Methylmercury Exposure in Adolescence: Effects on Delay Discounting and Sensitivity to *d*-Amphetamine in Mice

by

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Abstract

Methylmercury (MeHg) is an environmental neurotoxicant that can be used to study how cortical damage during early development affects behavior later in life. The consequences of MeHg exposure in adolescence, a developmental period in which the brain and behavior may be especially vulnerable to MeHg, is unknown. The current experiments were designed to assess the effects of low-level MeHg exposure during adolescence on delay discounting (i.e., preference for small, immediate reinforcers over large, delayed ones) and sensitivity to *d*-amphetamine (a dopamine agonist) using a mouse model. Thirty-six male C57BL/6n mice were exposed to 0, 0.3, or 3.0 ppm mercury (as MeHg) via drinking water from postnatal day 21 through 59, the murine adolescent period. As adults, mice lever-pressed for a 0.01-cc droplet of milk solution delivered immediately and four 0.01-cc droplets delivered after a series of delays for 35 sessions. A dose-response determination of *d*-amphetamine (i.p.; 0.1 – 1.7 mg/kg) followed. An information-theoretic analysis, which does not rely on traditional null-hypothesis testing, was employed to determine the most parsimonious model of the generalized matching equation to describe the data collected. Magnitude-sensitivity estimates were lower for mercury-exposed mice relative to controls, and delay-sensitivity estimates were reduced in the 0.3-ppm group compared to controls and the 3.0-ppm group. Further, *d*-amphetamine dose-dependently reduced delay-sensitivity estimates in all groups but decreased magnitude-sensitivity estimates only in the mercury-exposed groups. Adolescence is a developmental period during which the brain and behavior may be vulnerable to MeHg exposure.

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Chapter 1

Literature Review

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Abstract

Excessive delay discounting, or the preference for smaller-sooner reinforcers over larger-later ones, is implicated in a variety of behavioral disorders. The dopamine (DA) neurotransmitter system is heavily involved in delay discounting, and both acute and prolonged disruptions in DA signaling are associated with enhanced preference for smaller-sooner reinforcers. One way DA signaling may become compromised is by exposure to methylmercury (MeHg), an environmental neurotoxicant found in fish. Gestational MeHg exposure in rodent models is associated with impaired DA signaling and DA reuptake as well as distortions in choice and decision-making in adult animals. Whether this neurobehavioral toxicity is observed following MeHg exposure in adolescence, a developmental period during which DA signaling and choice are particularly plastic, is unknown. The importance of examining the vulnerability of the brain and behavior to adolescent MeHg exposure is reviewed, and two experiments designed to address the effects of adolescent MeHg exposure on delay discounting and sensitivity to *d*-amphetamine (a DA agonist) in a mouse model are described.

Delay discounting and its modulation by dopamine

Delay discounting, which describes the decrease in a reinforcer's effectiveness as a function of its delay, is a process that underlies a variety of health-related behavior disorders. In animal studies, delay discounting is often measured by systematically presenting to an organism a choice between a smaller, immediate reinforcer and a larger reinforcer available after a series of delays (e.g., Evenden & Ryan, 1996). A pattern of choosing the smaller-sooner reinforcer at the expense of the larger-later reinforcer is deemed "impulsive" (Ainslie, 1975), and impulsive choice is associated with disorders linked to maladaptive behavior, such as substance abuse (Kirby, Petry, & Bickel, 1999), drug taking (de Wit, 2009), gambling (Alessi & Petry, 2003), and obesity (Hendrickson & Rasmussen, 2013; Rasmussen, Lawyer, & Reilly, 2010). The distinction between impulsive choice and "self-controlled" choice is not dichotomous, but one of degree. The rate at which a reinforcer's efficacy decreases as a function of delay is a quantitative marker of impulsive choice and can vary along a continuum (Mazur, 1987), with higher rates of delay discounting corresponding to increased preference for smaller-sooner reinforcers (i.e., impulsive choice). The growing interest and relevance of delay discounting in health-related behavior has resulted in numerous preclinical studies on the factors that alter delay discounting (see Madden & Bickel, 2009) with particular focus on neurotransmitters and associated neural substrates that support motivation, such as dopamine (DA).

Though disruptions in many neurotransmitter systems can affect delay discounting (Pattij & Vanderschuren, 2008), dysregulation of DA plays a major role. For example, destruction of DA neurons in the rat prefrontal cortex result in more responses allocated to a smaller-sooner reinforcer (i.e., increased delay discounting) compared to controls (Kheramin et al., 2004). Genetic strain differences in the number of DA receptors are associated similarly with changes in

delay discounting. For example, both Lewis rats (Flores, Wood, Barbeau, Quirion, & Srivastava, 1998) and obese Zucker rats (Thanos, Michaelides, Piyis, Wang, & Volkow, 2008) have fewer DA D2 receptors in the striatum and choose smaller-sooner reinforcers more often in delay discounting tasks compared to controls (Anderson & Diller, 2010; Boomhower, Rasmussen, & Doherty, 2013; Huskinson, Krebs, & Anderson, 2012). Genetically-induced overexpression of the DA transporter (DAT), a presynaptic protein that removes DA from the synapse, in the rat nucleus accumbens also increases delay discounting (Adriani et al., 2009). Both lesion and genetic studies are important because they demonstrate that reduced DA activity, whether by destruction of DA neurons, underexpression of DA receptors, or removal of synaptic DA via DAT, can increase delay discounting and impulsive choice. These studies are limited, however, because genetic manipulation and lesioning of specific areas in the brain are irreversible and may alter other neurobiological processes (e.g., Thanos et al., 2008). Methods that reversibly and specifically target mechanisms of DA neurotransmission are necessary to gain a more detailed characterization of DA's role in delay discounting. One way to temporarily enhance (or diminish) DA signaling without drastically altering other neurobiological functions is through acute drug challenges.

Acute injections of drugs that block (i.e., antagonists) or enhance (i.e., agonists) DA neurotransmission induce transient changes in delay discounting. For example, DA D2-receptor antagonists (e.g., haloperidol, raclopride, and flupenthixol) increase delay discounting and preference for smaller-sooner reinforcers (Boomhower & Rasmussen, 2014; Denk et al., 2005; Wade, de Wit, & Richards, 2000), and DA agonists (e.g., amphetamine and methylphenidate) typically decrease delay discounting and preference for smaller-sooner reinforcers in rats (Richards, Sabol, & de Wit, 1999; van Gaalen, van Koten, Schoffelmeer, & Vanderschuren,

2006; Wade et al., 2000). Some have noted that the effects of DA agonists on delay discounting can depend on a variety of factors, including genetic (Huskinson et al., 2012; Pope, Newland, & Hutsell, 2015) and procedural variables (Cardinal, Robbins, & Everitt, 2000; Maguire, Henson, & France, 2014; Pope et al., in review; Slezak & Anderson, 2009; Tanno, Maguire, Henson, & France, 2014) as well as previous experience with DA agonists (Richards et al., 1999). Baseline dependency, however, may be one explanation of these discrepant findings. It is often the case that DA agonists reduce delay discounting in organisms with high rates of discounting under baseline, but enhance delay discounting when baseline rates of discounting are low (Barbelivien, Billy, Lazarus, Kelche, & Majchrzak, 2008; Huskinson et al., 2012; Perry, Stairs, & Bardo, 2008). The extent to which DAergic drugs alter delay discounting can change also as a result of exposure to certain environmental substances and chemicals, such as high-fat diets (Boomhower & Rasmussen, 2014) or neurotoxic pesticides (Cardona et al., 2006; Cardona, López-Crespo, Sánchez-Amate, Flores, & Sánchez-Santed, 2011). Though no research to date has examined its role in delay discounting, methylmercury (MeHg) is one neurotoxicant that can have life-long, profound effects on DAergic signaling and choice (Newland, Paletz, & Reed, 2008; Newland & Reile, 1999).

Neurobehavioral effects of early-life exposure to methylmercury

Mercury exists in various forms, but the most common way people are exposed is through the consumption of fish containing MeHg (AMAP/UNEP, 2013; EPA, 2001; Renzoni, Zino, & Franchi, 1998). Acute and chronic adult-onset exposure to high concentrations of MeHg has been associated with sizeable sensorimotor deficits in humans (Bakir et al., 1973; Harada, 1995; Kinjo, Higashi, Nakano, Sakamoto, & Sakai, 1993; Takaoka et al., 2008). Though adult-onset MeHg exposure is both harmful and expensive (Bellanger et al., 2013), MeHg exposure

during very early development presents a particular concern because of the continued development of the brain during this time (EPA, 1997; Rice & Barone, 2000). Gestational MeHg exposure can result in irreversible and life-long neurobehavioral effects at doses one to two orders of magnitude lower than those typically studied in adult-onset cases (see Newland, Donlin, Paletz, & Banna, 2006). Broadly, *in-utero* MeHg exposure is associated with learning and cognitive impairments in humans (Axelrad, Bellinger, Ryan, & Woodruff, 2007; EPA, 2013), which suggests that one mechanism of MeHg toxicity may include disruption of brain neurocircuitry.

In experimental models, early-life exposure to concentrations of MeHg that encompass typical exposures in humans can cause a variety of changes in the DA neurotransmitter system. Gestational exposure to MeHg increases DA neurotransmitter levels and DA uptake in the adult rat brain (Bartolome, Whitmore, Seidler, & Slotkin, 1984). During MeHg administration, DA levels increase and DAT function is inhibited in neonatal rat synaptosomes (i.e., isolated synapses *in vitro*) (Bartolome et al., 1982). Once MeHg exposure ends, however, synaptosomal DA uptake significantly increases relative to controls, suggesting an enduring effect on DAT function following MeHg exposure. Similar effects extend even to the pre-adolescent period in which MeHg inhibits DAT function in rat striatal synaptosomes but does not appear to affect total DA production (Dreiem, Shan, Okoniewski, Sanchez-Morrissey, & Seegal, 2009). Neuronal alterations caused by gestational MeHg exposure are accompanied by enhanced sensitivity to DAergic drugs *in vivo*. Several investigations document increased sensitivity to DAT blockers, such as amphetamine (Cagiano et al., 1990; Eccles & Annau, 1982; Rasmussen & Newland, 2001; Rossi, Ahlbom, Ogren, Nicotera, & Ceccatelli, 1997) and cocaine (Reed & Newland, 2009), as well as other DAergic agonists (Giménez-Llort et al., 2001) after gestational MeHg

exposure in rodents. These effects also appear to be relatively specific to DA signaling, as rats exposed to MeHg during gestation were indistinguishable from controls in their sensitivity to drugs that target acetylcholine, norepinephrine, and glutamate neurotransmission as controls (Rasmussen & Newland, 2001; Reed & Newland, 2009). Therefore, the DA neurotransmitter system seems especially vulnerable to MeHg exposure early in life. MeHg-induced changes in DA signaling represent one mechanism of MeHg's toxicity, but there are also functional (i.e., behavioral) manifestations of MeHg exposure—specifically, those related to reinforcement.

Gestational exposure to MeHg is associated with a variety of behavioral impairments in adulthood. For example, response allocation in both adult rats (Newland, Reile, & Langston, 2004; Reed, Paletz, & Newland, 2006) and monkeys (Newland, Yezhou, Logdberg, & Berlin, 1994) exposed to low concentrations of MeHg *in utero* is less sensitive to changes in reinforcement contingencies relative to controls. Gestational MeHg exposure is also associated with increased responding on fixed-ratio and progressive-ratio schedules and prolonged extinction from fixed-interval schedules of reinforcement in rats (Paletz, Craig-Schmidt, & Newland, 2006; Reed & Newland, 2007), which suggests MeHg may alter reinforcer efficacy (Newland, Reed, & Rasmussen, 2015). Though no research has examined the relation between early-life MeHg exposure and delay discounting directly, there is some evidence that gestational MeHg exposure can affect behavioral processes related to delay discounting and impulsivity, such as disrupting response inhibition in rats (Newland, Hoffman, Heath, & Donlin, 2013) and humans (Stewart et al., 2006). In light of the extant literature on MeHg's effects on the brain and behavior, identifying developmental periods of vulnerability to MeHg across the lifespan is particularly important (EPA, 1997, 2013). One vulnerable developmental period that is associated with not only the continued development of the DA neurotransmitter system, but also

increased delay discounting is adolescence. However, the neurobehavioral effects of MeHg exposure during adolescence are virtually unknown.

Adolescence: A period of vulnerability

Adolescence is marked by the continued development of the neural pathways that support choice and decision-making, such as the DA neurotransmitter system. In humans, the adolescent period—typically considered to range anywhere between 10 to 20 years (though this depends on a number of factors; Spear, 2000, 2007b)—is associated with the onset of a reduction in gray matter that is primarily confined to the frontal cortex and striatum (Giedd et al., 1999; Sowell, Thompson, Holmes, Jernigan, & Toga, 1999). Adolescent-onset neuroanatomical changes have been highly conserved phylogenetically and are paralleled in rodents and humans (Spear, 2007b). The age range that is typically considered to encompass the entire rodent adolescent period is postnatal day (PND) 21 through 59 (Laviola, Macrì, Morley-Fletcher, & Adriani, 2003), during which the number of postsynaptic DA receptors peaks around PND 28 and then decreases with use-dependent pruning into adulthood (Brenhouse, Sonntag, & Andersen, 2008; Tarazi & Baldessarini, 2000; Teicher, Andersen, & Hostetter, 1995). DA levels and DAT expression are elevated similarly in 21-day-old rat synaptosomes relative to pre-adolescent levels (Dreiem et al., 2009). Because the adolescent brain seems to be in a state of flux, many consider adolescence to be a vulnerable period that is susceptible to various neurochemical insults that could manifest as risky or impulsive behavior (Chambers & Potenza, 2003; Chambers, Taylor, & Potenza, 2003; Spear, 2007a). Indeed, DA-related neurobiological changes across the adolescent period are associated also with various maladaptive behavior related to delay discounting.

Adolescence coincides with increased risky and impulsive decision-making (Spear, 2000). For example, both adolescent humans (Olson, Hooper, Collins, & Luciana, 2007;

Steinberg et al., 2009) and mice (Pinkston & Lamb, 2011) prefer smaller-sooner reinforcers more often than adults. As the DA neurotransmitter system continues to develop during adolescence, decreases in delay discounting across adolescence and into adulthood co-occur with the maturation of the prefrontal cortex and striatum (Christakou, Brammer, & Rubia, 2011; Green, Fry, & Myerson, 1994). Because delay discounting appears to be highly plastic during adolescence, neurotoxic insults that occur in the adolescent period, such as MeHg exposure, could result in altered delay discounting that persists into adulthood.

The current study

The current experiments were designed to examine the effects of MeHg exposure during adolescence on delay discounting in adulthood in mice. No research to date has examined adolescence as a vulnerable period that is susceptible to the long-term, neurobehavioral effects of MeHg exposure. Because the DAT appears especially vulnerable to MeHg exposure (Dreiem et al., 2009), which can result in increased sensitivity to DAT inhibitors (Cagiano et al., 1990; Eccles & Annau, 1982; Rasmussen & Newland, 2001; Reed & Newland, 2009; Rossi et al., 1997), the effects of *d*-amphetamine (a DAT inhibitor) on delay discounting were assessed. We expected that MeHg-exposed mice would demonstrate increased delay discounting relative to controls and *d*-amphetamine would decrease delay discounting to a greater extent in MeHg-exposed mice compared to controls. Findings from the proposed study would not only add to the literature on MeHg by characterizing the extent of the adolescent period's susceptibility to the neurobehavioral effects of MeHg, but also offer a neurobiological (i.e., altered DA signaling) and behavioral (i.e., increased delay discounting) mechanism for MeHg's toxic effects during adolescence.

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Chapter 2

Effects of Adolescent Methylmercury Exposure on Delay Discounting in Mice

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Abstract

The developing fetus is vulnerable to the neurobehavioral effects of low-level exposure to methylmercury (MeHg), an environmental neurotoxicant, but the consequences of exposure during the adolescent period remain virtually unknown. The current experiments were designed to assess the effects of low-level MeHg exposure during adolescence on delay discounting (preference for small, immediate reinforcers over large, delayed ones) using a mouse model. Thirty-six male C57BL/6n mice were exposed to 0, 0.3, or 3.0 ppm mercury (as MeHg) via drinking water from postnatal day 21 through 59, encompassing the murine adolescent period. As adults, mice lever-pressed for a 0.01-cc droplet of milk solution delivered immediately and four 0.01-cc droplets delivered after a series of delays for 35 sessions. A generalized matching analysis indicated that sensitivity to reinforcer magnitude (i.e., larger-reinforcer preference under equal-delay conditions) was lower for MeHg-exposed mice relative to controls. Estimates of sensitivity to reinforcer delay were reduced (i.e., delay discounting was decreased) in the 0.3-ppm group, but not in the 3.0-ppm group, compared to controls. Adolescence is a developmental period during which the brain and behavior may be vulnerable to MeHg exposure.
Key words: methylmercury, adolescence, delay discounting, impulsivity, impulsive choice

Effects of Adolescent Methylmercury Exposure on Delay Discounting in Mice

Human exposure to methylmercury (MeHg), an environmental neurotoxicant, is a major public health concern in the United States and abroad (Ceccatelli & Aschner, 2012; EPA, 2001, 2013; National Research Council, 2000). Exposure to low doses of MeHg during gestation presents a particular concern because of the development of brain neurocircuitry during this period (Choi, 1991; Rice & Barone, 2000). Disruption of dopamine signaling by gestational MeHg exposure has received much attention (Newland, Reed, & Rasmussen, 2015) as dopamine pathways underlie reinforcement-based learning and decision-making later in life (Dalley, Cardinal, & Robbins, 2004; Robbins & Everitt, 1996). In laboratory models, acquisition of choice (Newland, Reile, & Langston, 2004; Newland, Yezhou, Logdberg, & Berlin, 1994) and behavior that underlies cognitive flexibility (Paletz, Day, Craig-Schmidt, & Newland, 2007; Reed, Paletz, & Newland, 2006) and executive functioning (Newland, Paletz, & Reed, 2008) are significantly impaired in adult animals that were exposed to MeHg during gestation.

It is unknown whether vulnerability to developmental MeHg exposure extends to adolescence, a period during which the brain and behavior continue to mature and develop in humans and nonhumans (Spear, 2000, 2007b). Many consider adolescence to be a period of sensitivity to pharmacological and neurotoxicological insults because of the high degree of neurobehavioral plasticity during this time (Chambers, Taylor, & Potenza, 2003; Spear, 2007a); however, specific behavioral effects of adolescent MeHg exposure remain virtually unexplored. Identifying developmental periods that are susceptible to MeHg exposure and the behavioral mechanisms through which MeHg exposure exerts its effects is not only crucial to public health, but also contributes to a greater understanding of the consequences of interfering with cortical development during critical periods.

Human and nonhuman adolescence are both marked by deficits in executive functioning and maladaptive decision making that may persist into adulthood after exposure to drugs or toxicants (Chambers & Potenza, 2003; Pope, Boomhower, Hutsell, Teixeira, & Newland, 2016; Green, Fry, & Myerson, 1994; Laviola, Macrì, Morley-Fletcher, & Adriani, 2003; Marco et al., 2011; Spear, 2000, 2007b). In rodent models, the adolescent period is considered to be wholly encompassed by the age range of postnatal day (PND) 21 through 59 (Laviola et al., 2003; Spear, 2007a). During this period there are profound changes in the prefrontal cortex and dopamine neurotransmitter systems, and these changes co-occur with increased risky and impulsive behavior (Brenhouse, Sonntag, & Andersen, 2008; Dreiem, Shan, Okoniewski, Sanchez-Morrissey, & Seegal, 2009; Tarazi & Baldessarini, 2000; Teicher, Andersen, & Hostetter, 1995). For example, adolescent mice demonstrate excessive delay discounting (preference for smaller-sooner reinforcers over larger-later ones) relative to adults (Pinkston & Lamb, 2011). High rates of delay discounting are associated with a variety of health-related outcomes in humans and nonhumans, including obesity (Boomhower, Rasmussen, & Doherty, 2013; Lawyer, Boomhower, & Rasmussen, 2015), drug use (de Wit, 2009), and drug addiction (Madden, Petry, Badger, & Bickel, 1997), but the impact of exposure to MeHg on delay discounting is virtually unknown. Though no research has examined the relation between MeHg exposure and delay discounting directly, there is some evidence that early developmental MeHg exposure can affect behavioral processes related to delay discounting and impulsivity, such as decreasing response inhibition in rats (Newland, Hoffman, Heath, & Donlin, 2013) and humans (Stewart et al., 2006). Because delay discounting is highly malleable during adolescence (Adriani et al., 2009; Laviola et al., 2003; Pinkston & Lamb, 2011), exposure to MeHg during the adolescent period could result in permanent alterations in delay discounting that extend into adulthood.

The present experiment was designed to assess the effects of adolescent MeHg exposure on delay discounting in mice. Following adolescent exposure to MeHg via drinking water, adult mice were trained on a rapid acquisition delay-discounting procedure (Pope, Newland, & Hutsell, 2015) in adulthood. Response allocation between a small, immediate reinforcer and a larger reinforcer delivered after a series of six delays were described using the generalized matching equation, a model of choice (Baum, 1974; Grace and Hucks, 2013; McCarthy and Davison, 1988) that has been applied to delay discounting to estimate sensitivity to both reinforcer magnitude and delay simultaneously (Grace, 1999).

Methods

Subjects and MeHg exposure

Thirty-six, 21-day-old, male C57BL/6n mice from twelve litters (three mice/litter) were purchased from Harlan (Indianapolis, IN) and pair-housed in an AAALAC-accredited animal facility under a 12-hr light/dark cycle (lights on at 0630 h) with temperature and humidity control. Upon arrival, mice were given free access to standard chow and the three littermates were randomly assigned to three exposure groups (n = 12 in each): 0 (control), 0.3, and 3 ppm mercury (as MeHg) in drinking water. These doses were selected because they produce approximately 0, 50, and 500 μg/kg/day of Hg (as MeHg), respectively, in mice. These exposures cause irreversible behavioral deficits, but no gross sensorimotor impairment, when exposure occurred during gestation in rats (Newland et al., 2015). MeHg was dissolved in the mice's drinking water as methylmercuric chloride. Exposure lasted through PND 59, and on PND 60 MeHg exposure ceased and all mice were given tap water. Beginning two weeks before and during experimental sessions, mice were maintained at a body mass of 26 (±1) grams.

An additional 21 mice were exposed to MeHg from PND 21 through 59 in an identical fashion as above (n = 3, 0 ppm MeHg; n = 9, 0.3 ppm MeHg, and n = 9, 3.0 ppm MeHg) for brain mercury analyses. Three mice in the 0.3- and 3.0-ppm MeHg groups were euthanized via CO_2 on PND 40, 59, and 90. The three control mice were euthanized via CO_2 on PND 59. Whole brains were dissected out and stored below 0º C. Total brain mercury was measured via inductively coupled plasma (ICP) mass spectrometry at Michigan State University. All procedures were approved by Auburn University's Institutional Animal Care and Use Committee.

Apparatus

Eleven Med Associates® (St. Albans, VT) operant chambers modified for mice were used for data collection. Each chamber was equipped with two retractable levers on the front wall panel with an LED light above each lever and a houselight centered between two Sonalert® tone generators (high tone: 4500 Hz, low tone: 2700 Hz) at the top of the wall. Centered between each lever was an alcove for liquid-reinforcement delivery, in which one or more 0.01-cc presentations of a 3:1 water and sweetened-condensed milk solution (hereafter, milk) were presented when response criteria were met. Each chamber was enclosed in a sound-attenuating cubicle with an air-circulating fan in the upper left corner of the right wall. A Windows® computer in an adjacent room controlled all experimental contingencies with 0.01-sec resolution. Each mouse was assigned a particular chamber for the duration of the experiment and was assigned to one of four squads that were run in sessions at approximately the same time of day (±15 min) Monday through Friday. The number of mice exposed to 0, 0.3, and 3.0 ppm MeHg in each squad was counterbalanced across squads and chambers.

Procedure

Lever-press training. At PND 90, lever-pressing was established using an autoshaping procedure described in Reed et al. (2006). Briefly, a lever (left for half of the mice, right for the others) was extended into the chamber with its corresponding stimulus light illuminated for 30 sec or until the lever was pressed. Upon 30 sec or a lever-press, the lever was retracted, the stimulus light was extinguished, and sweetened condensed milk was delivered as the high tone (4500 Hz) sounded. A 5-min intertrial interval separated each lever presentation. After ten lever-presses occurred, the timed-delivery of reinforcement was removed and a fixed-ratio (FR) 1 schedule of reinforcement was imposed. Lever-pressing was considered trained when 40 responses occurred on the FR1 schedule during a single 60-min session. Lever-press training for the opposite lever occurred in a similar manner, and whether the left or right lever was trained first was counterbalanced across subjects. If fewer than 40 responses occurred on a lever for five sessions in a row, hand shaping (i.e., reinforcement of successive approximations) was employed until 40 responses occurred on a lever in a single session.

Delay discounting. After lever-press training, mice began a two-lever choice procedure as described in Pope et al., (2015). Briefly, sessions were structured such that (a) subjects could lever-press for access to a small or large reinforcer, (b) the smaller reinforcer was delivered after a 1.26-s delay, (c) six different delays to the larger reinforcer were presented in a random order across a session, with each delay signaled by a unique auditory stimulus, and (d) mice experienced the smaller and larger reinforcers an equal number of times—an important variable to control in delay discounting procedures (see Cardinal, Daw, Robbins, & Everitt, 2002; Maguire, Henson, & France, 2014; Tanno, Maguire, Henson, & France, 2014).

Preliminary training began immediately after autoshaping ended. Sessions consisted of six blocks, and each block comprised twelve choice trials during which mice could select one

lever that resulted in one 1.2-sec milk presentation and another lever that produced four 1.2-sec milk presentations, each separated by 0.5-sec intervals. At the start of a trial, both levers extended into the chamber, and the stimulus lights above each lever illuminated. The computer then selected randomly which lever-press would be reinforced on that trial. The same lever was selected for reinforcement no more than three trials in a row. Assigning which lever was active ensured that a mouse was allowed to respond freely between the two levers, and that it experienced each alternative six times in a twelve-trial block. After a single lever-press on the computer-selected lever, the opposite lever retracted and its associated stimulus light extinguished, the stimulus light above the selected lever began blinking (0.5 sec on/off), and one or four presentations of milk occurred after 1.26 sec. Responses on the other lever were recorded but had no other consequences. Following reinforcer delivery, a 3-sec intertrial interval occurred during which both levers retracted and both stimulus lights were extinguished until the next trial. A 10-sec interblock interval separated each block of twelve trials during which both levers were retracted and both stimulus lights were extinguished. A session ended after the completion of six blocks (72 trials) or 75 min, whichever came first. To reduce lever bias, each mouse completed preliminary training with both levers (left and right) being associated with the larger reinforcer for 10 sessions each. The lever that was first associated with the larger reinforcer was counterbalanced across mice and exposure groups. One purpose of preliminary training was to assess the effects of reinforcer magnitude on response allocation when the delay to both reinforcers was equal (i.e., 1.26 sec).

The delay-discounting procedure began after preliminary training. These sessions were identical to the preliminary-training procedure with the exception that six, differentially signaled, geometrically-spaced delays (1.26, 2.82, 6.31, 14.13, 31.62, and 70.79 sec) to the larger

reinforcer were interposed. At the start of a block, the computer selected randomly (without replacement) one of the six delays to the larger reinforcer to be in effect throughout a block. The delay to the smaller reinforcer remained 1.26 sec throughout the session. Each delay to the larger reinforcer had a specific low/high-tone combination pulsating on and off for varying durations associated with it (see Table 1). The delay-specific tones remained present throughout a trial, but not during interblock intervals. The lever associated with the larger reinforcer (left or right) remained constant throughout the experiment. The delay discounting procedure was in place for 35 sessions to allow behavior to stabilize.

Data analysis

The primary dependent variable was the ratio of responses on the larger-later reinforcer lever to those on the smaller-sooner reinforcer lever. All data were taken from the last block of four trials. This measure of preference was quantified using the generalized matching equation (Grace, 1999), which describes the ratio of responses allocated to two alternatives as a function of the ratios of delay to and magnitude of the reinforcers derived from those two alternatives:

$$\log\left(\frac{B_l}{B_s}\right) = s_M \log\left(\frac{M_l}{M_s}\right) - s_D \log\left(\frac{D_l}{D_s}\right) \quad (1)$$

where B is the number of responses, M is the magnitude of reinforcement, D is the delay to reinforcement, and the subscripts l and s refer to the larger and smaller alternatives, respectively. The coefficients s_M and s_D are free parameters and represent sensitivity to magnitude (s_M) and sensitivity to delay (s_D). s_D is the slope of the linear relation between log response ratio and log delay ratio, $D_l/1.26$, because the shorter delay is always 1.26 s. s_M affects the Y-intercept of the line because M_l/M_s was always equal to 4/1. From Eq. 1, we would expect preference for the larger reinforcer to decrease as the delay to it increases, hence the negative sign. There is not a separate term for unexplained bias in this equation because bias is incorporated into s_M. Position

biases were minimized, however, by verifying that both response devices require equivalent amounts of force and by providing a history in which both the left and right levers are associated with the larger reinforcer in preliminary training (Pope et al., 2015).

An information-theoretic approach was used to assess differences in responding among the three MeHg exposure groups (Burnham & Anderson, 2002). This model-comparison analysis has been growing in popularity in the behavioral and neural sciences to model drug and neurotoxicant effects (e.g., Avila, Reilly, Sanabria, Posadas-Sanchez, Chavez, Banerjee, et al., 2009; Pope & Newland, under review; Sanabria, Acosta, Killeen, Neisewander, & Bizo, 2008). It does not rely on traditional null-hypothesis testing. Instead a model-comparison approach allows the user to determine the best, most parsimonious model of all ones investigated to describe the data collected. In this analysis, the best model given the data is determined by calculating the corrected Akaike information criterion (AICc), which takes into account the degree of fit of a model (i.e., residual sum of squares, RSS) and penalizes additional free parameters (*k*) in the model for a given number of data points (*n*) (Burnham & Anderson, 2002). The lowest AICc value indicates a better account of the data given the degrees of freedom in the model. Because the calculation of the AICc for one model is independent of another model's, multiple models can be tested simultaneously in this approach without the need for corrective measures (e.g., Bonferroni).

Log_{10}-transformed response ratios, calculated by dividing the responses on the larger-later (L) reinforcer lever by the responses on the smaller-sooner (S) reinforcer lever, from the last five sessions of the baseline period were averaged for each subject. Similarly, log_{10}-transformed delay ratios were calculated by dividing the larger-reinforcer delay by the smaller-reinforcer delay of 1.26 s. Response ratios from the final block of four trials were compared across

exposure groups by fitting Eq. 1 to individual-subject data using least squares regression. First, the full model that allowed s_M and s_D to vary across exposure groups was evaluated. Then, reduced models, in which s_M and/or s_D remained constant for two or all exposure groups was assessed. The model that resulted in the lowest AICc was considered the best model. To ensure that the best of the hypothesized models actually provided a reasonable fit to the data, the line from that model was plotted against the actual data, and AICc weights (w)—the probability that a given model was the best model given the data (Burnham & Anderson, 2002)—were calculated.

Results

Figure 2.1 shows mean dose of mercury, body mass, and water consumption as a function of postnatal day for the 0.3- and 3.0-ppm MeHg groups during the adolescent exposure period. Doses were estimated for individual mice, and spillage was accounted for using a sham water bottle. Overall, MeHg dose was highest for both groups at the beginning of adolescence (PND 21 to 26) and then stabilized to approximately 50 and 500 μg/kg/day for the 0.3- and 3.0-ppm exposure groups, respectively. This was accompanied by a rapid increase in body mass between PND 21 and 36 and a relatively constant level of water intake.

Figure 2.2 shows mean brain mercury content midway and at the end of the exposure period as well as at the time of behavioral testing, 31 days after exposure ended. Both exposure groups showed increasing brain mercury across the exposure regimen. At PND 90, brain mercury was undetectable (i.e., <0.1 ppm) in the 0.3-ppm MeHg group and approximately 0.11 ppm in the 3.0-ppm MeHg group.

Following exposure, all groups acquired lever-pressing at similar rates, with approximately 1-2 mice in each group requiring hand shaping. During preliminary training, estimates of magnitude sensitivity were similar for all groups both before (range of s_M: 0.28 -

0.39) and after (range of s_M: 0.34 - 0.46) the larger reinforcer was switched to the opposite lever, suggesting there was no bias for responding on one lever over another. Magnitude-sensitivity estimates at the end of preliminary training also were similar across exposure groups (range of s_M: .33 - .42).

Table 2.2 shows the seven models that resulted in the lowest AICc values of 25 that were tested. The full models in which only one or both parameters varied across all exposure groups resulted in lower AICc values relative to the model in which both s_M and s_D remained constant (not shown; AICc = -658.33). The best full model was model 3 in which both parameters varied across all exposure groups (AICc = -836.18). Further modeling revealed that the best reduced model was one in which s_M varied across dose of MeHg, and one s_D was used for the 0.3 ppm MeHg-exposed mice and a separate s_D was used for controls and 3.0 ppm MeHg-exposed mice (model 1). Of all 25 models, the likelihood that model 1 was the best model given the data was 84% with the second-best model only being 7% likely given the data.

Figure 2.3 shows log response ratio as a function of log delay ratio for each mouse. Each panel shows responding from three littermates. For all mice, larger-reinforcer preference decreased as a function of delay. The best model of Eq. 1 (model 1) was fit to individual-subject data (shown as lines) and described the data well for 0 ppm (M_{RSS} = 0.06, *SEM* = 0.01), 0.3 ppm (M_{RSS} = 0.03, *SEM* = 0.01), and 3.0 ppm MeHg-exposed mice (M_{RSS} = 0.07, *SEM* = 0.01). The constraints of the best model, which specified that s_M vary across dose of MeHg and a separate s_D for the 0.3 ppm MeHg-exposed mice, are evident in Figure 2.2: Y-intercepts vary for each littermate, reflecting changes in s_M, and slopes (s_D) remain constant (i.e., parallel) for control and 3.0 ppm MeHg-exposed littermates and vary for the 0.3 ppm MeHg-exposed littermate.

To better visualize how group responding changed as a function of MeHg dose, Figure 2.3 shows mean larger-reinforcer choice as a function of delay (left panel) and log response ratio as a function of log delay ratio (right panel). The data are shown in two formats. The left panel displays the delay-discounting data in a more traditional format, with proportion of larger-reinforcer choice shown as a function of delay. The right panel shows the data expressed as in Eq. 1, and shows how using the log response ratio and log delay ratios linearizes the function. Mean predictions of Eq. 1 according to the best model are shown as lines. Overall, larger-reinforcer choice decreased as a function of delay for all groups. MeHg-exposed mice on average displayed lower preference for the larger-reinforcer under equal-delay conditions relative to controls.

Figure 2.4 shows mean estimates of magnitude and delay sensitivity for MeHg-exposed mice. According to the best model (model 1), magnitude sensitivity (s_M) decreased as MeHg dose increased. Delay sensitivity (s_D) was lowest for the 0.3-ppm MeHg-exposed mice and equivalent for controls and 3.0 ppm MeHg-exposed mice.

Discussion

Mice were exposed to MeHg during the adolescent period and delay discounting was measured in adulthood. Mice exposed to either 0.3 or 3.0 ppm MeHg during adolescence displayed an overall similar pattern of dosing (Fig. 2.1). The dose of MeHg consumed was highest during the first week of exposure and then decreased, stabilizing at approximately 50 and 500 μg/kg/day for the 0.3 and 3.0 ppm groups, respectively. The high dosing at the beginning of adolescence was due to elevated fluid consumption immediately after weaning or during early adolescence (see also Adriani et al., 2002; Hefner & Holmes, 2007) coupled with low body mass in these young mice. Decreases in dosing occurred as body mass increased and fluid

consumption stabilized across adolescence. The exposure regimen employed here resulted in brain mercury concentrations at the end of exposure that were approximately 0.3 ppm and 1.5 ppm mercury in the 0.3-ppm and 3.0-ppm exposure groups, respectively (Fig. 2.2). Brain mercury at the time of behavioral testing (PND 90) was undetectable in the 0.3-ppm MeHg exposed mice and approximately 0.11 ppm in the 3.0-ppm MeHg exposed mice. The brain mercury concentrations at the end of exposure were slightly lower than brain mercury levels following gestational MeHg exposure in rats (Newland & Reile, 1999; Newland et al., 2006).

The present study employed a model-comparison analysis (Burnham & Anderson, 2002), a robust statistical approach that does not rely on null-hypothesis testing and has been growing in popularity in the neurobehavioral sciences (e.g., Avila, Reilly, Sanabria, Posadas-Sanchez, Chavez, Banerjee, et al., 2009; Pope & Newland, under review; Sanabria, Acosta, Killeen, Neisewander, & Bizo, 2008), to examine responding following adolescent MeHg exposure. The model that allowed magnitude sensitivity (s_M) to vary across all exposure groups and delay sensitivity (s_D) to vary only for the 0.3 ppm MeHg group was the best model (Table 2.2). Relative to the other model variants tested, this final model (model 1) was 84% likely given the data and fit the data well (Fig. 2.3). The next-best models were 7% likely or lower given the data, and these models either drastically misfit the data (e.g., model 2) or did not justify the additional parameters estimated (i.e., had a more positive AIC value; see model 3). Thus, all behavioral data were interpreted using the constraints of model 1 on parameter estimates.

The differences in responding among the exposure groups were present. Adolescent MeHg exposure was associated with reductions in magnitude sensitivity (Fig. 2.5), which was evidenced in increased preference for the smaller reinforcer under equal-delay conditions relative to controls (Fig. 2.4). Two accounts of the low magnitude sensitivity in the exposed mice can be

offered. First, adolescent MeHg exposure may have diminished the efficacy of reinforcement. Past studies have demonstrated gestational MeHg exposure is associated with enhanced breakpoints and higher response rates for food in rats (Paletz, Craig-Schmidt, & Newland, 2006; Reed, Banna, Donlin, & Newland, 2008), suggesting gestational MeHg exposure increases the impact of reinforcement on responding (Newland et al., 2015). The present results suggest that MeHg's effects on reinforcer efficacy are in the opposite direction following adolescent exposure and represent a distinction between the behavioral effects of gestational and adolescent MeHg exposure. A second possibility is that adolescent MeHg exposure enhanced perseveration and behavioral inflexibility. Gestational MeHg exposure increases perseverative errors in spatial discrimination reversal tasks in rats (Newland, Paletz, & Reed, 2008; Reed, Paletz, & Newland, 2006). Because magnitude-sensitivity estimates decreased due to MeHg exposure in the current study, this would suggest enhanced perseveration on the smaller-reinforcer lever in exposed mice. In this account, the impact of adolescent MeHg exposure would resemble that of gestational exposure.

Estimates of delay sensitivity were also reduced in mice exposed to 0.3 ppm MeHg (Fig. 2.5). This indicated that delay discounting was reduced in these mice. Stated differently, preference for the larger reinforcer decreased more slowly with increases in delay in that exposure group as compared with the other two groups. This experiment is the first to date to demonstrate that MeHg exposure affects delay discounting in adulthood. Previous reports have shown that gestational MeHg exposure decreases response inhibition—another facet of impulsivity—in adult rats (Newland et al., 2013). It is unclear why delay discounting was reduced in the 0.3-ppm group, but not the 3.0-ppm group; however, some evidence suggests that the effects of different doses of MeHg exposure on behavior are non-monotonic. For example,

Bourdineaud et al. (2008) found that diets containing less than 0.3 ppm MeHg enhanced anxiety-like behavior in adult mice exposed for one month beginning on postnatal day 21, but a diet containing 0.52 ppm MeHg resulted in no behavioral change. Bourdineaud et al. (2008) demonstrated this discrepancy may be due to a dose-specific, non-monotonic, MeHg-induced alteration in gene expression; lower levels of MeHg resulted in one pattern of hippocampal gene expression and higher levels of MeHg result in a different pattern.

Many studies have noted that the effects of gestational MeHg exposure on choice are revealed by challenges to the nervous system, such as aging or acute exposure to stimulants (Newland & Rasmussen, 2000; Rasmussen & Newland, 2001; Reed & Newland, 2009). These challenges might reveal "silent damage" by early-life MeHg exposure and explain why some signs and symptoms of exposure only present themselves in the geriatric period (Weiss, Clarkson, & Simon, 2002). Mice in the present experiment were tested as adults (approximately PND 90 to 140), and it could be that the 3.0-ppm dose would alter delay discounting if subjects were allowed to age further.

Results from the present experiment should be interpreted with some limitations in mind. First, the observed impairments in delay discounting may not have been specific to adolescent exposure but may be a result of chronic exposure to MeHg during the lifespan. No reports to date have systematically compared the effects of adolescent and adult MeHg exposure on behavior. Second, exposure began on PND 21 and continued through 59 in the present study, an age range selected because it is generally considered to encompass the entirety of murine adolescence (Laviola et al., 2003; Spear, 2007a). More nuanced estimates of different periods of rodent adolescence exist (see Adriani & Laviola, 2004), and it is not clear whether MeHg's effects are

confined to windows within adolescence, for example, pre- (or late juvenile), mid-, and post-adolescence (Adriani & Laviola, 2004), or whether exposure must span the entire period.

Limitations aside, the present experiment extends the literature on the behavioral consequences of low-dose MeHg exposure. Specifically, a novel behavioral mechanism (i.e., delay discounting) of MeHg's effects was examined following exposure during adolescence, an underlooked developmental window in the MeHg literature. Results from the present experiment suggest adolescent MeHg exposure alters delay discounting— behavior that underlies executive functioning and decision making—in adult mice, which may have important implications for public health. The current experiment represents a first attempt to assess the behavioral toxicity of adolescent MeHg exposure. More research is needed to determine the extent of this toxicity and the underlying neurobiological mechanisms that permit it.

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Tables

Table 2.1

Low/high tone combinations associated with each delay in the delay-discounting procedure.

Delay to larger reinforcer (sec)	Low/high tone durations (sec)
1.26	0.15/1.19
2.82	0.74/0.60
6.31	0.92/0.42
14.13	1.04/0.30
31.62	1.13/0.21
70.79	1.19/0.15

Table 2.2

Results of the model-comparison approach using the generalized matching equation (Eq. 1). The seven models that resulted in the lowest AICc are shown. The free parameters s_M and s_D were allowed to vary across all exposure groups ("vary"), vary for one exposure group (e.g., "0.3 ppm"), or remain constant across all exposure groups ("constant"). The best model is bolded.

Model	RSS	k	n	AICc	Δ AICc	w_i
1. s_M (vary), s_D (0.3 ppm)	**1.99**	**61**	**216**	**-841.52**	**0.00**	**0.84**
2. s_M (3.0 ppm), s_D (0 ppm)	2.49	49	216	-836.42	5.10	0.07
3. s_M (vary), s_D (vary)	1.61	73	216	-836.18	5.34	0.06
4. s_M (3.0 ppm), s_D (vary)	2.06	61	216	-834.11	7.41	0.02
5. s_M (vary), s_D (3.0 ppm)	2.07	61	216	-833.18	8.35	0.01
6. s_M (vary), s_D (constant)	2.61	49	216	-826.09	15.43	≈ 0
7. s_M (3.0 ppm), s_D (3.0 ppm)	2.65	49	216	-822.86	18.67	≈ 0

RSS = residual sum of squares; k = number of parameters; n = number of data points; AICc = corrected Akaike Information Criterion; Δ AICc = difference in AICc relative to the best model's AICc; w_i = probability that the i^{th} model is the best model given the data

Figures

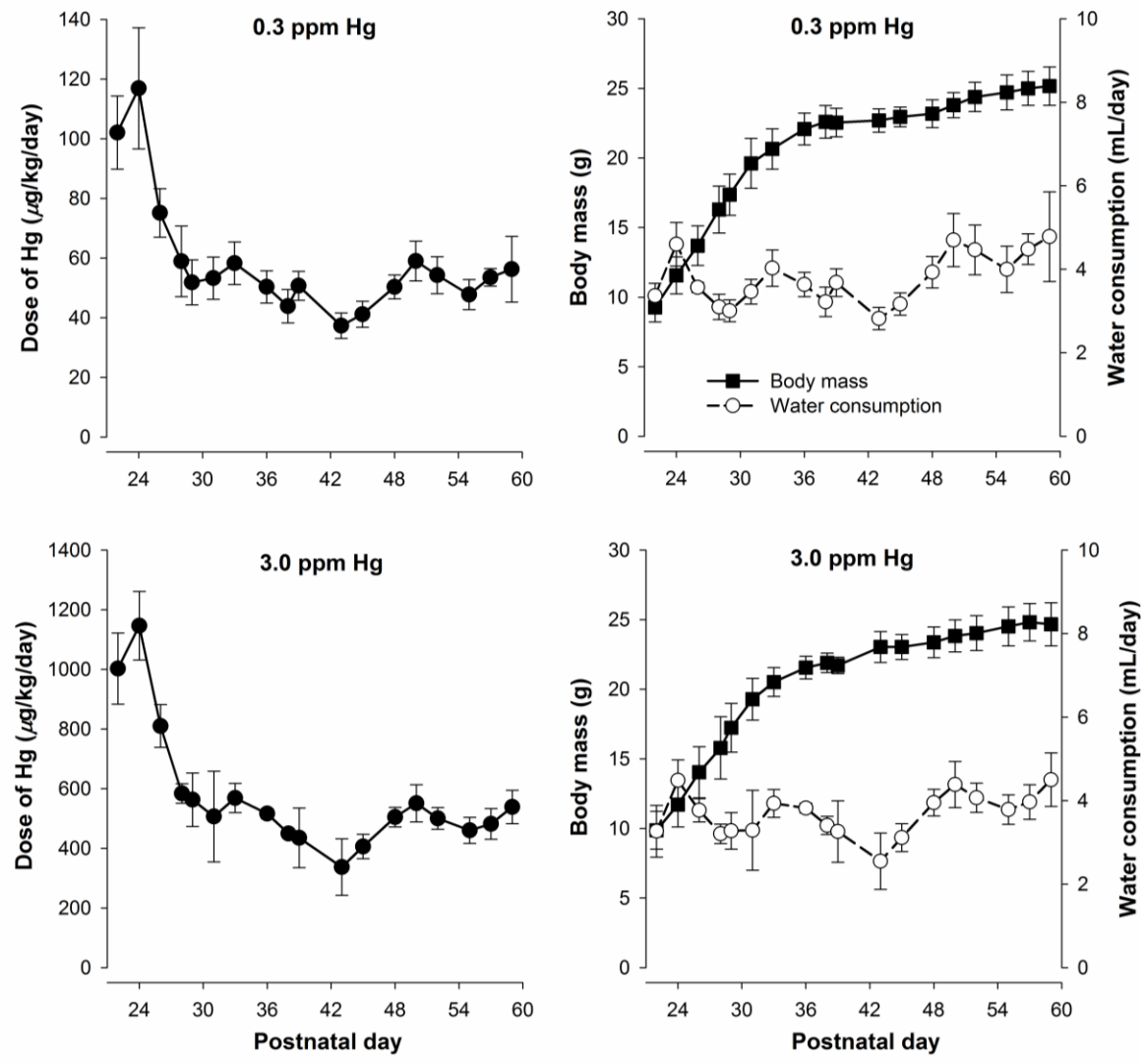

Figure 2.1

Left panels: Mean (±SD) dose of MeHg as a function of postnatal day for mice exposed to 0.3 (top) and 3.0 ppm MeHg (bottom) during the adolescent period (postnatal day 21 through 59). Doses were estimated for an individual mouse as mice were pair-housed. Right panels: Mean (±SD) body mass and water consumption throughout adolescence. Note the y-axes in the left panels differ by an order of magnitude and all error bars represent one SD rather than one SEM.

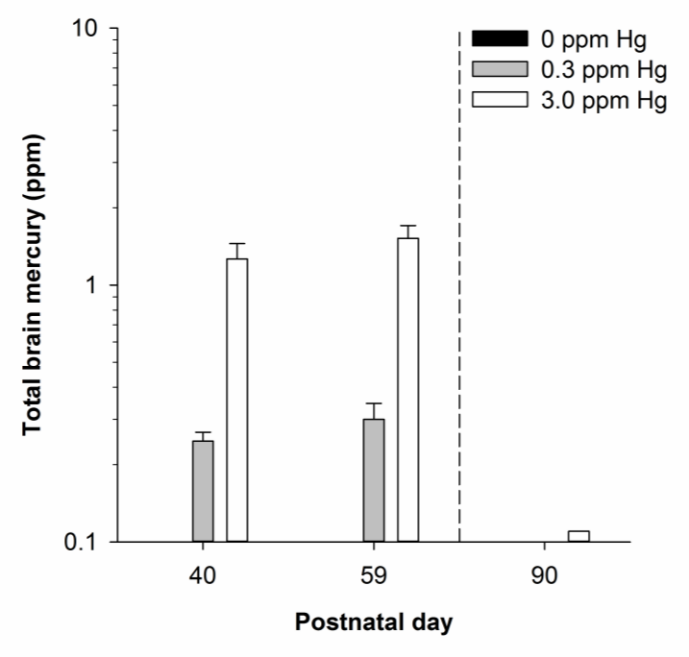

Figure 2.2

Mean (+SD) total brain mercury at postnatal day (PND) 40, 59, and 90 for 0.3- and 3.0-ppm MeHg exposed mice. Total brain mercury in control animals were assessed at PND 59 only. Exposure lasted from PND 21 through 59. Note errors bars represent one SD rather than one SEM and may be too small to visualize in some cases.

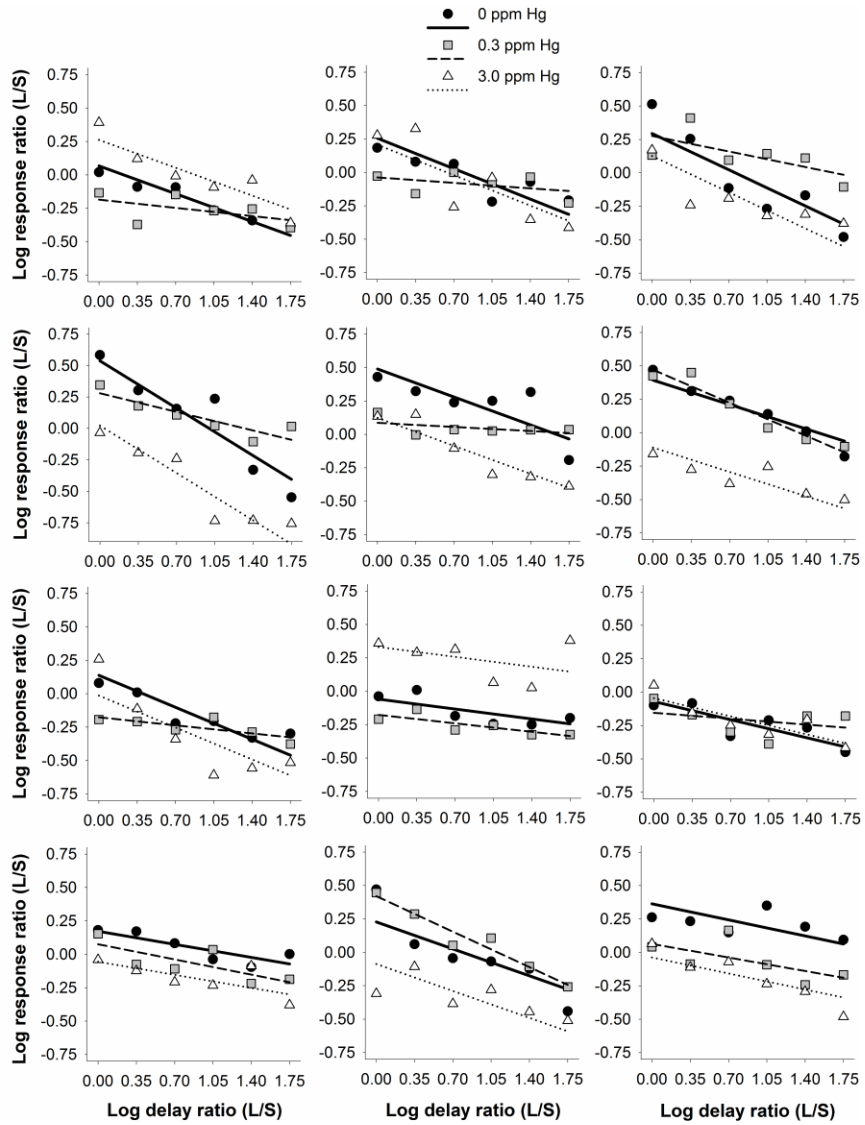

Figure 2.3

Log response ratio as a function of log delay ratio for individual mice. Each panel shows data from three littermates exposed to 0, 0.3, and 3.0 ppm MeHg during adolescence. Lines represent the best model of Eq. 1 according to the model-comparison analysis in which s_M (affecting the line's intercept) varied across all exposure groups, and s_D (affecting the line's slope) varied for 0.3 ppm MeHg-exposed mice and remained constant for both controls and 3.0 ppm MeHg-exposed mice. Log response and log delay ratios were calculated by dividing those of the larger-reinforcer lever (L) by the smaller (S).

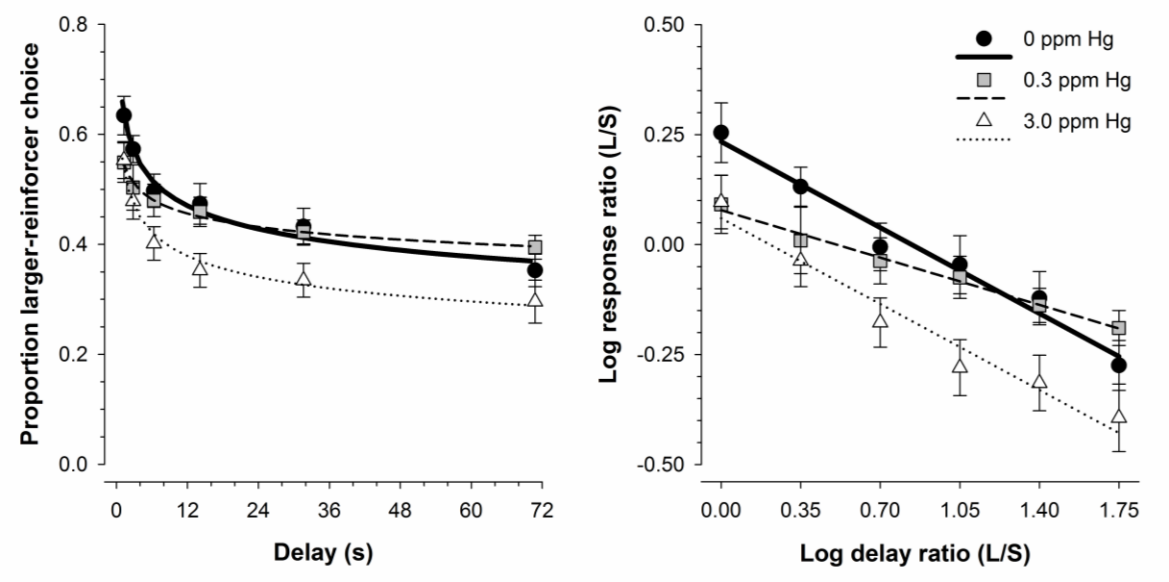

Figure 2.4

Left panel: Mean (±SEM) proportion larger-reinforcer choice as a function of delay for MeHg-exposed mice. Right panel: Mean (±SEM) log response ratio as a function of log delay ratio for MeHg-exposed mice. All lines represent the mean predictions of Eq. 1 according to the best model.

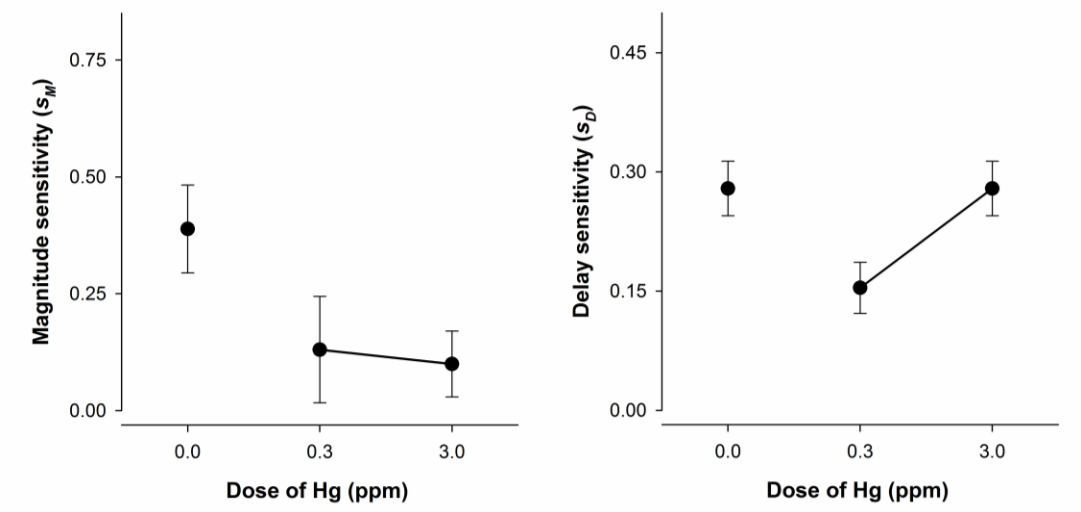

Figure 2.5

Mean (±SEM) parameter estimates of magnitude (left) and delay sensitivity (right) for MeHg-exposed mice. The best model indicated s_M varied across all exposure groups, and s_D varied for only 0.3 ppm MeHg-exposed mice and remained constant for both controls and 3.0 ppm MeHg-exposed mice.

Chapter 3

Effects of Adolescent Methylmercury Exposure on Sensitivity to *d*-Amphetamine in Mice

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Abstract

Gestational exposure to methylmercury (MeHg), an environmental neurotoxicant, results in a variety of neurochemical perturbations, including disruption of dopamine (DA) neurotransmission, but the consequences of exposure during the adolescent period remain virtually unknown. The current experiments were designed to assess the effects of low-level MeHg exposure during adolescence on sensitivity to *d*-amphetamine (a DA agonist) in adulthood using a delay-discounting task in mice. Thirty-six male C57BL/6n mice were exposed to 0, 0.3, or 3.0 ppm mercury (as MeHg), with one littermate assigned to each condition. MeHg exposure via drinking water spanned postnatal day 21 through 59, encompassing the murine adolescent period. As adults, mice lever-pressed for a 0.01-cc droplet of milk solution delivered immediately and four 0.01-cc droplets delivered after a series of delays under acute *d*-amphetamine (i.p.; 0.1 – 1.7 mg/kg) and vehicle. A model-comparison analysis using the generalized matching equation indicated that *d*-amphetamine dose-dependently reduced estimates of delay sensitivity (i.e., decreased delay discounting) for all mice. *d*-Amphetamine also dose-dependently decreased magnitude sensitivity (i.e., larger-reinforcer preference under equal-delay conditions) for MeHg-exposed mice, but *d*-amphetamine did not affect magnitude-sensitivity estimates in controls. The present experiment indicates that adolescent MeHg exposure is associated with alterations in dopaminergic function.

Key words: methylmercury, adolescence, delay discounting, impulsive choice, *d*-amphetamine

Effects of Adolescent Methylmercury Exposure on Sensitivity to *d*-Amphetamine in Mice

Gestational exposure to methylmercury (MeHg) in humans is estimated to increase the risk of learning deficits in newborns and represents a significant public health concern worldwide (Ceccatelli & Aschner, 2012; EPA, 2001, 2013; National Research Council, 2000). In animal models, gestational MeHg exposure impairs behavior that falls under the umbrella term of executive functions, including the acquisition of choice (Newland et al. 1994; Newland et al. 2004), impaired intradimensional shift (Reed et al. 2006; Paletz et al. 2007), and sensitivity to reinforcement (Newland & Rasmussen 2000; Paletz et al. 2006; Reed et al. 2008). These behavioral impairments are thought to result in part from altered dopamine (DA) signaling (Newland et al. 2008; Newland et al. 2015), as gestational MeHg exposure selectively increases sensitivity to amphetamine (Eccles & Annau 1982; Cagiano et al. 1990; Rossi et al. 1997; Rasmussen & Newland 2001) and cocaine (Reed & Newland 2009), both of which are DA transporter (DAT) inhibitors. The variety of neurobehavioral alterations that result from gestational MeHg exposure most likely come from the mammalian brain's vulnerability to neurotoxic insult during this time (Rice & Barone 2000). However, it is unclear if this vulnerability to MeHg exposure extends past the gestational period. One sensitive developmental window during which the brain and behavior is particularly plastic after birth is adolescence (Spear, 2000; Spear, 2007a).

The adolescent period is marked by the continued development of the neurochemical substrates that support executive functions and decision-making in adulthood (Spear, 2000; Chambers et al. 2003). Human adolescence is associated with pruning of frontal and striatal gray matter (Giedd et al. 1999; Sowell et al. 1999) as well as risky and impulsive behavior (Spear 2000; Chambers & Potenza 2003), particularly delay discounting, a preference for smaller-

sooner reinforcers over larger-later ones (Olson et al. 2007; Steinberg et al. 2009). Adolescent-onset neurobehavioral changes in humans are paralleled in rodents (Spear, 2007a), whose entire adolescent period is considered to span postnatal day (PND) 21 through 59 (Laviola, Macrì, Morley-Fletcher, & Adriani, 2003; Spear, 2000). During this time, DA levels and DAT function are elevated (Bartolome et al. 1982; Dreiem et al. 2009), and the number of postsynaptic DA receptors peaks and then decreases with use-dependent pruning into adulthood (Teicher et al. 1995; Tarazi & Baldessarini 2000; Brenhouse et al. 2008). Adolescent mice also display increased delay discounting relative to adults (Pinkston & Lamb 2011), suggesting that both DA signaling and delay discounting are particularly plastic during adolescence (e.g., Pope, Boomhower, Teixeira, Hutsell, & Newland, 2016).

The adolescent brain and behavior are sensitive to neurotoxic insults (Chambers et al. 2003; Spear 2007b), but few studies have examined the neurobehavioral effects of adolescent MeHg exposure. Some evidence shows that MeHg inhibits DAT in 21-day-old rat striatal synaptosomes but does not affect total DA production (Dreiem et al. 2009). Further, pre-adolescent MeHg administration similarly inhibits synaptosomal DAT but increases DA uptake once exposure ends (Bartolome et al. 1982). It is unclear though whether these MeHg-induced changes in DAergic signaling are permanent, which could be detected by alterations in sensitivity to DAT inhibitors, such as *d*-amphetamine, later in life.

The present experiment was designed to assess the extent to which adolescent MeHg exposure affected sensitivity to *d*-amphetamine in mice using a delay-discounting task. Adolescent mice were exposed to MeHg via drinking water and then trained on a rapid acquisition delay-discounting procedure (Grace, 1999; Pope et al. 2015) as adults. Response allocation between a larger-later and smaller-sooner reinforcer were described with the

generalized matching equation (Pope et al., 2015—a model of choice that estimates sensitivity to reinforcer magnitude and delay—following acute injections of *d*-amphetamine. As no studies to date have examined the consequences of adolescent MeHg exposure on sensitivity to *d*-amphetamine, the present experiment enhances understanding of the neurobehavioral mechanisms of MeHg's effects.

Methods

Subjects and MeHg exposure

The subjects and exposure regimen have been reported elsewhere (Experiment 1). Briefly, 21-day-old male C57BL/6n mice (n = 36) were pair-housed with free access to standard chow in an AAALAC-accredited animal facility. Upon arrival, mice were equally and randomly assigned to three exposure groups: 0 (control), 0.3, and 3 ppm mercury (as MeHg) dissolved in the mice's drinking water as methylmercuric chloride. Exposure lasted through PND 59 at which time they were returned to normal drinking water. When the mice reached 25 g, their food was rationed to maintain this body mass (±1 g). All procedures were approved by Auburn University's Institutional Animal Care and Use Committee.

Apparatus

Eleven Med Associates® (St. Albans, VT) operant chambers were used for data collection. Each chamber was equipped with two retractable levers on the front wall panel with an LED light above each lever and a houselight centered between two Sonalert® tone generators (high tone: 4500 Hz, low tone: 2700 Hz) at the top of the wall. An alcove for liquid-reinforcement delivery, in which one or more 0.01 cc presentations of a 3:1 water and sweetened-condensed milk solution were presented when response criteria were met, was centered between the two levers. Each chamber was enclosed in a sound-attenuating cubicle with

an air-circulating fan in the upper left corner of the right wall. A Windows® computer in an adjacent room controlled all experimental contingencies with 0.01-sec resolution. Each mouse was assigned to a chamber for the duration of the experiment, and sessions were run Monday through Friday.

Procedure

After lever-press training by autoshaping (see Experiment 1), mice began a two-lever choice procedure (Pope et al., 2015). Sessions consisted of six blocks, and each block was composed of twelve choice trials in which mice could choose between two levers that resulted in one and four 1.26-sec presentations of milk (separated by .5-sec intervals in the case of four presentations). At the start of a trial, both levers extended into the chamber, and the stimulus lights above each lever illuminated. The computer selected randomly which lever led to reinforcement once pressed, the active lever. The same lever could not result in reinforcement more than three trials in a row. Thus, a mouse was allowed to respond freely between the two levers, but it experienced each alternative an equal number of times (i.e., six trials resulted in the smaller reinforcer and six trials resulted in the larger reinforcer). After a single lever-press on the active lever, the opposite lever retracted and its associated stimulus light extinguished, the stimulus light above the selected lever began blinking (.5 sec on/off), and one or four presentations of milk occurred after 1.26 sec. Following reinforcer delivery, a 3-sec intertrial interval occurred in which both levers retracted and both stimulus lights were extinguished until the next trial. Similarly, a 10-sec interblock interval separated each block of twelve trials during which both levers were retracted and both stimulus lights were extinguished. A session ended after the completion of six blocks (i.e., 72 trials) or 75 min, whichever came first.

Following 10 sessions of preliminary training on equal-delay trials, six differentially-signaled, geometrically-spaced delays (1.26, 2.82, 6.31, 14.13, 31.62, and 70.79 sec) to the larger reinforcer were imposed. At the start of a block, the computer selected randomly (without replacement) one of the six delays to the larger reinforcer, and this delay was in effect throughout a block. The delay to the smaller reinforcer was always 1.26 sec. Each delay to the larger reinforcer had a specific low/high-tone combination pulsating on and off for varying durations associated with it (see Table 3.1; Krägeloh & Davison, 2003; Pope et al., 2015). The delay-specific tones remained present throughout a trial, but terminated during interblock intervals. The lever associated with the larger reinforcer (left or right) remained constant throughout the experiment, but was counterbalanced across subjects. The delay-discounting procedure was in place for 35 sessions to allow behavior to stabilize. See Boomhower and Newland (in preparation) for effects of MeHg exposure during this period.

Drug administration

d-Amphetamine sulfate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a 0.9% saline solution (2 ml/kg volume). Upon completion of 35 sessions under baseline, mice were injected *i.p.* with *d*-amphetamine (0.1, 0.3, 0.56, 1.0, and 1.7 mg/kg as the salt) or saline vehicle five minutes prior to sessions. Injections of *d*-amphetamine occurred on Tuesdays and Fridays and saline injections occurred on Thursdays. Mondays and Wednesdays served as non-injection control days. Each mouse received all doses of *d*-amphetamine in ascending order and two saline injections. Before the determination of a dose-response curve, each mouse also received an injection of 1.0 mg/kg *d*-amphetamine in its homecage. This allowed mice to behave under the drug before operant sessions were conducted and was meant to minimize any behavioral

disruption (e.g., suppressed responding) following the first drug injection for the dose-response determination.

Data analysis

The primary dependent variable was the ratio of responses on the larger-later reinforcer lever to those on the smaller-sooner reinforcer lever. Log_{10}-transformed response ratios (calculated by dividing the responses to the larger-later, "L", reinforcer lever by the responses to smaller-sooner, "S", reinforcer lever) from the last four trials of each delay were used in drug analyses. Similarly, log_{10}-transformed delay ratios were calculated by dividing the larger-reinforcer delay by the smaller-reinforcer delay. This measure of preference was quantified using the generalized matching equation (Grace, 1999; Rachlin & Green, 1972), which describes the ratio of responses allocated to two alternatives as a function of the ratios of delay to and magnitude of the reinforcers derived from those two alternatives:

$$\log\frac{B_l}{B_s} = s_M \log\frac{M_l}{M_s} - s_D \log\frac{D_l}{D_s} \quad (1)$$

where B is the number of responses, M is the magnitude of reinforcement, D is the delay to reinforcement, and the subscripts l and s refer to the larger and smaller alternatives, respectively. s_M and s_D were free parameters of interest and represent sensitivity to magnitude (s_M) and sensitivity to delay (s_D). s_D is the slope of the linear relation between log response ratio and log delay ratio, $D_l/1.26$. Because M_l/M_s was always equal to 4/1, s_M is the Y-intercept of the line. We would expect preference for the larger reinforcer to decrease as the delay to it increases, hence the negative sign in Eq. 1.

To assess the effects of *d*-amphetamine on responding among the three MeHg exposure groups, an information-theoretic approach was used (Burnham & Anderson, 2002). This model-comparison analysis has been growing in popularity in the behavioral and neural sciences (e.g.,

Avila, Reilly, Sanabria, Posadas-Sanchez, Chavez, Banerjee, et al., 2009; Pope & Newland, in review; Sanabria, Acosta, Killeen, Neisewander, & Bizo, 2008) because it does not rely on traditional null hypothesis testing. Instead it allows the user to determine the best, most parsimonious model (of all models investigated) to describe the data collected. In this analysis, the best model given the data is determined by calculating the corrected Akaike information criterion (AICc), which takes into account the degree of fit of a model (i.e., residual sum of squares, RSS) and penalizes additional free parameters (k) in the model for a given number of data points (n) (Burnham & Anderson, 2002). The lowest AICc value indicates a better account of the data given the degrees of freedom in the model. Because the calculation of the AICc for one model is independent of another model's, multiple models can be tested simultaneously in this approach without the need for corrective measures (e.g., Bonferroni).

Modeled after Pope et al. (under review), the effects of MeHg and *d*-amphetamine on the parameter estimates of Eq. 1 were determined by allowing s_M and s_D to remain constant or vary across dose of MeHg and *d*-amphetamine for each subject. That is, a model where s_M and s_D were fixed, s_M varied, s_D varied, and both s_M and s_D varied across dose were examined. A control model was also examined in which parameter estimates obtained under non-injection control conditions were applied to data from all doses. Eq.1 was fit to individual-subject response data by least squares regression. The model that resulted in the lowest AICc was considered the best model. To exemplify interpretation of the results of this approach, if a model that includes dose is more likely given the data than a model that is based only on baseline values, then that would imply that the drug affected one or more parameters. AICc weight (w), which is the probability that a given model is the best model given the data (Burnham & Anderson, 2002), was also calculated to aid in interpretation of the data.

Results

Baseline differences among exposure groups have already been reported on (Experiment 1). For all but one mouse under the two high-dose conditions (1.0 and 1.7 mg/kg), the number of trials (out of 72 maximum) completed under *d*-amphetamine equaled the number completed under vehicle for all exposure groups. One mouse in the 0.3-ppm MeHg group did not complete any trials under the 1.0- and 1.7-mg/kg dose of *d*-amphetamine so that mouse was excluded from analysis of these doses.

Figure 3.1 shows mean log response ratio as a function of log delay ratio for each dose of *d*-amphetamine with a separate exposure group in each panel. Lines represent mean estimates of the best model of Eq. 1 as determined by the model-comparison approach. Table 3.2 shows the best models (bolded) for each of the exposure groups. For all exposure groups, the best models allowed delay sensitivity (s_D) to vary across dose of *d*-amphetamine. This is reflected in progressively shallower slopes of the fitted lines as *d*-amphetamine dose increases (Figure 3.1). For control animals, the best model held magnitude sensitivity (s_M) constant across dose of *d*-amphetamine and was 78% likely given the data. In terms of AICc, the best model (model 3) was marginally better (AICc = -1301.25) than the second best model (model 4; AICc = -1298.6), which was 21% likely given the data. The relative stability of s_M and drastic decrease in s_D as a function of *d*-amphetamine dose in controls does not support the conditions of the second-best model, though (see Figure 3.1), since that model holds s_D constant and varies s_M across *d*-amphetamine dose. The best models for both MeHg-exposure groups allowed s_M to vary across dose of *d*-amphetamine. These models were 96% and 100% likely given the data, compared to the second-best models which were only 4% and 0% likely for the 0.3- and 3.0-ppm MeHg groups, respectively. The model differences among the three exposure groups are reflected in a

Y-intercept of the fitted lines that remains constant for all *d*-amphetamine doses in control mice, and a Y-intercept that decreases as a function of *d*-amphetamine dose in MeHg-exposed mice (Figure 3.1).

To visualize the effect of MeHg exposure better, Figure 3.2 shows log response ratios as a function of dose of *d*-amphetamine for each exposure group (lines), with delays to the larger reinforcer represented in different panels. According to the best models (Table 3.2), magnitude-sensitivity estimates varied across *d*-amphetamine dose for MeHg-exposed subjects but were invariant for controls. This is reflected in a dose-dependent decrease in larger-reinforcer preference under the equal-delay condition for the 0.3- and 3.0-ppm group (top left panel), and relatively stable preference across *d*-amphetamine doses for controls. *d*-Amphetamine's dose-dependent changes in delay sensitivity are evident in Figures 3.3. For controls, *d*-amphetamine increased larger-reinforcer preference as the delay interval lengthened. For MeHg-exposed mice, *d*-amphetamine decreased larger-reinforcer preference under equal delays and increased larger-reinforcer preference under long delays. These concomitant effects on larger-reinforcer preference resulted in the shallower functions shown in Figure 3.1.

Figure 3.3 summarizes these effects by showing mean parameter estimates of magnitude and delay sensitivity as a function of dose of *d*-amphetamine. The parameter estimates that are shown were derived from the best model (Table 3.2). For control animals, the best model held s_M constant across *d*-amphetamine dose, hence the invariant mean s_M estimates (closed circles) shown in Figure 3.3. This does not imply that there was no variance in s_M estimates across *d*-amphetamine dose; rather, the model in which s_M remained constant was more parsimonious (AICc = -1301.25) than the model in which s_M varied (AICc = -1291.73). To demonstrate the small degree of variability in s_M, Figure 3.4 also shows mean estimates of s_M when it was

allowed to vary across *d*-amphetamine dose for controls (open circles; left panel). Although the evidence derived from AICc implied a marginal difference between the two models, a comparison of the filled and open circles shows that the model in which magnitude sensitivity is held constant is clearly better. The open circles do not show systematic changes with dose, and even if the open circles are accepted, it does not change the overall conclusion that *d*-amphetamine's effects on s_M for controls were qualitatively different from its effects on MeHg-exposed mice. Thus, *d*-Amphetamine dose-dependently decreased s_M estimates for MeHg-exposed mice, but not controls. *d*-Amphetamine also dose-dependently decreased s_D estimates similarly for all exposure groups.

Discussion

The current experiment was designed to assess the effects of acute *d*-amphetamine injections on choice and delay discounting in mice exposed to MeHg during adolescence. We employed a model-comparison analysis (Burnham & Anderson, 2002) to examine these effects because it is a robust statistical approach that does not rely on null-hypothesis testing and has been growing in popularity in the neurobehavioral sciences (e.g., Avila, Reilly, Sanabria, Posadas-Sanchez, Chavez, Banerjee, et al., 2009; Pope & Newland, under review; Sanabria, Acosta, Killeen, Neisewander, & Bizo, 2008). In all cases except one, the second-best models were 4% likely or lower given the data. The second-best model of *d*-amphetamine's effects on the parameter estimates of Eq. 1 for control subjects was 21% likely given the data, but the constraints of this model (varying s_M and holding s_D constant) did not accurately describe the actual data (Figs. 3.2 & 3.4). Thus, the models that resulted in the lowest AICc values (i.e., the best models) were used to interpret the effects of *d*-amphetamine on delay discounting.

The model-comparison analysis captured MeHg-related differences in responsiveness to *d*-amphetamine. The most dramatic difference was the ability of *d*-amphetamine to reduce magnitude sensitivity in both the 0.3- and 3.0-ppm exposed mice but not in controls. This differential sensitivity was further exacerbated by *d*-amphetamine in a dose-related fashion for exposed mice, but not for controls. Thus, for MeHg-exposed mice, the preference for the larger reinforcer at isodelay conditions was blunted in comparison to controls, and this diminished sensitivity was increased by *d*-amphetamine. The present findings lend support to mounting evidence that developmental MeHg exposure alters DAT function and extends these effects from very early development to adolescence. The finding that adolescent MeHg exposure enhanced susceptibility to *d*-amphetamine-induced reductions in magnitude sensitivity relative to controls suggests an enduring change in DAT function following MeHg exposure in adolescence. Indeed, gestational MeHg exposure was associated with an increased sensitivity to *d*-amphetamine (Eccles & Annau 1982; Cagiano et al. 1990; Rossi et al. 1997) and a leftward shift in the dose-effect curve by a factor of two for *d*-amphetamine (Rasmussen & Newland, 2001) and cocaine (Reed & Newland, 2009). These effects appear to be specific to DAT, as sensitivity to DA D_1 and D_2 agonism as well as norepinephrine transporter inhibition was unchanged in rats gestationally exposed to MeHg (Reed and Newland, 2009). MeHg inhibits DAT but does not affect total DA production in 21-day-old rat striatal synaptosomes (Dreiem et al. 2009). Striatal DAT function also is inhibited in the presence of MeHg but then is increased relative to control levels after exposure ceases in adult rats (Faro et al. 1997; Faro et al. 1998; Faro et al. 2002). Whether MeHg-induced inhibition of DAT results in functional changes in DAT expression (e.g., reduced DAT trafficking, removal of DAT from the plasma membrane, or alterations in DAT phosphorylation) is presently unclear.

For all exposure groups, *d*-amphetamine dose-dependently reduced delay discounting, or the rate at which larger-reinforcer preference decreased as a function of delay. This was evidenced in a delay-discounting curve that flattened and in estimates of delay sensitivity that decreased under increasing doses of *d*-amphetamine. This is consistent with previous reports demonstrating that *d*-amphetamine (as well as other DA agonists) reduces delay discounting (Richards et al. 1999; Wade et al. 2000; Pitts & McKinney 2005; van Gaalen et al. 2006; Ta et al. 2008; Huskinson et al. 2012; Krebs & Anderson 2012) and delay sensitivity in rodents and pigeons (Pitts & Febbo, 2004; Pope & Newland, under review; Ta et al. 2008). The manner by which *d*-amphetamine decreased delay sensitivity, however, differed between MeHg-exposed and control mice. In controls, *d*-amphetamine reduced delay sensitivity by increasing larger-reinforcer preference at long delays only (Fig. 3.3). In contrast, *d*-amphetamine reduced delay sensitivity in MeHg-exposed mice by both decreasing larger-reinforcer preference under equal-delay conditions and modestly increasing larger-reinforcer preference under long delays.

Some alternative interpretations of the present results may exist. First, high doses of *d*-amphetamine reduced magnitude-sensitivity estimates in MeHg-exposed mice to such a degree that the smaller reinforcer was sometimes preferred in the equal-delay condition. Though similar patterns of responding have occurred in monkeys gestationally exposed to MeHg (Newland et al. 1994), this finding could be interpreted as a deficit in discrimination between the smaller- and larger-reinforcer alternatives. If this were the case, one would expect non-discriminated responding across all delay conditions, but this was not observed. Positive estimates of delay sensitivity were present under high doses of *d*-amphetamine, suggesting that preference was to some degree discriminated across the delays. Second, the degree to which the stimuli present during the delays were modulating the effects of *d*-amphetamine in the present study is unclear

because it was not examined directly. Previous reports have demonstrated that stimuli during the delays can modify the effects of *d*-amphetamine (and other DA agonists) on delay discounting (Cardinal, Robbins, & Everitt, 2000; Pope et al., in review; Slezak & Anderson, 2009). Future research might examine this question by omitting stimuli during the delay (see Pope, Newland, & Hutsell, 2015).

Limitations aside, the present experiment documents altered sensitivity to the behavioral effects of *d*-amphetamine in mice exposed to MeHg during adolescence. These findings provide important evidence in support of the hypothesis that adolescence represents a period of neurobehavioral susceptibility to MeHg exposure and propose altered DAT function as a potential mechanism of these effects. As very little is known about adolescent MeHg exposure, future research is needed to determine the extent of neurobehavioral toxicity that results from adolescent MeHg exposure across the lifespan.

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Tables

Table 3.1

Low/high tone combinations associated with each delay in the delay-discounting procedure.

Delay to larger reinforcer (sec)	Low/high tone durations (sec)
1.26	0.15/1.19
2.82	0.74/0.60
6.31	0.92/0.42
14.13	1.04/0.30
31.62	1.13/0.21
70.79	1.19/0.15

Table 3.2

Results of the model-comparison approach across MeHg exposure groups when magnitude and delay sensitivity were held constant or varied across dose of *d*-amphetamine. The control model used estimates derived from non-injection control days for all doses. The best model is bolded for each exposure group.

	Model	RSS	*k*	*n*	AICc	Δ AICc	w_i
0 ppm Hg:	1. Control	38.80	25	432	-987.92	313.33	≈ 0
	2. s_M (constant), s_D (constant)	22.87	25	432	-1216.27	84.98	≈ 0
	3. s_M (constant), s_D (vary)	**13.00**	**85**	**432**	**-1301.25**	**0.00**	**0.78**
	4. s_M (vary), s_D (constant)	13.08	85	432	-1298.60	2.65	0.21
	5. s_M (vary), s_D (vary)	7.88	145	432	-1291.73	9.52	0.01
0.3 ppm Hg:	1. Control	41.87	25	420*	-915.09	148.28	≈ 0
	2. s_M (constant), s_D (constant)	29.87	25	420	-1056.93	6.44	0.04
	3. s_M (constant), s_D (vary)	25.23	83	420	-973.63	89.74	≈ 0
	4. s_M (vary), s_D (constant)	21.33	83	420	-1044.16	19.21	≈ 0
	5. s_M (vary), s_D (vary)	**12.11**	**141**	**420**	**-1063.37**	**0.00**	**0.96**
3.0 ppm Hg:	1. Control	28.97	25	432	-1114.13	151.02	≈ 0
	2. s_M (constant), s_D (constant)	20.95	25	432	-1254.15	11.00	≈ 0
	3. s_M (constant), s_D (vary)	15.54	85	432	-1224.15	41.00	≈ 0
	4. s_M (vary), s_D (constant)	16.42	85	432	-1200.35	64.80	≈ 0
	5. s_M (vary), s_D (vary)	**8.38**	**145**	**432**	**-1265.15**	**0.00**	**1.00**

RSS = residual sum of squares; *k* = number of parameters; *n* = number of data points; AICc = corrected Akaike Information Criterion; Δ AICc = difference in AICc relative to the best model's AICc; w_i = probability that the i^{th} model is the best model given the data

*Note one mouse in the 0.3 ppm Hg group did not complete any trials under the 1.0 and 1.7 mg/kg *d*-amphetamine doses. Thus, his data are not included in analysis.

Figures

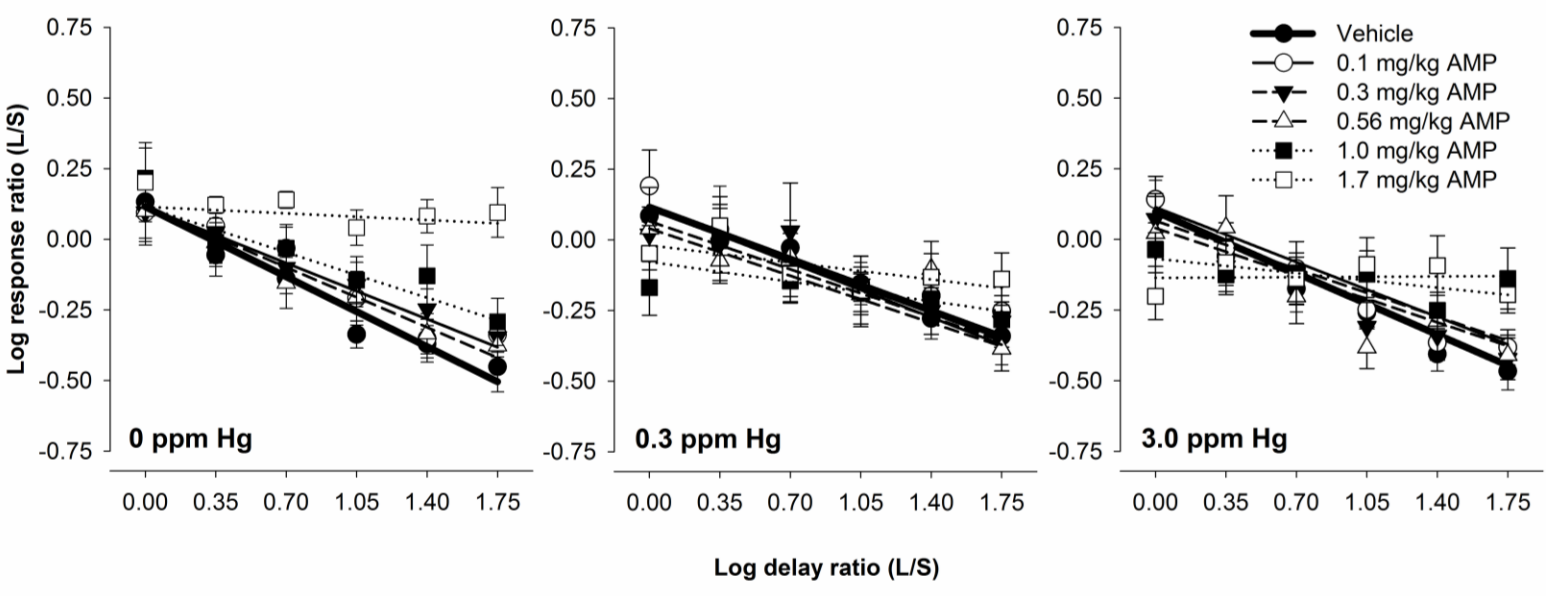

Figure 3.1

Mean (±SEM) log response ratio as a function of log delay ratio under vehicle and each dose of *d*-amphetamine. Data for mice exposed to 0, 0.3, and 3.0 ppm mercury (as MeHg) are in the left, middle, and right panels, respectively. Lines are the mean predictions of the best model according to the model-comparison analysis.

AMP = *d*-amphetamine.

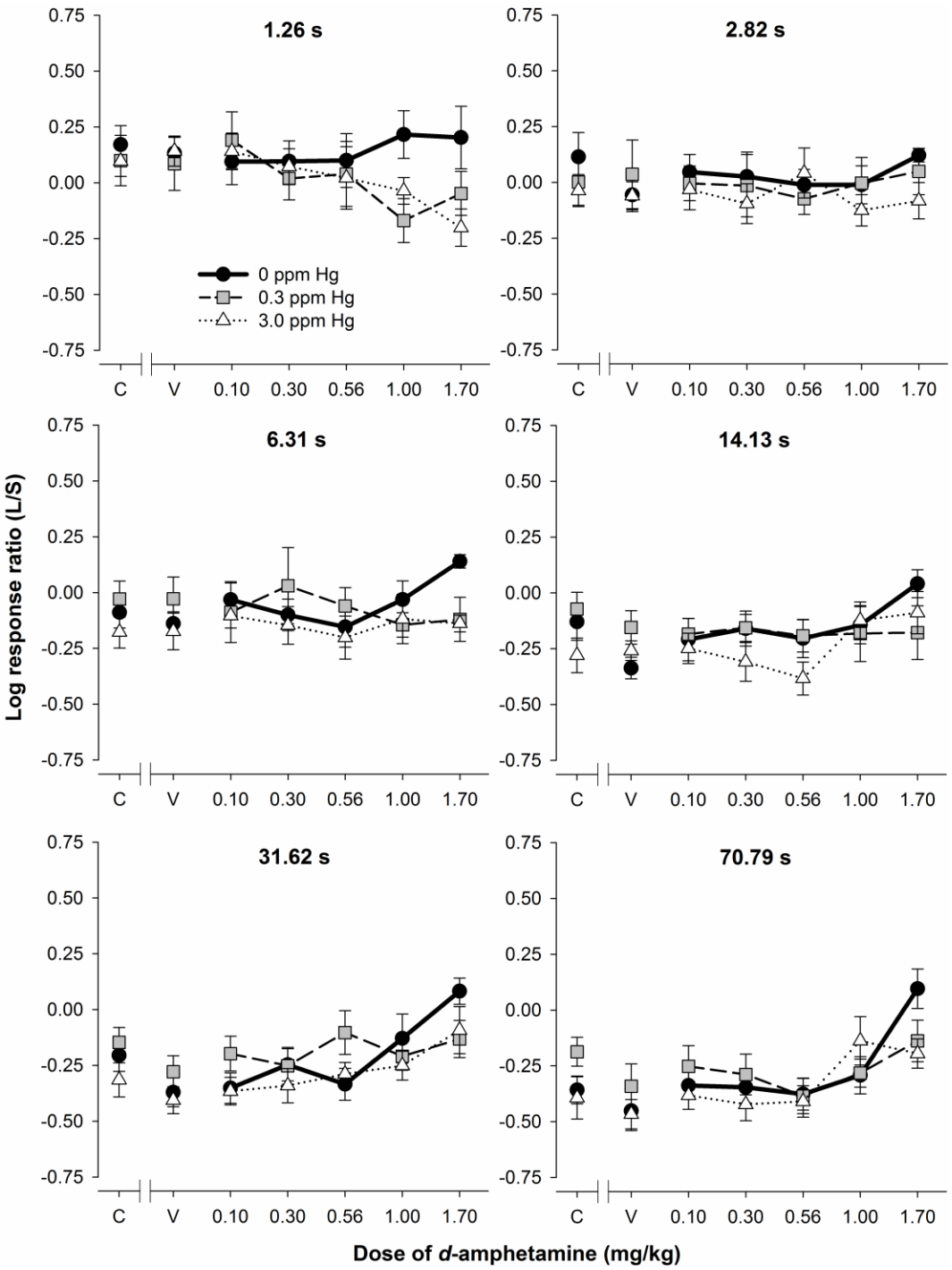

Figure 3.2

Mean (±SEM) log response ratio as a function of dose of *d*-amphetamine for MeHg-exposed mice. Each panel shows dose-response data for each of the six delays.

C = non-injection control; V = vehicle

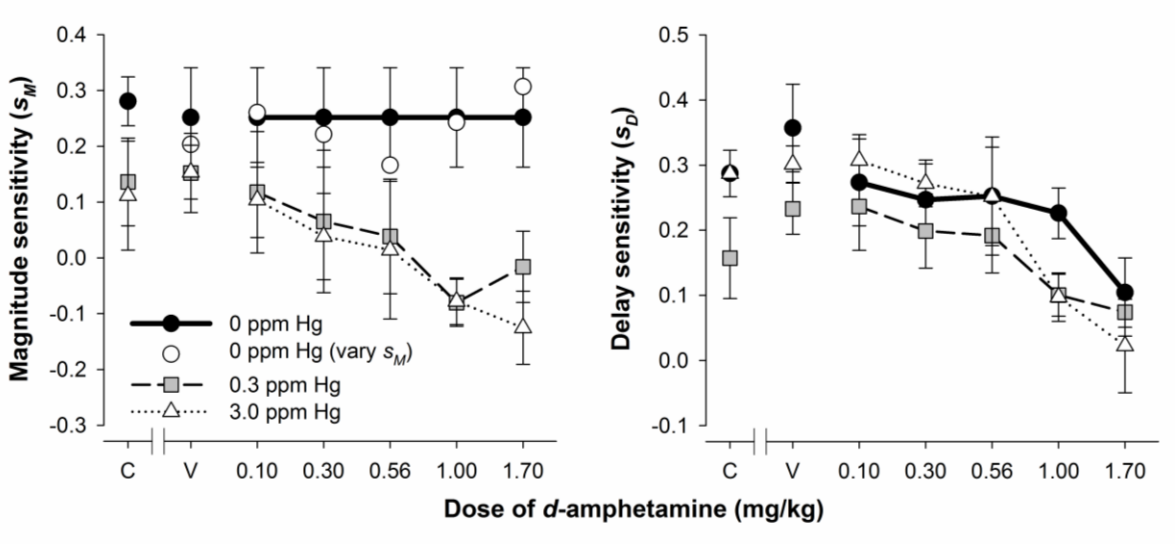

Figure 3.3

Mean (±SEM) estimates of magnitude (left) and delay (right) sensitivity according to the best model as a function of dose of *d*-amphetamine for MeHg-exposed mice. For magnitude sensitivity, the best model specified that s_M vary across *d*-amphetamine dose for MeHg-exposed mice and remain constant for controls (filled circles) rather than vary (open circles). For delay sensitivity, the best model specified that s_D vary for all groups.

C = non-injection control; V = vehicle