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Elevated mercury exposure and neurochemical alterations in little brown bats (*Myotis lucifugus*) from a site with historical mercury contamination

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Abstract Despite evidence of persistent methylmercury (MeHg) contamination in the South River (Virginia, USA) ecosystem, there is little information concerning MeHg-associated neurological impacts in resident wildlife. Here we determined mercury (Hg) concentrations in tissues of insectivorous little brown bats (*Myotis lucifugus*) collected from a reference site and a MeHg-contaminated site in the South River ecosystem. We also explored whether neurochemical biomarkers (monoamine oxidase, MAO; acetylcholinesterase, ChE; muscarinic acetylcholine receptor, mAChR; *N*-methyl-D-aspartate receptor, NMDAR) previously shown to be altered by MeHg in other wildlife were associated with brain Hg levels in these bats. Concentrations of Hg (total and MeHg) in tissues were significantly higher (10–40 fold difference) in South River bats when compared to reference sites. Mean tissue mercury levels (71.9 ppm dw in liver, 7.14 ppm dw in brain, 132 ppm fw in fur) in the South River bats exceed (sub)-clinical thresholds in mammals. When compared to the South River bats, animals from the reference site showed a greater ability to demethylate MeHg in brain (33.1% of total Hg was MeHg vs. 65.5%) and liver (8.9% of total Hg was MeHg vs. 50.8%) thus suggesting differences in their ability to detoxify and eliminate Hg. In terms of Hg-associated neurochemical biomarker responses, interesting biphasic responses were observed with an inflection point between 1 and 5 ppm dw in the brain. In the reference bats Hg-associated decreases in MAO ($r = -0.61$; $p < 0.05$) and ChE ($r = -0.79$; $p < 0.01$) were found in a manner expected but these were not found in the bats from the contaminated site. Owing to high Hg exposures, differences in Hg metabolism, and the importance of the aforementioned neurochemicals in multiple facets of animal health, altered or perhaps even a lack of expected neurochemical responses in Hg-contaminated bats raise questions about the ecological and physiological impacts of Hg on the bat population as well as the broader ecosystem in the South River.

Keywords Methyl mercury · South River · Ecotoxicology · Neurochemical biomarkers · Wildlife

Introduction

Methylmercury (MeHg) is a persistent and ubiquitous environmental pollutant that is of neurotoxic concern to both humans (Mergler et al. 2007) and wildlife (Scheuhammer et al. 2007). The neurotoxicity of MeHg is due to its efficient transport across the blood–brain barrier and high affinity for protein thiols which ultimately leads to the disruption of key structural and functional neurological processes. Many studies have established that MeHg, often at environmentally relevant exposure levels, can disrupt the function of the nervous system, cause brain lesions, and impair animal neurobehavior (Clarkson and Magos 2006).

Historic examples of gross MeHg contamination have been documented near industrial point sources, such as the Chisso Corporation chemical factory in Minamata Bay,

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Japan (Eto 2000) or the Dryden chlor-alkali facility and paper mill in the English-Wabigoon River, Ontario (Kinghorn et al. 2007). Despite closure of these facilities, MeHg contamination still persists in the immediately surrounding regions. Another site of note is the former Dupont textile facility located in Virginia. Between 1929 and 1950 it is estimated that up to 100 pounds of mercuric sulfate may have been released daily into the local South River (Carter 1977). Though six decades have passed since the closure of this facility, recent studies have demonstrated that MeHg contamination persists. For example, tissue residue levels in terrestrial riparian organisms (e.g., songbirds, toads) are deemed to be still excessive (Cristol et al. 2008; Bergeron et al. 2010a, b). Another recent study found a moribund river otter that apparently died directly from mercury intoxication (Sleeman et al. 2010). It has been reported that continuous Hg contamination in the South River ecosystem is suspected to have detrimental effects on endocrine and reproductive systems on resident wildlife (Cristol et al. 2008; Wada et al. 2009; Bergeron et al. 2010a).

In addition to the aforementioned wildlife, another species in the South River Ecosystem that may be at risk of MeHg exposure are little brown bats (*Myotis lucifugus*). Although this species was formerly one of the most common bats throughout the northern United States and Canada, white-nose syndrome (Meteyer et al. 2011) and potentially other stressors (Pybur et al. 1986) have significantly reduced its population where it is being considered for listing under the U.S. Endangered Species Act. Bats typically consume between 40 and 100% of their body mass in prey each night (Hickey and Fenton 1996), and prey consists primarily of aquatic insects. Though research in the South River ecosystem has documented that Hg transfer via the terrestrial ecosystem represents an important yet under-studied exposure pathway (Cristol et al. 2008), this has yet to be explored in bats. Consequently, little is known about the potential neurological effects of MeHg on any species of bat.

The present study was carried out to characterize Hg (total and methyl) concentrations in tissues of little brown bats collected from reference (Moscow, VA, Barn, Middle River) and Hg-contaminated (Grottoes, VA, Barn, South River) sites in Virginia (United States). We also explored brain Hg levels associated with several neurochemical biomarkers (monoamine oxidase, MAO; acetyl cholinesterase, ChE; muscarinic acetyl choline receptor, mAChR; *N*-methyl-D-aspartate receptor, NMDAR) that were previously demonstrated as useful for determining sublethal effects in mammals (i.e., mustelids) (Basu et al. 2005, 2007a, 2008) and birds (Scheuhammer et al. 2008; Rutkiewicz et al. 2011).

Materials and methods

Sample collection

Twenty-six adult post-lactating female little brown bats (*Myotis lucifugus*) were sampled on August 12, 2008. Fifteen female bats were collected from the Renkin Barn (South River, VA), which is 25 km downstream from the original textile facility. Reference female bats ($n = 11$) were obtained from the Moscow Barn which is located on the nearby Middle River (VA), on an adjacent river system about 25 km from the contaminated site. The barn in the contaminated site was 0.1 km from the river and the barn of the reference site was 2.0 km from the river. Both rivers have similar flows, bathometry, and chemistry. Both barns were near residential areas, cow pastures, and cornfields. The roosting sites of maternal colonies were either known previously or located by radio-tracking females caught in mist nets on the South River. Bats were captured as they emerged at dusk from two maternity colonies using harp traps and mist-nets or were collected by hand immediately before emergence. Thoracic compression was used to euthanize the bats which is a commonly accepted method for small mammals. We followed guidelines for the capture, handling and care of mammals as approved by the American Society of Mammalogists and our permit provided by the State of Virginia (Animal Care and Use Committee 1998).

Animals were examined for sex, relative age, and reproductive condition. Adult female reproductive condition was determined by examining mammary glands for evidence of pregnancy and lactation. To qualitatively distinguish between young and adult bats, rates of closure of the cartilaginous epiphyseal growth plates in the wings were estimated. For all 26 bats, total body weight, and liver and brain weight were recorded (Table 1). Liver, brain, and fur (<0.1 g fur was sampled from the dorsal and ventral areas) samples were next extracted, placed in sterile polyethylene sample containers and frozen for subsequent analyses of metals and neurochemical biomarkers.

Mercury (Hg) analyses

Fur samples were washed with acetone and then with deionized water and air-dried to constant weight. Subsamples of liver and brain were studied as a 'wet' tissue, and some of them were dried (60°C, 2–3 days) to calculate average moisture content in both tissues. Total Hg (THg) content in each sample was measured as previously established by Nam et al. (2011) using a Direct Mercury Analyzer 80 (Milestone Inc., Shelton, CT). Methyl Hg was measured in liver and brain tissues using a micro-scale

Table 1 Body mass and mercury (Hg) concentrations in tissues from reference and Hg-contaminated South River bats

		Reference ($n = 11$)			South River ($n = 15$)			P value
		Mean ± SD	Median	Range	Mean ± SD	Median	Range	
Whole body	Weight (g)	7.2 ± 1.2	6.7	5.6–9.1	7.8 ± 1.1	7.8	6.2–9.1	0.157
Liver	Weight (g)	0.28 ± 0.10	0.30	0.20–0.50	0.31 ± 0.07	0.30	0.20–0.50	0.360
	THg (μg/g dw)	5.86 ± 2.72	5.40	2.57–12.3	71.9 ± 35.8	69.2	14.0–151	<0.001
	MeHg (μg/g dw)	0.52 ± 0.31	0.41	0.27–1.34	38.6 ± 24.6	38.0	5.9–84.6	<0.001
	MeHg (%)	8.9 ± 2.5	8.9	5.0–12.0	50.8 ± 14.7	49.4	27.5–77.7	<0.001
Brain	Weight (g)	0.12 ± 0.10	0.10	0.05–0.40	0.11 ± 0.04	0.10	0.05–0.20	0.658
	THg (μg/g dw)	0.34 ± 0.17	0.32	0.17–0.66	7.14 ± 4.63	6.35	0.41–18.7	<0.001
	MeHg (μg/g dw)	0.11 ± 0.08	0.09	0.05–0.32	4.72 ± 4.10	3.59	0.37–17.0	<0.001
	MeHg (%)	33.1 ± 16.3	29.6	13.7–66.1	65.5 ± 19.5	70.2	33.7–91.4	<0.001
Fur	THg (μg/g fw)	3.09 ± 1.28	2.84	1.39–5.50	132 ± 94	96	7.3–274	<0.001

Moisture contents: liver (72.8% for reference and 75.0% for South River bats) and brain (72.6% for reference and 75.1% for South River bats)

method that we have previously described in detail (Nam and Basu 2011). As Hg in mammalian fur is comprised nearly of organic Hg (Dietz et al. 2011), here we report on THg values in fur.

For both THg and MeHg, analytical accuracy and precision were determined through the use of Certified Reference Materials (DOLT-3 and TORT-2 from National Research Council of Canada) and intermittent analysis of duplicate samples. Average recovery rates of DOLT-3 and TORT-2 for total Hg were 98.9 ± 4.3% ($n = 61$) and 97.5 ± 5.5% ($n = 23$), respectively. Average recovery rates of DOLT-3 and TORT-2 for MeHg were 98.6 ± 5.7% ($n = 47$) and 97.9 ± 4.7% ($n = 19$), respectively. For measures of THg and MeHg, the relative standard deviation (RSD) was lower than 6% for all replicate measures. The detection limit for the direct Hg analyzer was 0.047 ng, and no sample fell below this limit. Mercury concentrations are expressed for liver and brain as μg/g (ppm) dry wt (dw) and fur as μg/g (ppm) fresh wt (fw).

Neurochemical enzyme activity and receptor binding analyses

Approximately 0.1–0.5 g of whole brains were homogenized for 30 s in ice-cold phosphate buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4). Cellular membranes and tissue homogenates were then isolated for neurochemical analyses as detailed elsewhere (Nam et al. 2010). In all neurochemical analyses outlined below, intra- and inter-plate variation was determined by use of internal controls and less than 20%.

Neurochemical enzyme activities of acetylcholinesterase (ChE) and monoamine oxidase (MAO) were measured according to procedures and technical equipment detailed elsewhere (Adams et al. 2010; Nam et al. 2010). For both assays, fluorescence ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 590$ nm) of the end product resorufin was measured every 5 min, between 30 and 60 min (HTS7000Plus, PerkinElmer). Enzyme activities were expressed as nmol of resorufin formed per min per unit (μg or mg) protein and each sample was assayed in triplicate.

For neurochemical receptor binding assays, cellular membranes were prepared from whole brain tissues of bats. Binding to the mACh receptor (phosphate buffer: 50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) and NMDA receptor (Tris: 50 mM Tris, 100 μM glycine, 100 μM L-glutamic acid, pH 7.4) were performed in the buffers indicated using methods we have described elsewhere (Adams et al. 2010; Nam et al. 2010). Specific binding to both receptors was defined as the difference in radioligand bound in the presence and absence of 100 μM unlabelled atropine and MK-801 for mACh and NMDA receptors, respectively. Binding was reported as fmol of radio-isotope bound per mg of membrane protein (fmol/mg). All samples were assayed in quadruplicate for total and non-specific binding.

Statistical analyses

Normality and homoscedasticity of data were tested by using the Kolmogorov–Smirnov test and the Levene's test (F_{max}-test), respectively. Parametric T-tests after data normalization through log transformation were used to compare two test groups. The relationships among tissue Hg, MeHg, and brain neurochemical data were determined using Pearson correlation tests after data normalization. A p value of less than 0.05 was considered statistically significant in all analyses.

Results and discussion

In the present study, there were significant differences in tissue Hg concentrations between the reference and Hg-contaminated South River bats (Table 1). Hepatic total Hg levels (dw) in South River bats ranged between 14.0 and 151 ppm (mean 71.9 ppm), and were 12-times greater than mean values in the reference bats. These hepatic concentrations were substantially higher than levels (<6 ppm) observed in wild bat populations collected from other known Hg-contaminated sites (Miura et al. 1978; Powell 1983; Gerell and Lundberg 1993; Allinson et al. 2006). These hepatic levels were also greater than values measured in Japanese human residents that succumbed to Minamata disease (Tokuomi 1968; Eto 2000; Ekino et al. 2007). In fur, total Hg levels (fw) ranged between 7.3 and 274 ppm (mean 132 ppm), and these were 42-times greater than mean values in the reference bats. These values were greater than levels found in other Hg-exposed bat populations (Miura et al. 1978; Hickey et al. 2001; Wada et al. 2010) and other mammalian species (e.g., muskrat *Ondatra zibethicus*, mink *Mustela vison*) (Cumbie 1975; Stevens et al. 1997; Basu et al. 2007c). These fur Hg levels were above the toxicity thresholds (e.g., >10 ppm dw) at which neurobehavioral disorders have been reported in rodents (Burton et al. 1977) and captive mink (Wobeser et al. 1976). Of the 15 individual bats sampled from South River, 9 bats had brain Hg that exceeded sub-clinical threshold values (5–20 ppm dw), which may be enough to cause subtle neurological (Wolfe et al. 1998) and neurochemical (Basu et al. 2006, 2007b) effects. One of these bats from the South River had much lower Hg levels, the reasons being unclear. Collectively, these exposure data suggest that South River bats are being exposed to high levels of Hg, and in relation to other studies, these environmental exposures may be associated with (sub)-clinical effects.

When studying Hg risk it is important to resolve the proportion found as organic versus inorganic. Here we found increased levels of organic MeHg in relation to THg in the liver and brain of bats (Fig. 1). Significantly higher percentages of MeHg (of THg) were measured in tissues from South River bats compared to reference ones (Fig. 1). Methyl Hg accounted for 50.8 and 65.5% of the THg in liver and brain, respectively, of contaminated-bats and these percentages were significantly greater than reference bats (8.9% for liver, 33.1% for brain) (Fig. 1). Greater demethylation in bats from reference areas (8.9% MeHg in liver) compared to South River bats (50.8% MeHg in liver) may reflect an adaptive ability to detoxify MeHg given that inorganic Hg binds with selenium (Se) to form an inert crystalline Hg-Se complex (Khan and Wang 2009). Alternatively, high MeHg burdens in South River bats may exceed their demethylation capacity, resulting in a higher percentage of Hg in their livers found in the MeHg form unable to be demethylated via endogenous biotransformation pathways. This is of neurotoxicological relevance because MeHg actively penetrates the blood–brain barrier by conjugating with L-cysteine and exploiting the methionine-uptake pathway in mammals (Aschner and Aschner 1990). This becomes evident when considering that hepatic MeHg levels correlates well with brain MeHg and THg (Fig. 2).

The distribution of Hg in tissue compartments provides useful information in resolving the body burden of Hg and identifying susceptible organs. Such inter-tissue studies also allow the design of monitoring strategies using non-lethal sampling techniques. Here, significant positive correlations were measured between internal tissues (liver and brain) and fur Hg, and the directionality of these relationships were similar between reference and Hg-contaminated bats (Fig. 3). Given that fur Hg is comprised mainly of MeHg (Dietz et al. 2011), fur may be a crucial excretion route for intracellular MeHg as well as a useful predictor to determine approximate internal tissue Hg concentrations.

In regards to the brain, there were several noticeable differences between the two groups of bats in terms of Hg exposure. Mean levels of THg and MeHg were 21- and 43-fold higher, respectively, in the South River bats compared to the reference population (Table 1). The percentage of THg found as MeHg was about twofold greater in the South River bats. As Hg has a high affinity for protein thiols (Castoldi et al. 2001), multiple neural components in the nervous system may be vulnerable to its toxic action in Hg-contaminated South River bats.

When the mean values of the four neurochemical biomarkers were compared between the South River and reference populations, there were no significant differences. However, when data were stratified and analyzed via correlative methods, several interesting results were found. Though Hg-contaminated South River bats had similar enzyme activities and receptor densities that were 93–96 and 84–106%, respectively, of values measured in reference bats, opposing correlations were described for (in brackets, data from reference bats is shown first, followed by MeHg-contaminated bats): MAO ($r = -0.610$; $p < 0.05$ vs. $r = 0.616$; $p < 0.05$), ChE ($r = -0.793$; $p < 0.01$ vs. $r = 0.337$; $p > 0.05$), and mAChR ($r = 0.258$; $p > 0.05$ vs. $r = -0.672$; $p < 0.01$) (Fig. 4). An inflection point being around 1–5 ppm dw total Hg in the brain was observed. These findings suggest that perhaps Hg-associated neurochemical changes differ between individual bats from reference areas and those chronically exposed to high MeHg levels; the latter of which may have either adapted to chronic MeHg exposures or possibly succumbed to long-term exposures with neurochemical systems that have become exhausted. While we are unable to draw firm

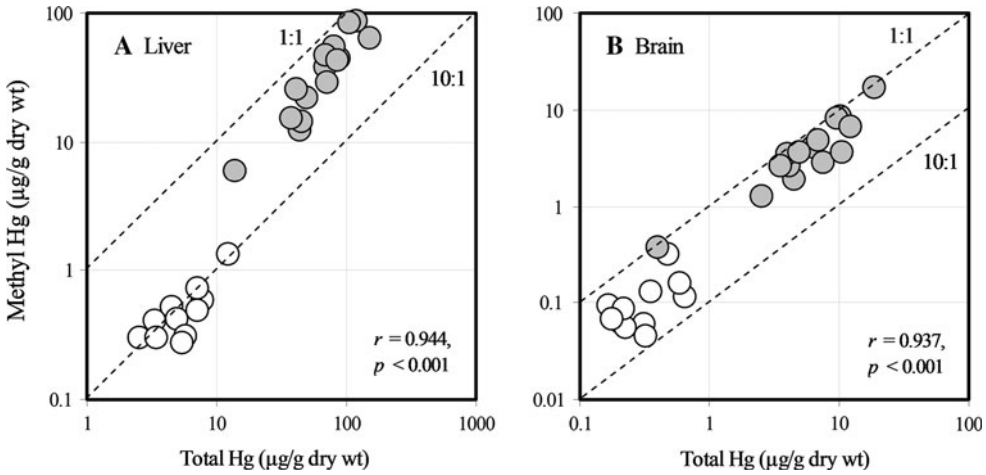

Fig. 1 Relationships between total Hg and methyl Hg in liver and brain of control (*open circles*) and Hg-contaminated (*shaded circles*) bats. As indicated, *dashed lines* refer to theoretical total Hg:methyl Hg ratios of 1:1 and 10:1

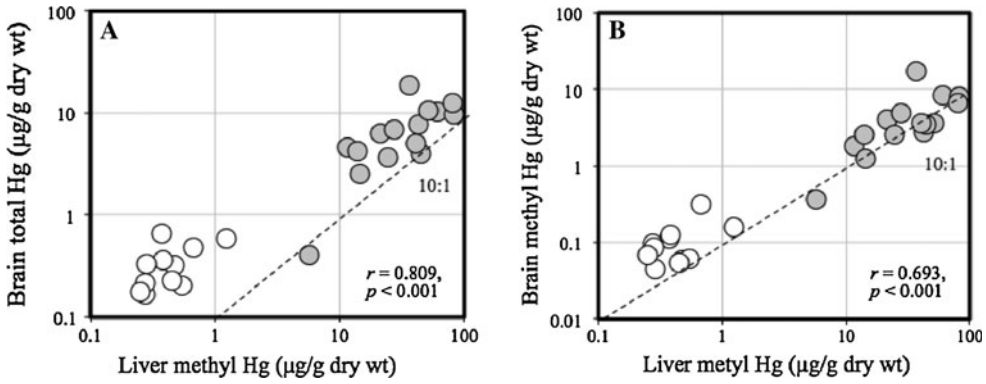

Fig. 2 Relationships of Hg (methyl, total) between liver and brain in control (*open circles*) and Hg-contaminated (*shaded circles*) bats. *Dashed line* refers to a theoretical liver:brain Hg ratio of 10:1

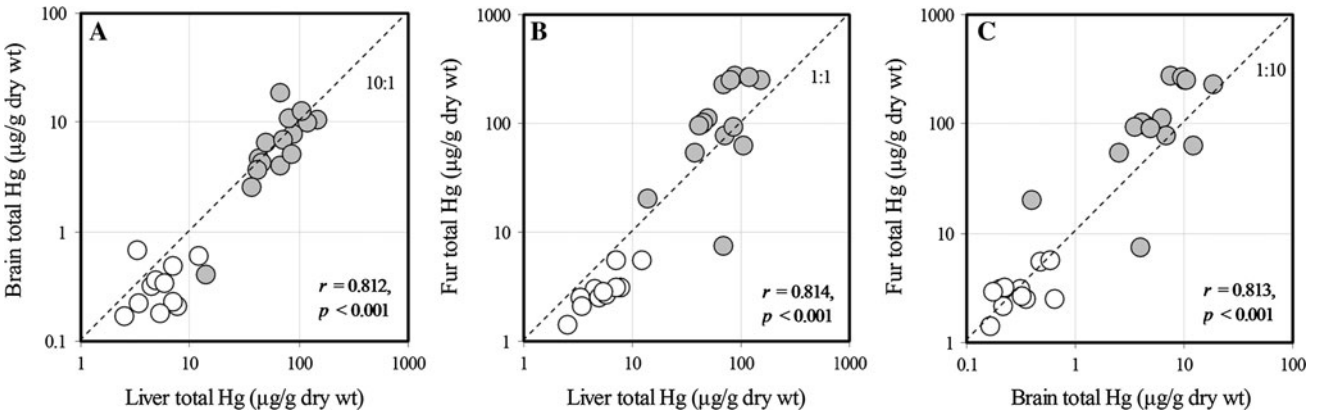

Fig. 3 Relationships among tissue total Hg in control (*open circles*) and Hg-contaminated (*shaded circles*) bats. *Dashed line* refers to a theoretical X–Y ratio, as indicated on the *upper-right side* of each graph

conclusions as to the significance of these neurochemical findings due to a limited sample size, it is established that the studied neurochemical pathways facilitate a range of physiologically important neurobehaviors, including learning and memory, motor function, thermoregulation, and cognition (Missale et al. 1998; Wess 2004), and that chronic exposure to environmentally relevant MeHg levels has the potential to cause significant changes in brain neurochemistry ultimately leading to functional behavioral deficits.

Some interesting comparisons may be made between our findings here and the literature. Consistent with our observations of bats from reference areas, previous studies in river otters also demonstrated Hg-associated decreases in brain MAO activity (Basu et al. 2007a). This reduction in MAO activity is expected to result in a net increase of

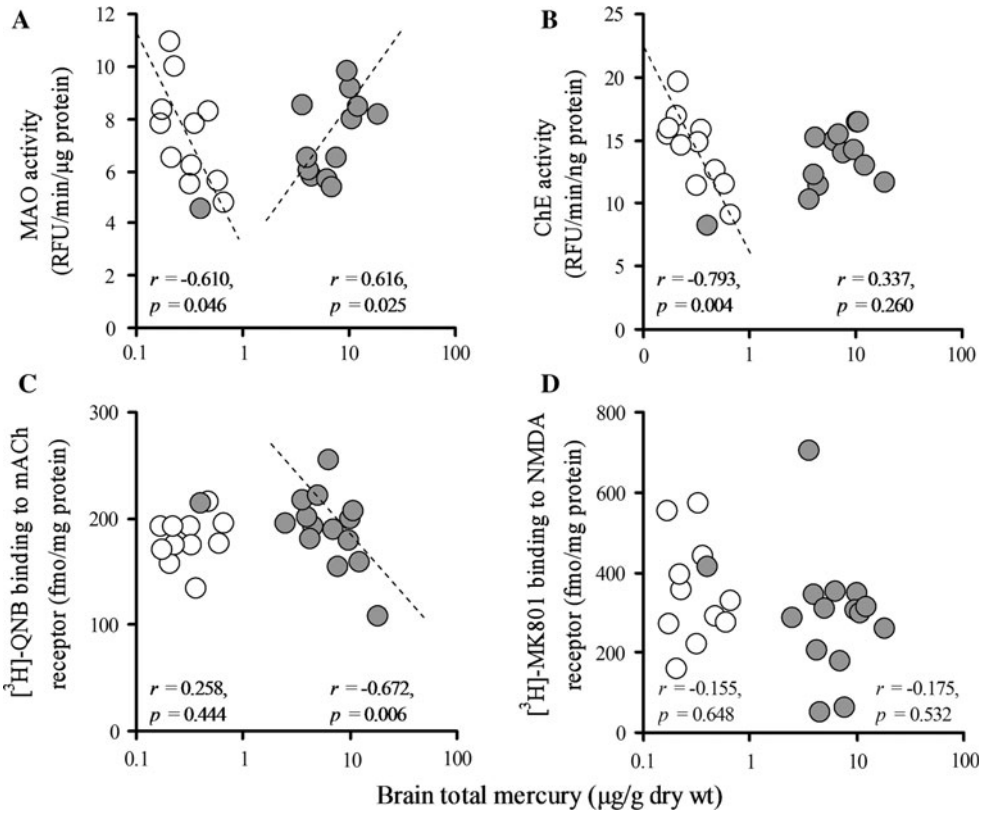

Fig. 4 Comparisons of neurochemical enzyme activities (**a**, **b**) and receptor densities (**c**, **d**) associated with brain total Hg levels of control (*open circles*) and South River (*shaded circles*) bats. Significant *lines* of best fit for a given study site is indicated with *dashes*

synaptic monoamines such as dopamine, and these changes may represent a strategy to ensure that brain dopamine levels remain at homeostatic levels. In contrast to the reference area bats, Hg-associated increases in MAO activity were found in the South River bats. Human epidemiological studies indicate that MAO (specifically MAO-B) activity may increase in concurrence with the progression of Parkinson's disease by enhancing the neurotoxic effects of environmental chemicals (Bonuccelli et al. 1990; Weinstock et al. 2003). To our knowledge, such an increase has not yet been documented in association with Hg exposure in wildlife, though a previous study on common loons (*Gavia immer*) and bald eagles (*Haliaeetus leucocephalus*) found non-significant increases in MAO activity in relation to Hg exposure (Scheuhammer et al. 2008).

Significant changes in cholinergic function were associated with Hg exposure in bats. Specifically, Hg exposure was related to reduced ChE activity in the reference bats (as previously documented in Hg-exposed river otters; Basu et al. 2007a), while there was a lack of enzymatic response in the contaminated bats (Fig. 4). Just like the MAO findings above, Hg-associated reductions of ChE activity may represent a strategy to maintain neurotransmitter homeostasis, and the lack (or inverse finding) of Hg-associated ChE activity or mAChR changes in the contaminated bats (Fig. 4) may be due to exhaustion of this cholinergic system. These biphasic responses of cholinergic systems have also been reported in experimental models exposed to other neurotoxic chemicals, where prolonged exposures superseded the cells inherent ability to overcome cellular stress (Tomlinson et al. 1981; Kumar 1998; Calabrese and Baldwin 2003).

The NMDAR is the main excitatory neurotransmitter receptor in the vertebrate nervous system and plays an important role in synaptic plasticity and in the consolidation of learning and memory (Meldrum 2000). Glutamate-induced excitotoxicity results from prolonged impairment of ion (e.g., Na^+, Ca^{2+}) balance coupled with NMDAR hyperstimulation. Here we did not find a significant association between Hg exposure and NMDAR levels in either the reference or contaminated bats (Fig. 4). However, when data from the two study groups were combined, NMDAR levels were significantly correlated with brain Hg levels ($r = -0.394$; $p < 0.01$). While there is inherent bias in grouping the two groups into one (high and low Hg exposed groups), a negative correlation has also been previously found between Hg and NMDAR levels in a range of wild (e.g., mink, polar bear *Ursus maritimus*, bald eagle) and laboratory (e.g., captive mink) animals (Scheuhammer et al. 2008; Basu et al. 2007b, 2009). Such NMDAR changes were associated with broad ranges of brain Hg exposures (e.g., 0.1–1 ppm dw for polar bear; 1–10 ppm dw for wild mink; 0.1–15 ppm dw for captive mink; 0.1–30 ppm for bald eagle; 0.1–70 ppm dw for common loon), and these could represent one of the most consistent

biochemical responses to Hg exposure in wild animals including bats.

In conclusion, there is increasing concern over exposure of wildlife to MeHg in the South River ecosystem. Here we found that bats from the Hg-contaminated South River ecosystem accumulated extensively higher tissue Hg (total and methyl) levels than bats from a reference site. The tissue Hg (total and methyl) levels found here exceed (sub)-clinical thresholds from studies on other mammalian species. There were differences in the two groups in terms of Hg de-methylation (i.e., higher de-methylation in control bats which is generally associated with an improved capacity to metabolize and eliminate Hg). We also documented evidence of Hg-associated neurochemical changes, with biphasic responses observed for the dopaminergic and cholinergic systems. When compared to reference bats, altered or a lack of neurochemical responses in South River bats may reflect the loss of homeostatic control due to prolonged extensive Hg exposure.

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