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The effects of methylmercury exposure on behavior and biomarkers of oxidative stress in adult mice

Meg Kirkpatrick [a,*], Janina Benoit [b], Wyll Everett [a], Jennifer Gibson [a], Michael Rist [b], Nicholas Fredette [b]

[a] Psychology Department and Neuroscience Program, Wheaton College, 26 East Main Street, Norton, MA 02766, United States
[b] Chemistry Department, Wheaton College, United States

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ABSTRACT

Methylmercury (MeHg) is a widely distributed environmental neurotoxin with established effects on locomotor behaviors and cognition in both human populations and animal models. Despite well-described neurobehavioral effects, the mechanisms of MeHg toxicity are not completely understood. Previous research supports a role for oxidative stress in the toxic effects of MeHg. However, comparing findings across studies has been challenging due to differences in species, methodologies (*in vivo* or *in vitro* studies), dosing regimens (acute *vs.* long-term) and developmental life stage. The current studies assess the behavioral effects of MeHg in adult mice in conjunction with biochemical and cellular indicators of oxidative stress using a consistent dosing regimen. In Experiment 1, adult male C57/BL6 mice were orally administered 5 mg/kg/day MeHg or the vehicle for 28 days. Impact of MeHg exposure was assessed on inverted screen and Rotor-Rod behaviors as well as on biomarkers of oxidative stress (thioredoxin reductase (TrxR), glutathione reductase (GR) and glutathione peroxidase (GPx)) in brain and liver. In Experiment 2, brain tissue was immunohistochemically labeled for 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of DNA oxidation and an indicator of oxidative stress, following the same dosing regimen. 8-OHdG immunoreactivity was measured in the motor cortex, the magnocellular red nucleus (RMC) and the accessory oculomotor nucleus (MA3). Significant impairments were observed in MeHg-treated animals on locomotor behaviors. TrxR and GPx was significantly inhibited in brain and liver, whereas GR activity decreased in liver and increased in brain tissue of MeHg-treated animals. Significant MeHg-induced alterations in DNA oxidation were observed in the motor cortex, the RMC and the MA3.

1. Introduction

Mercury is an environmental contaminant that has become widely dispersed *via* atmospheric transport and deposition. In aquatic environments, inorganic mercury is converted to methylmercury (MeHg), a potent neurotoxin that readily bioaccumulates in food webs. Humans and wildlife are exposed to MeHg primarily through diet, especially through fish consumption; therefore, coastal and riverine human populations with fish-focused diets are at risk from environmental Hg contamination. Although *in utero* exposure to MeHg produces the most profound neurological effects, more subtle impacts have also been observed in adults. For example, substantial dietary MeHg exposure has been documented for riverine people of the Amazonian Basin, and deficits in motor, visual and memory function are related to hair mercury levels in adults from some communities in this region (Lebel et al., 1998; Dolbec et al., 2000; Yokoo et al., 2003).

Laboratory studies using animal models confirm the behavioral changes observed following MeHg exposure, especially locomotor deficits (Bellum et al., 2013; Dietrich et al., 2005). Dietrich et al. (2005) reported that 20 mg/L MeHg in drinking water caused coordinated motor and activity deficits in adult mice measured through hind limb clasp, beam walking, and open field tasks after three weeks of treatment. Also, Bellum et al. (2013) observed reduced latency to fall from a rotating rod (Rotor-Rod) and increased angle of foot placement in aged mice exposed to 1 mg MeHg/kg body weight for five days in mouse chow. MeHg-induced deficits in coordinated movements are in keeping with reports that MeHg causes neurodegeneration in brain areas involved in locomotor behaviors in adult mice, specifically the cerebral cortex (Glaser et al., 2010; Liu et al., 2014; Xu et al., 2005) and cerebellum (Bellum et al., 2013). Locomotor effects, such as impairments in

* Corresponding author.
E-mail address: Kirkpatrick_meg@wheatoncollege.edu (M. Kirkpatrick).

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strength and coordination, are consistent across human and animal populations (Al-Tikriti and Al-Mufti, 1976; Castoldi et al., 2008; Yokoo et al., 2003; Eto, 2000; Karagas et al., 2012) and can therefore serve as a useful experimental model for studying the mechanisms of MeHg toxicity.

A variety of biochemical mechanisms have been implicated in MeHg neurotoxicity including induction of oxidative stress (Farina et al., 2011), calcium dis-homeostasis (Denny and Atchison, 1996), disruption of glutamate pathways (Aschner et al., 2007), and inhibition of neuronal Na+/K+-ATPases (Huang et al., 2008). These mechanisms are likely connected; for example, Huang et al. (2008) found that low-dose oral MeHg exposure in mice led to a concomitant increase in oxidative stress and reduction in Na+/K+-ATPase enzyme activity in brain cells, perhaps due to free radical attack of transporter ATPases. Similarly, uncontrolled release of Ca^{2+} from mitochondria can occur in response to oxidative stress (Duchen, 2000) and reactive oxygen species (ROS) can inhibit glutamate transporters (Aschner et al., 2007). Therefore, oxidative stress appears to be an underlying process in MeHg toxicity that directly harms brain cells and/or promotes other cell-damaging pathways.

Two selenium-dependent redox enzymes, thioredoxin reductase (TrxR) and glutathione peroxidase (GPx), provide targets for MeHg neurotoxicity through the promotion of oxidative stress. TrxR is a key enzyme in the thioredoxin system, which functions in a number of mammalian tissues to regulate cellular redox status (Holmgren and Bjornstedt, 1995). Inhibition of mammalian TrxR by MeHg has been demonstrated both *in vitro* (Carvalho et al., 2008) and *in vivo* (Wagner et al., 2010). Glutathione peroxidase (GPx) mediates the oxidation of glutathione (GSH) and the reduction of H_2O_2 into H_2O. In assays using both mouse tissue and brain cell isolates, MeHg was shown to inhibit GPx and promote lipid peroxidation, a hallmark of oxidative stress (Franco et al., 2009). Therefore, MeHg toxicity is likely facilitated by inhibition of TrxR and GPx, resulting in oxidative stress that disrupts multiple biochemical pathways, causes brain cell death, and ultimately, produces behavioral effects.

Measurement of ROS-mediated oxidative damage in the brain provides evidence of a direct role of oxidative stress in MeHg neurotoxicity. Cellular nucleotide oxidation occurs when ROS react with guanosine nucleotides within DNA strands, producing 8-hydroxyguanosine (8-OH-Gua). To defend against oxidative damage, 8-OH-Gua is removed from intact DNA strands by 8-OH-Gua glycosylase and metabolized by nucleotidases, producing 8-hydroxyl-2′-deoxyguanosine (8-OHdG) as a waste product (reviewed in Evans et al., 2004). 8-OHdG is therefore a product of DNA oxidation caused by ROS that can be used to measure oxidative damage in cells and may also be associated with DNA repair (Bessho et al., 1993). Several studies have shown dose-dependent increases in 8-OHdG-immunoreactivity (8-OHdG-IR) and ROS, along with decreases in antioxidant activity, in MeHg exposed mice (Glaser et al., 2013; Liu et al., 2014; Xu et al., 2012). Collectively, these studies support use of 8-OHdG as a reliable indicator of MeHg-induced cellular damage associated with oxidative stress in rodent brain tissue.

The purposes of this investigation were to use multiple methodologies for examining biochemical and behavioral impacts of methylmercury exposure in an *in vivo* animal model and to use this platform to further examine specific targets in the nervous system. Although previous studies have assessed the impact of MeHg exposure on behavior, measured exposure biomarkers in mammals, and/or investigated the toxicity pathway using histopathology, few studies have combined these strategies. In this study we expand on previous research by simultaneously: (1) assessing behavioral impacts in mice exposed to MeHg through food, (2) assessing MeHg impacts on key redox enzymes, (3) measuring Hg levels in the brain and liver of MeHg-exposed animals, and (4) tying biochemical changes and behavioral deficits associated with MeHg toxicity to specific areas of neural damage (DNA-oxidation) in the brain.

2. Materials and methods

2.1. Animals

The subjects were adult male C57BL/6N mice obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in the Wheaton College Vivarium (Norton, MA, USA). The male mice were approximately 40 days old and 30 g at the beginning of the study. All animals ($n = 50$) were housed in polycarbonate caging and provided Harlan mouse chow, 2016 Teklad Global 16% Protein Rodent Diet and water *ad libitum*. The mice were kept on a 12:12 h light:dark cycle (Lights ON 9:30 pm). All procedures used in these studies adhere to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Wheaton College.

2.2. MeHg treatment

Methylmercury solution (Alfa Aesar, Ward Hill, MA) was diluted in ethanol and a 5 mg/kg/day dose pipetted onto a half of a Mini Teddy Graham cookie (Nabisco; Liang et al., 2009). The cookies were allowed to air-dry overnight to allow the ethanol vehicle to evaporate. Control cookies were treated with the ethanol vehicle only. In each study, all animals were given one cookie (MeHg or control) around the time of Lights OUT for a total of 28 days. Each cage was checked daily to verify that the cookie was ingested. The dose of MeHg (5 mg/kg/body weight) used in the present study was chosen because behavioral impairments were observed in adult mice when 20 mg/L MeHg was administered daily in drinking water (Dietrich et al., 2005). The comparable dose used here (5 mg/kg/body weight) was calculated based on a water concentration of 20 mg/L and an average daily water consumption rate of 7.7 mL/day for a 30 g mouse (Bachmanov et al., 2002). Body weights were recorded each week on the Test Day and the dose was adjusted accordingly. On Day 28 of treatment, all animals were euthanized *via* decapitation. Brain and livers were removed immediately and stored at −20 °C until use.

2.3. Experiment 1: effect of MeHg on locomotor behavior and biochemical biomarkers

All tests were conducted at Baseline, and after 1, 2, 3 and 4 weeks of treatment. All behavioral tests were carried out between 3 and 6 h after Lights OUT and were conducted in the dark under dim red light.

2.3.1. Rotor-Rod

Coordination was assessed using a Rotor-Rod apparatus (San Diego Instruments). Prior to testing, a week-long training phase was implemented. On each training day, each mouse was placed on the stationary rod for 1-min to acclimate, and then underwent three 3-min trials, each separated by a 5-min rest period. Following the training phase, all animals were tested twice a week for 4 weeks. During baseline and treatment testing, each mouse underwent two 3-min trials separated by a 5-min rest period. For all trials, the mice were placed on a stationary rod, and upon start of the trial, rod rotation was set to accelerate to a maximum speed of 16 rpm over the first 20 s of the trial (Carratu et al., 2006). Latency to fall was measured by photo beams in the apparatus and the data were collected using SDI's Rotor-Rod software.