous wildlife and laboratory studies.

Methods

Sample collection

Appropriate research permits and licenses were obtained from the Aurora Research Institute (Licenses #14357R and #14717) and Department of Fisheries and Oceans (DFO) and the sampling program was approved and supported by the Tuktoyaktuk and Inuvik Hunters and Trappers Committees. Twenty whales were sampled in 2008 (16 male, 4 female) and 15 beluga whales were sampled in 2010 (15 males, 0 females) on Hendrickson Island, NT, Canada; samples were collected from hunter-harvested beluga whales from the EBS population, as described elsewhere (Ostertag et al. 2013). Typically, sampling commenced 30–45 min postmortem, and subsamples were collected and preserved within 3 h of animal death. Subsamples of cerebellum and temporal cortex were collected and frozen at ~−20 °C (~0.5–3 g) for THg, Se, and speciation analyses; and flash-frozen and stored in nitrogen vapour (approximately −176 °C) for neurochemical analyses (~0.5–1 g). Samples were flash-frozen in 2008 (~0.07 g) for mRNA expression assays and were placed in RNALater™ at approximately 4 °C for 24 h prior to freezing at −20 °C in 2010. Sample numbers varied for the different assays due to subsample availability for the separate analyses. Animal age estimates were based on counting individual growth layer groups (GLG) in the dentine from a thin section of tooth at the Freshwater Institute, Winnipeg, MB, with the assumption that one GLG was deposited per annum (Stewart 2006; Luque and Ferguson 2007). Animal sex was determined by palpating the genitalia of harvested whale (American Society of Mammalogists 1961).

Mercury and selenium analyses

The concentrations of THg, iHg, MeHg, and Se were determined previously for the brain regions analyzed using freeze-dried samples (Ostertag et al. 2013). In brief, approximately

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10 mg of freeze-dried samples were analyzed by inductively coupled plasma mass spectrometry (Agilent Technologies, 7500 CX) following modified acid digestion (Armstrong and Uthe 1971). One blank, DOLT-4 in triplicate, and one sample in triplicate were included within each batch of 36 samples. Recovery of THg and total Se was 88% and 92%, respectively ($n = 18$). Concentrations of THg and Se in blanks were approximately 0.30 ± 0.05 mg kg^{-1} and 0.21 ± 0.06 mg kg^{-1} for a 10 mg sample ($n = 17$), and detection limits were 0.001 and 0.01 mg kg^{-1} for THg and Se, respectively.

The chemical forms of Hg were analyzed via high-performance liquid chromatography (HPLC; Agilent 1200 series HPLC system, Agilent Technologies Canada Inc., Mississauga, ON, Canada), equipped with autosampler, quaternary pump, and 100 mL injection loop. A ZORBAX Eclipse XDB-C18 Column (2.1 mm × 50 mm, 5 μm) was connected to an inductively coupled mass spectrometer (ICP-MS; Agilent Technologies™, 7500 CX), following extraction using a method developed by Krey et al. (2012). Each sample batch ($n = 33$) included one blank and DOLT-4 (dogfish liver, NRC; triplicate) to ensure quality within and between batches. With this method, the labile fraction of iHg (iHg_{labile}) bound weakly to proteins or thiols was extracted; however, iHg associated with strong structural proteins was not be extracted efficiently. Recovery of MeHg and iHg_{labile} in DOLT-4 was 129.8 ± 4.7% and 98.9 ± 5.8% ($n = 28$), respectively. Both MeHg and iHg concentrations were below the level of detection (0.5 mg kg^{-1}) in all blanks analyzed ($n = 7$).

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Receptor binding assays

The mAChR binding assay was adapted for beluga brain tissue from previous studies on brain tissue from mink by Basu et al. (2007*b*, 2006*b*), following the preparation of membrane homogenate as previously described (Ostertag et al. 2014). Protein concentration was determined with the Bradford assay and bovine serum albumin was used as the standard (Bradford 1976). In brief, 30 μg of membrane preparation was resuspended in buffer and added to a 96-well plate (Costar™). Membrane protein was suspended in 100 μL of 50 mM sodium phosphate buffer (5 mM KCl, 120 mM NaCl, 50 mM HaH_2PO_4; pH 7.4) and incubated with [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB; 1.6 nM for cerebellum, 3.2 nM temporal cortex) for 60 min. Binding affinity curves (0.32–20 nM [^{3}H]-QNB were used to determine the appropriate concentrations of [^{3}H]-QNB for each brain region (data not shown)). Incubation was carried out with gentle shaking and binding reactions were terminated by vacuum filtration. Sample was filtered through glass fiber (Inotech) using a cell harvester (Inotech), the filter was washed four times with ice-cold NaK buffer. The filter was set in an Omnifilter microplate (PerkinElmer), dried for 60 min under a heat lamp before 25 μL scintillation cocktail (Universol™, MP Biomedicals) was added. Radioactivity retained by the filters was quantified immediately by a Chameleon™ liquid scintillation counter (Hidex, Turku, Finland) with approximately 19% counting efficiency. Specific binding was calculated as the difference between radioligand binding in the presence and absence of 100 μM atropine, a mAChR inhibitor. Homogenized mouse cerebrum was used as a control to monitor inter-plate variation. Receptor binding was reported as femtomole of radioisotope bound per miligram of membrane protein (fmol mg^{-1}).

Enzyme activity

Total monoamine oxidase activity was analyzed according to a protocol developed by Zhou et al. (1997) and described for polar bear (*Ursus maritimus*) by Basu et al. (2009). In brief, brain tissue samples were homogenized in sodium phosphate buffer (10 mL g^{-1} tissue), 0.05% Triton-X-100 was added to the homogenate and the sample was sonicated for 30 s (Sonic dismembrator: Model 100, Fisher Scientific), prior to centrifugation (12 000×*g*, 8 °C for 10 min). Homogenate (25 μg protein) was incubated with buffer for 30 min and

enzyme activity was quantified following 15 min incubation with 10 mM 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent), 5 mM tyramine, 200 mU horseradish peroxidase. Fluorescence (540 nm excitation and 590 nm emission) of resorufin (end product) was read every 5 min for 75 min with a Chameleon™ spectrofluorometer (Hidex). Enzyme activities were expressed as picomol resorufin formed per minute per microgram protein (pM min^{-1} μg^{-1}) based on the standard curve of resorufin (range: 0.25–1.75 μM resorufin; $R^2 \geq 0.99$. Each sample was assayed in triplicate. Sodium phosphate buffer was used as a negative control and hydrogen peroxide (1.25 μM) was used as a positive control.

Expression of mRNA

Species-specific primers and fluorogenic probes (IDT, Coralville, IA, USA) were designed using Genscript RT-PCR primer design (Table 1). As described previously by Ostertag et al. (2014), primer pairs were designed for conserved nucleotide regions of the genes of interest based on the alignment of genes from multiple species [human, cow, goat, pig, primate (chimpanzee and macaque), dog, cat, and sheep] using the National Centre for Biotechnology Information (NCBI) website. Species-specific probes were then developed by using the nonspecific primers in polymerase chain reaction (PCR) reactions on complementary DNA (cDNA) from beluga cerebellum (n = 2). The PCR products were direct-sequenced at UBC Okanagan (Fragment Analysis and DNA Sequencing Services, Kelowna, CA, USA) and used to design species-specific primers. Chromatograms were edited and the retrieved sequences were run through the NCBI website (Standard Nucleotide BLAST) to ensure they matched the conserved sequences of genes.

Quantitative real-time PCR (qRT-PCR) expression of mRNA for mAChR subtype m1 (mAChR m1) and MAO-A target genes and the s9 reference gene were analyzed followed the RNA extraction and cDNA archive procedures. Total RNA was extracted using Trizol with the Qiagen RNeasy™ Lipid Tissue kit. Total RNA was treated with Ambion™ DNase | buffer (6 μL), rDNAse | (1 μL), and DNase inactivation reagent (6 μL), prior to quantification of RNA-40 using a spectrophotometer (Nanodrop, ND-1000). Taqman reverse transcription reagents (Applied Biosystems) were used for the generation of cDNA for all samples. The identical reaction was performed concurrently without reverse transcriptase, to ensure the absence of genomic DNA. The thermocycler parameters for the generation of cDNA archive were as follows: 25 °C for 10 min, 37 °C for 60 min, and 95 °C for 5 min (DYAD™, DNA Engine).

All PCR runs were performed under identical conditions using the 7300 Real-Time PCR System (Applied Biosystems). Simplex assays were run with iTaq™ Supermix with ROX kit (Biorad). The thermocycle program included an enzyme activation step at 95 °C (10 min) and 40 cycles of 95 °C (15 s) and 60 °C (1 min).

Inter-plate variability of s9 expression (reference gene) was monitored through the inclusion of pooled sample in triplicate for each plate. The absence of genomic DNA was ensured through the inclusion of samples that had not undergone reverse transcription (no reverse transcription, NRT), in each plate. A NanoDrop (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) was used to evaluate RNA purity and quality. Optical density (OD) ratios (260:280 nm wavelengths) >1.8 indicated good RNA quality (Fleige and Pfaffl 2006) The ratio of 28S:18S and RNA quality indicator (RQI) value were used to evaluate degradation via Experion (Bio-Rad Laboratories, Hercules, CA, USA); a 28S:18S ratio of 2.0 (Fleige and Pfaffl 2006), or RQI of 10 indicated perfect integrity (Taylor et al. 2009).

Data analysis

Mercury concentrations, fold-change ratios, receptor density, and enzyme activity were log-transformed when necessary to meet assumptions of normal distribution and

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Table 1. Sequences of primers and probes for monoamine oxidase A (MAO-A), muscarinic acetylcholine receptor (mAChR) subtype m1, and the housekeeping gene S9 used for real time PCR.

	Primer sequence			
Target gene	Fw	Rvs	Probe	Efficiencies (%)
MAO-A	GGCCAGGAACGGAAGTTTGT	CCCCGAGGAGGTGCATTA	TGGATCTGGTCAAGTAAGCGAGCGG	102
mAChR subtype m1	GCAACGCCTCGGTCATG	GCCGGGTCACGGAGAAGTA	CGGTCAACAACTACTTCCTGCTGAGCCTG	103
S9	GCTGCTGACGCTGGATGAG	CGCAGCAGGGCATTGC	AAGACCCGCGGCGTCTGTTTGAA	106

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homogeneity of variance. The molar ratio of total Hg to Se (Hg:Se) was determined according to eq. (1).

$$\text{(1)}\quad \text{Hg:Se molar ratio} = ([\text{THg}\ \mu\text{g kg}^{-1}\text{dw}/\text{MW}_{Hg}])/([\text{Se}\ \mu\text{g kg}^{-1}\text{dw}/\text{MW}_{Se}])$$

where MW_{Hg} is 200.59 and MW_{Se} is 78.96.

Dry weight concentrations were converted to ww concentrations for THg and Se to ease comparison to literature values, using the sample-specific moisture content and equation as described previously (Ostertag et al. 2013). To correct for poor to fair RNA integrity arising from sub-optimal sampling conditions, transcription levels were normalized to the s9 reference gene and transcription efficiencies were utilized to calculate fold changes in mRNA expression (Fleige and Pfaffl 2006). The efficiencies (*E*) for target genes and reference gene (s9) were calculated using standard curves generated for all genes from serial dilutions of cDNA (six 10-fold dilutions per target or reference gene). Fold changes for each target gene were calculated relative to the reference gene (s9) (Pfaffl 2001), for each animal, using the mean Ct (cycle threshold) values for the three lowest Hg-exposed animals as the control. The lowest Hg-exposed animals were included in reported results and data analysis. Normalizing the Ct values for samples with an internal reference gene reduces the effect of RNA quality on the mRNA values presented (Fleige and Pfaffl 2006).

Paired *t*-tests were used to compare THg, Se, iHg_{labile}, and MeHg concentrations in the two brain regions analyzed. The relationship between neurochemical and molecular biomarker, and the concentration of THg, Hg species, Se and molar ratio of Hg:Se was explored using Pearson's correlations followed by multiple regression analyses. A backwards-stepwise approach was used to evaluate the following predictor variables in the multiple regression analysis: age, sampling year, and THg, iHg_{labile}, MeHg, Se, or molar ratio of Hg:Se. The outcome variables used were mAChR binding, MAO activity, and MAO-A mRNA expression in both brain regions, and mAChR m1 mRNA expression in the cerebellum. For biomarkers that varied between sample years, regression analyses were conducted on pooled data and on 2008 data separately. Models were compared using the Akaike Information Criterion (AIC). The effect of animal sex on biomarkers could not be determined due to the small sample size; therefore, statistical analyses were conducted on pooled data (males and females), followed by multiple regression analyses on males only (only data for males presented). Pearson's correlation coefficients were calculated for the expression of target genes and corresponding receptor or enzyme. Correlations (Pearson and Spearman), *F*-values, and *t*-tests were considered to be statistically significant if $p \leq 0.05$. Data in tables and graphs are displayed in the original scale of measurement.

Results

Mercury and selenium analyses

Estimated animal age for both females and males sampled ranged from 16 to 60 yo (28 ± 10 yo; Table 2). Mercury concentration (mean, SD) in the temporal cortex was 21.0 ± 22.6 mg kg^{-1} dw (4.1 ± 4.5 mg kg^{-1} ww) and 14.9 ± 18.9 mg kg^{-1} dw (3 ± 3.8 mg kg^{-1} ww) in the cerebellum for male and female whales (Table 2). Mean MeHg concentration was 2.2 ± 1.1 mg kg^{-1} dw (0.44 ± 0.22 mg kg^{-1} ww) in the temporal cortex and 1.6 ± 0.86 mg kg^{-1} dw (0.32 ± 0.17 mg kg^{-1} ww) in cerebellum (Table 2). Inorganic Hg_{labile} concentrations were 3.2 ± 2.1 mg kg^{-1} dw (0.64 ± 0.42 mg kg^{-1} ww) in the temporal cortex and 2.7 ± 1.5 mg kg^{-1} dw (0.32 ± 0.17 mg kg^{-1} ww) in the cerebellum (Table 2). Total Se concentrations were 11.2 ± 9.7 mg kg^{-1} dw (2.2 ± 1.9 mg kg^{-1} ww) in the temporal lobe and 9.1 ± 8.5 mg kg^{-1} dw (1.8 ± 1.7 kg^{-1} ww) in cerebellum; and the molar ratio of Hg:Se was 0.61 ± 0.21 in the temporal lobe and 0.57 ± 0.16 in the cerebellum (Table 2). There was a significant difference in mean

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Table 2. Descriptive statistics (mean ± s.d., n) are presented for male and female beluga whales sampled; total mercury (THg), methylmercury (MeHg), labile inorganic mercury (iHg) and selenium (Se) concentrations (dry weight, dw; wet weight, ww) and stoichiometric ratio of THgT to Se are provided for the cerebellum and temporal cortex from beluga whales sampled at Hendrickson Island, NT, Canada in 2008 and 2010.

	Descriptive statistics	
Variable	Temporal cortex	Cerebellum
Age	29.9 ± 11.3 (n = 30)	
THg concentration (mg kg^{-1})	dw: 21.0 ± 22.6 (31) ww: 4.1 ± 4.5	dw: 14.9 ± 18.9 (35) ww: 3.0 ± 3.8
MeHg concentration (mg kg^{-1})	dw: 2.2 ± 1.1 (31) ww: 0.44 ± 0.22	dw: 1.6 ± 0.86 (35) ww: 0.32 ± 0.17
iHg concentration (mg kg^{-1})	dw: 3.2 ± 2.1 (31) ww: 0.64 ± 0.42	dw: 2.7 ± 1.5 (35) ww: 0.55 ± 0.30
Se_T concentration (mg kg^{-1})	dw: 11.2 ± 9.7 (31) ww: 2.2 ± 1.9	dw: 9.1 ± 8.5 (35) ww: 1.8 ± 1.7
THg:Se molar ratio	0.61 ± 0.21 (31)	0.57 ± 0.16 (35)
mAChR binding (fmol mg^{-1} protein)	1428 ± 360 (25)	89.1 ± 13.5 (29)
Total MAO activity (pM min^{-1} μg^{-1} protein)	378.9 ± 943.8 (25)	286.1 ± 227.1 (30)
MAO-A mRNA (fold change)	1.1 ± 0.2 (30)	1.1 ± 0.28 (30)
mAChR mRNA (fold change)	NA	2.6 ± 1.8 (30)

Hg_T concentration in the temporal cortex (17.5 ± 2.8 mg kg^{-1} dw) compared with cerebellum (12.4 ± 1.6 mg kg^{-1} dw; t(28), $p < 0.05$). There was a significant difference in mean MeHg concentration in the temporal cortex (2.1 ± 0.2 mg kg^{-1} dw) compared with cerebellum (1.5 ± 0.1 mg kg^{-1} dw; t(28), $p < 0.05$). There was a significant difference in mean Se concentration in the temporal cortex (9.1 ± 1.2 mg kg^{-1} dw) compared with cerebellum (8.0 ± 0.8 mg kg^{-1} dw; t(28), $p < 0.05$). There was not a significant difference in mean iHg_{labile} concentrations in the temporal cortex (3.0 ± 0.4 mg kg^{-1} dw) compared with cerebellum (2.6 ± 0.3 mg kg^{-1} dw; t(28), $p > 0.05$). The molar ratio of Hg:Se was correlated to THg concentration in both the cerebellum ($r = 0.87$, $p < 0.0001$) and temporal cortex ($r = 0.92$, $p < 0.0001$).

Cholinergic signaling pathway

Nonspecific binding represented 60% and 15% of total binding in the cerebellar and temporal cortex, respectively. Inter-plate variability ranged from 21% to 23% for mAChR assays. Receptor binding levels were greater in the temporal cortex than cerebellum for mAChR ($z = -4.1$, $p < 0.0001$). Muscarinic AChR binding was negatively correlated to age in the temporal cortex ($r = -0.53$, $p < 0.05$, $n = 18$), but not cerebellum (Fig. 1*a*). Binding of [^{3}H]-QNB to the mAChR in the temporal cortex was predicted ($p < 0.05$) by sampling year but none of the other independent variables, when data were pooled for both sampling years (Table 3). For 2008 samples, mAChR binding was predicted by models that included MeHg ($F_{2,7} = 6.76$; adj $R^2 = 0.56$; $p < 0.05$): log(mAChR) = 3.19 + 0.26(log(MeHg)) – 0.13(log(age)); or Hg:Se molar ratio ($F_{2,7} = 4.7$; adj $R^2 = 0.57$): log(mAChR) = 3.5 + 0.42(log(HgSe)) – 0.18(log(age)). Age, MeHg, and the molar ratio of Hg to Se were significant predictors of mAChR binding ($p < 0.05$) and both models predicted mAChR binding equally well (difference in AIC < 10). Models that included age and THg, iHg_{labile}, or Se did not predict mAChR binding levels ($p > 0.05$). In contrast, in the cerebellum, mAChR binding levels were not predicted by any of the models explored for pooled data (Table 3) or samples from 2008 (data not shown).

Dopaminergic signaling pathway

Total MAO activity was greater in temporal cortex (378.9 ± 943.8 pM min^{-1} μg^{-1}) than cerebellum (286.1 ± 227.1 pM min^{-1} μg^{-1}; $z = -2.5$; $p < 0.05$). Animal age was not correlated

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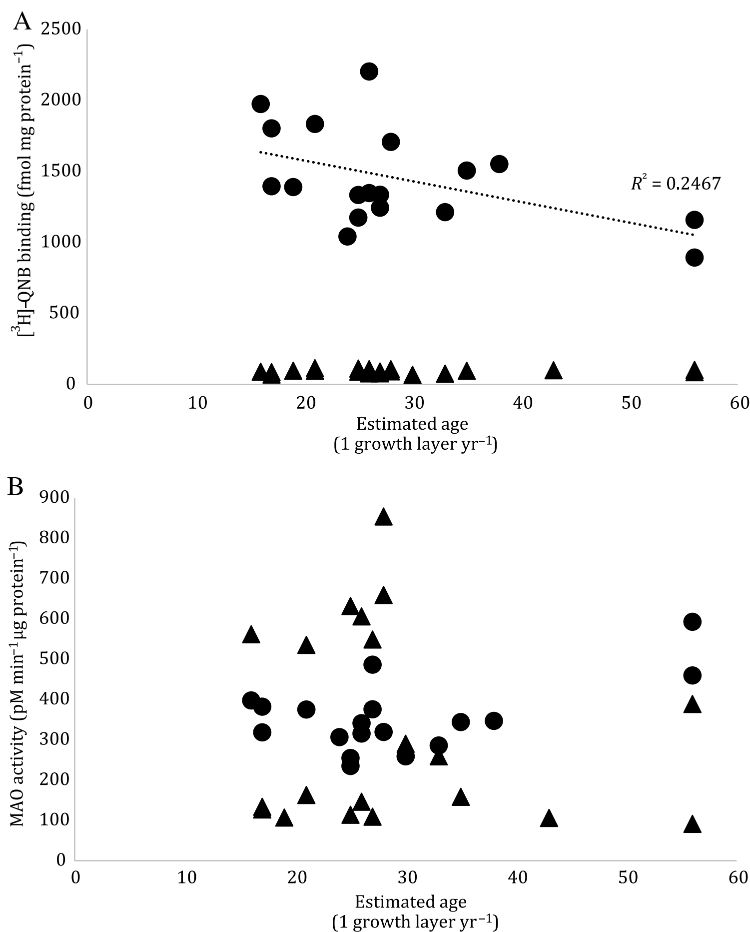
Fig. 1. The correlation between muscarinic acetylcholine receptor binding to [^{3}H]-QNB and estimated age (Panel A), based on tooth analysis (one growth layer per year), in the cerebellum (triangles) and temporal cortex (circles) of beluga whales (*Delphinapterus leucas*). Monoamine oxidase activity and estimated animal age (Panel B) in the cerebellum (triangles) and temporal cortex (circles).



to MAO activity (Fig. 1*b*). In the temporal cortex, MAO activity was significantly predicted ($p < 0.05$) by models that included the following variables: THg ($F_{2,16} = 3.7$, Adj. $R^2 = 0.22$): log(MAO) = 2.0 + 0.5(log(age)) – 0.1 (log(THg)), MeHg ($F_{2,16} = 4.4$, Adj. $R^2 = 0.28$): log (MAO) = 2.1 + 0.4(log(age)) – 0.3(log(MeHg)); iHg_{labile} ($F_{2,16} = 4.0$, Adj. $R^2 = 0.25$): log (MAO) = 2.0 + 0.4(log(age)) – 0.2(log(iHg_{labile})); or THg to Se molar ratio ($F_{2,16} = 5.4$, Adj. $R^2 = 0.33$): log(MAO) = 1.7 + 0.5(log(age)) – 0.4(log(HgSe)). However, the only independent variables that predicted MAO activity were animal age, and MeHg or the Hg:Se molar ratio ($p < 0.05$; Table 4). Models that included animal age and MeHg or Hg:Se molar ratio had similar AIC scores (difference in AIC < 10). In the cerebellum, all models explained variability in MAO activity (Table 4); however, year was the only significant predictor of MAO activity when all samples were pooled (Table 4). When cerebellum samples from 2008 were analyzed separately, MAO activity was significantly predicted by age, but not the other independent variables included in the models (i.e., THg, Hg:Se molar ratio, iHg_{labile}, MeHg or Se; $p < 0.05$).

Table 3. Results from backward stepwise multiple regressions conducted for both brain regions (males only), with binding of [^{3}H]-QNB to the muscarinic acetylcholine receptor (mAChR) and messenger RNA (mRNA) expression of mAChR subtype m1 as the outcome variables. Concentrations of mercury (total mercury (THg), methylmercury (MeHg), labile inorganic mercury (iHg)), molar ratio of Hg to Se (HgSe), or selenium (Se) concentration, and sampling year and animal age were tested as predictor variables.

Dependent variable	Cerebellum				Temporal cortex			
	Adjusted R^2	Intercept	Slope	*F*-value	Adjusted R^2	Intercept	Slope	*F*-value
mAChR	−0.08	1.7	0.05(year) + 0.03(log(THg)) + 0.12(log(age))	$F_{3,16} = 0.53$	0.39	−122	0.06(year)* + 0.07(log(THg)) – 0.23(age)	$F_{3,14} = 4.6^*$
	−0.03	1.8	0.04(year) + 0.09(log(MeHg)) + 0.1(log(age))	$F_{3,16} = 0.79$	0.42	−113.5	0.06(year)* + 0.15(log(MeHg)) – 0.19(age)	$F_{3,14} = 5.1^*$
	−0.09	1.8	0.04(year) – 0.02(log(iHg)) + 0.1(log(age))	$F_{3,16} = 0.49$	0.37	−117.8	0.06(year)* + 0.07(log(iHg)) – 0.21(age)	$F_{3,14} = 4.3^*$
	−0.06	1.8	0.05(year) + 0.08(log(HgSe)) + 0.1(log(age))	$F_{3,16} = 0.61$	0.41	−116	0.06(year)* + 0.21(log(HgSe)) – 0.24(age)	$F_{3,14} = 5.0^*$
	−0.08	1.7	0.05(year) + 0.02(log(Se)) + 0.1(log(age))	$F_{3,16} = 0.48$	0.36	−116	0.06(year)* + 0.07(log(Se)) – 0.21(age)	$F_{3,14} = 4.3^*$
mAChR m1 mRNA	0.7	−0.86	1.0(log(age)) – 0.3(log(Hg))^t	$F_{2,19} = 3.1^t$	NA			
	0.14	−0.95	0.09(log(age))^t – 0.5(log(MeHg))	$F_{2,19} = 2.70^t$				
	0.19	−0.86	1.0(log(age))* – 0.6(log(iHg))^t	$F_{2,19} = 3.4^*$				
	0.30	−1.5	1.0(log(age)) – 1.3(log(HgSe))*	$F_{2,19} = 5.5^*$				
	0.10	−0.72	1.0(log(age))^t – 0.3(log(Se))	$F_{2,19} = 2.2$				

$^*p \leq 0.05$; $^tp \leq 0.10$.

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Table 4. Results from backward stepwise multiple regressions conducted for both brain regions (males only), with total monoamine oxidase (MAO) activity and mRNA expression of MAO A as the outcome variables. Concentrations of mercury (total mercury (THg), methylmercury (MeHg), labile inorganic mercury (iHg)), molar ratio of Hg to Se (HgSe), or selenium (Se) concentration, and sampling year and animal age were tested as predictor variables.

Dependent variable	Cerebellum				Temporal cortex			
	Adjusted R^2	Intercept	Slope	F-value	Adjusted R^2	Intercept	Slope	F-value
Total MAO	0.27	0.97	0.5(year)* + 0.2(log(THg)) + 0.7(log(age))	$F_{3,17}$ = 3.4*	0.22	2.0	0.5(log(age))* − 0.1(log(THg))^t	F_{216} = 3.7*
	0.26	1.1	0.5(year)* − 0.1(log(MeHg)) + 0.7(log(age))	$F_{3,17}$ = 3.3*	0.28	2.1	0.4(log(age))* − 0.3(log(MeHg))*	$F_{2,16}$ = 4.4*
	0.26	1.0	0.5(year) + 0.2(log(iHg)) + 0.7(log(age))	$F_{3,17}$ = 3.4*	0.25	2.0	0.4(log(age))* − 0.2(log(iHg))^t	$F_{2,16}$ = 4.0*
	0.25	1.2	0.5(year)* + 0.1(log(HgSe)) + 0.7(log(age))	$F_{3,17}$ = 3.3*	0.33	1.7	0.5(log(age))* − 0.4(log(HgSe))*	$F_{2,16}$ = 5.4*
	0.28	0.7	0.5(year)* + 0.3(log(Se)) + 0.7(log(Se))	$F_{3,17}$ = 3.6*	0.16	2.1	0.4(log(age))* − 0.1(log(Se))	$F_{2,16}$ = 2.7^t
MAO-A mRNA	−0.1	−0.03	−0.002(year) − 0.09(log(THg)) − 0.2(log(age))	$F_{3,18}$ = 0.77	0.05	−73	0.04(year) + 0.07(log(THg))	$F_{2,22}$ = 1.6
	−0.1	−0.14	0.01(year) − 0.02(log(MeHg)) + 0.1(log(age))	$F_{3,18}$ = 0.2	0.01	−47	0.02(year) + 0.1(log(MeHg))	$F_{2,23}$ = 1.2
	−0.06	−0.1	−0.005(year) − 0.1(log(iHg)) + 0.1(age)	$F_{3,18}$ = 0.63	0.09	−80	0.03(year) + 0.1(log(iHg))	$F_{2,23}$ = 2.2
	−0.08	−0.2	0.01(year) − 0.2(log(HgSe)) − 0.2(age)	$F_{3,18}$ = 0.51	0.12	−67	0.03(year) + 0.2(log(HgSe))	$F_{2,23}$ = 2.7
	0.0002	−0.03	−0.007(year) − 0.12(log(Se)) + 0.16(log(age))	$F_{3,18}$ = 0.8	−0.04	−71	0.03(year) + 0.09(log(Se)) − 0.05(age)	$F_{3,19}$ = 0.69

*$p \leq 0.05$; $^t p \leq 0.10$.

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Messenger RNA transcription levels

The OD ratio was ≥1.8 for all RNA samples; therefore, RNA purity was considered acceptable. However, the median and range of 28S:18S and RQI were 0.83 (0.3–1.87) and 6.9 (3.7–8.9), respectively, for a subset of samples ($n = 12$); therefore, RNA integrity was considered poor to fair. The Ct values for the internal control gene (s9) did not vary with age or THg concentration in the cerebellum or temporal cortex. There was no amplification of NRT control following RT PCR.

In the cerebellum, age was not correlated to mRNA expression for mAChR m1 ($p < 0.05$) and there were no significant differences in mRNA expression for mAChR m1 with sampling year ($p < 0.05$; temporal cortex samples unavailable). Messenger RNA transcription levels for mAChR m1 were predicted by models that included iHg_{labile} ($F_{2,19} = 3.4$, Adj. $R^2 = 0.19$; $p < 0.05$): log(mAChR m1 mRNA) = −0.86 + 1.0(log(age)) – 0.6(log(iHg_{labile})), Hg:Se molar ratio ($F_{2,19} = 5.5$, Adj. $R^2 = 0.30$; $p < 0.05$): log(mAChR m1 mRNA) = −1.5 + 1.0(log(age)) – 1.3(log (HgSe)), (Table 3). Only animal age and the molar ratio of Hg:Se significantly predicted mAChR m1 mRNA transcription levels in the cerebellum ($p < 0.05$). The expression of mRNA for mAChR m1 was not correlated to mAChR binding to [^{3}H]-QNB in the cerebellum ($p > 0.05$).

Total Hg, MeHg, iHg_{labile}, Se, and Hg:Se molar ratio were not significant predictors of MAO-A mRNA levels when samples were pooled for both years, in either brain region (Table 4). The analysis of 2008 samples separately indicated that MAO-A mRNA levels in the cerebellum were only predicted by the concentrations of iHg_{labile} ($F_{1,11} = 6.6$; adj. $R^2 = 0.32$; $p < 0.05$): log(MAO-A-mRNA) = 0.2 – 0.34(log(iHg)); and, Se ($F_{1,11} = 4.8$; adj. $R^2 = 0.2$; $p = 0.05$): log(MAO-A-mRNA) = 0.3 – 0.2(log(Se)). MAO-A mRNA expression was predicted equally well by either model explored (difference in AIC < 10). The models explored did not significantly explain variation in MAO-A mRNA expression in the temporal cortex when samples were pooled (Table 4) or 2008 samples were analyzed separately (data not shown).

Discussion

Mercury and selenium

Total Hg, Hg species, and Se were analyzed in the cerebellum and temporal cortex and the results suggested that the temporal cortex may be at greater risk of MeHg-associated toxicity than the cerebellum, based on greater concentrations of THg and MeHg observed in this brain region. This could have implications for beluga whales as the temporal lobe is involved in primary auditory perception such as hearing (Schacter et al. 2010). Our results suggest that the molar ratio of Hg:Se is a better predictor than THg for mAChR binding levels and MAO activity in the temporal cortex. As the molar ratio of THg to Se approached 1, MAO activity decreased and mAChR binding levels increased. This can be explained by the potential protective effect of Se in the brain; previous analyses of brain tissue from EBS beluga identified a HgSe complex, which was hypothesized to contribute to demethylation and detoxification of MeHg in beluga organs (Lemes et al. 2011). Our findings support the hypothesis that Se may detoxify MeHg. We did not investigate potential deficiencies of Se due to the formation of the HgSe complex; however, Se deficiency could lead to increased oxidative stress in the brain due to decreased glutathione peroxidase activity (Khan and Wang 2009). These results support previous recommendations that molar ratio of Hg:Se be included in wildlife studies to evaluate the biological effects of MeHg exposure (Burger et al. 2013).

Cholinergic signaling pathway

Radioligand-binding assays for the mAChR receptor indicated that mAChR binding in the temporal cortex but not cerebellum was positively associated with MeHg and Hg:Se molar ratio. This was consistent with results from earlier *in vivo* studies that found a

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significant increase in mAChR binding in the occipital cortex and brain stem of captive mink associated with MeHg exposure but no effect on cerebellum in mink (Basu et al. 2006*b*; Basu et al. 2008) or cerebellum, frontal lobes, and occipital lobes in polar bear (Krey et al. 2014). The positive relationship between MeHg and mAChR in mink was attributed to a regulatory response to the accumulation of MeHg in the brain (Basu et al. 2008). The mAChR may play a critical role in physiological processes including thermoregulation, motor function, and feeding (Bymaster et al. 2003; Wess 2004). Therefore, the response of mAChR binding levels to MeHg and the molar ratio of Hg:Se in the temporal lobe of beluga whales raises concerns about whether this exposure may be affecting physiological processes.

Dopaminergic signaling pathway

Monoamine oxidase activity was negatively associated with MeHg in the cerebellum and temporal cortex, and with the molar ratio of Hg:Se in the temporal cortex. Previous in vivo studies have found similar relationships between MeHg exposure and MAO activity, although wildlife studies are uncommon in the literature. In wild river otters, a negative correlation between MAO activity and THg or MeHg concentration was observed in the cerebral cortex but not the cerebellum (Basu et al. 2007*a*), and in wild polar bears, MAO activity in the occipital lobe showed a negative correlative relationship with total Hg concentration (Krey et al. 2014). In laboratory studies, decreased MAO activity was observed in the brain stem of rat pups exposed to MeHg chloride in utero (Beyrouty et al. 2006), and in the cortex and cerebellum of rats exposed to MeHg (Chakrabarti et al. 1998). In addition, blood platelet MAO-B activity was negatively associated with blood THg concentration in fish-eating adults from Lake St. Pierre, Quebec, CA (Stamler et al. 2006). Disruption of MAO activity could lead to downstream impacts on neuronal signaling pathways that involve monoamines that are associated with fight-or-flight response, emotion, motor activity, and cognition (Beyrouty et al. 2006). Therefore, the observed relationship between MAO activity and MeHg in both the cerebellum and the temporal cortex of EBS beluga suggests that MeHg exposure may adversely affect the dopaminergic system in this population of beluga whales.

Messenger RNA transcription

The major findings from this study were that MAO-A mRNA levels in the cerebellum but not temporal cortex were predicted by iHg_{labile} and Se concentrations, and mAChR m1 mRNA expression in the cerebellum was predicted by the Hg:Se molar ratio (temporal cortex not available). The negative relationship between Hg:Se molar ratio and mAChR mRNA expression in cerebellar samples was consistent with the down regulation of mAChR associated with MeHg binding to mAChR in rat cerebellum (Limke et al. 2004). The physiological significance of the relationships between mRNA transcription levels in beluga brain tissue and MeHg exposure is difficult to predict, based on the lack of correlation between mRNA transcription levels and mAChR receptor binding or MAO activity observed in this study. The lack of correlation could be due to varied post-transcriptional mechanisms that convert mRNA to protein, variation in in vivo half-lives of proteins, and the error and noise associated with protein and mRNA experiments (Greenbaum et al. 2003). To fully understand the relationship between mRNA and protein expression, requires a better understanding of the dynamics of protein synthesis and degradation (Greenbaum et al. 2003).

Conclusions

Although previous research suggested that Se may detoxify MeHg in brain tissue of EBS beluga (Lemes et al. 2011), our results suggest that Se is not fully protecting beluga from changes to the cholinergic and dopaminergic signaling pathways associated with MeHg

exposure. In addition, previous research indicated that THg, MeHg, and Se were associated with variation in γ-amminobutyric acid type A receptor binding levels, and THg, iHg_{labile}, and MeHg were associated with the expression of mRNA for $GABA_A$-R subunit α2 in EBS beluga (Ostertag et al. 2014). Therefore, there is increasing evidence that chronic MeHg exposure in beluga whales is associated with variation in components of diverse neurosignaling pathways. The impact of chronic MeHg exposure for beluga health requires further study, especially in light of climate change and increasing global Hg emissions.

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