A bout analysis reveals age-dependent methylmercury neurotoxicity and nimodipine neuroprotection: Implications for the role of calcium homeostasis in aging

by

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Abstract

Learning and sensorimotor function decline in normal aging and these deficits may be related to elevations in intracellular calcium (Ca^{2+}) levels [Ca^{2+}]i. The neurotoxicity of methylmercury (MeHg), a ubiquitous environmental contaminant, also appears to be mediated, in part, by elevated [Ca^{2+}]i. Calcium channel blockers (CCBs), which can lower [Ca^{2+}]I, may confer neuroprotection against aging and/or MeHg-induced dysfunction. Studying the effects of chronic MeHg and/or CCB exposure presents a unique way by which to study potential mechanisms of aging. Experiments 1 and 2, conducted simultaneously, chronically exposed two age cohorts (adults and retired breeders) of BALB/c mice to 0 or 10 ppm MeHg and 0 or 200 ppm nimodipine, a CCB, for approximately 8.5 months. Experiment 1 investigated high-rate nose-poking meanwhile Experiment 2 investigated wheel-running and rotarod performance. A bout analysis approach was used to estimate motor and motivational contributions to both nose-poking and wheel-running.

Methylmercury produced age-independent mortality and nimodipine afforded protection in an age-dependent manner; there was greater protection in younger animals. Reliably, MeHg-induced motor impairment of nose-poking, wheel-running, and rotarod performance appeared early into exposure while motivational deficits appeared only near mortality. Nimodipine delayed the onset of MeHg-induced behavior deficits and this protection was more pronounced in younger animals. For nose-poking, latency to motor impairment was shorter in older animals than in younger animals. These results provide a comprehensive profile of adult-onset MeHg exposure and also provide support for the use of a bout analysis approach as an analytical tool to delineate between motor and motivational components of behavior.

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Chapter 1

Neurobehavioral consequences of aging and methylmercury exposure

Declines in learning, memory, sensory, and motor function are all associated with the normal aging process (Verkhratsky, 2004). Individuals that age in the absence of a major disease express subtle deficits in behavior that fall under the executive functions, including attention, planning, behavior flexibility, working memory, and inhibitory control (Buckner, 2004; Salthouse et al., 2003), information-processing speed (Salthouse, 1996, 2000), and different facets of remembering (Erickson & Barnes, 2003), as well as declines in physical fitness (Spirduso et al., 2004). A better understanding of the mechanisms that control these age-related changes may help address neurodegenerative processes, e.g., Parkinson's disease (PD) and Alzheimer's disease (AD), as these diseases may resemble forms of accelerated or perturbed aging.

The following review will discuss the behavioral consequences of normal aging and of adult-onset MeHg exposure. Then, in an effort to marry these two sets of consequences together, the review will focus on the neurobiological impact of aging and of MeHg on different neural mechanisms, specifically Ca^{2+} regulation and homeostasis. To elucidate the importance of these neural mechanisms, the efficacy of nimodipine, an L-type calcium channel antagonist, to antagonize Ca^{2+}-mediated CNS insults will be reviewed. Finally, an approach to modeling behavior, bout analysis, that may be sensitive to aging and neurotoxicant exposure is discussed.

Behavioral consequences of normal aging

Normal aging is typically characterized by subtle behavior impairments that manifest gradually in time rather than a rapid onset of gross cognitive and physical deficits. In many

cognitive and motor tasks young adults outperform older adults and older adults outperform the very geriatric but no single time point demarcates one age group from another. While some deficits in motor function during aging are relatively clear (e.g., musculoskeletal deficits), others are more ambiguous, e.g., reaction time (RT), as they may be mediated by executive functions. Aside from strict motor deficits, information-processing speed and remembering, as well as behavioral flexibility are among the most prominent behavioral domains affected. Numerous animal models of aging (non-human primate and rodent being the most popular) have been developed to characterize neurobiological and behavioral changes that occur during normal and disordered aging. The following review focuses mostly on rodent models of aging and potential links to findings from human research.

Numerous behavior deficits in normal aging, some of the most prominent being decreased motor speed have been described in rodent models (Marriott & Abelson, 1980; Medin et al., 1974; Bartus et al, 1974; Burwell & Gallagher, 1993; Houx & Jolles, 1993; Wilkinson & Allison, 1989); reduced accuracy on learning and remembering procedures, e.g., conditioned eye-blink (Graves & Solomon, 1985; Woodruff-Pak et al., 1987), maze performance (Doyere et al., 2000); behavioral rigidity, i.e., perseveration (Means & Holsten, 1992). Reports also indicate that aged animals exhibit deficits in temporal processing or time estimation (Lustig & Meck, 2004) and alterations in sensitivity to reinforcement contingencies (Tripp & Alsop, 1999; Sanford, 1978), both of which may underlie age-related deficits across several domains of behavior.

Neuromuscular functioning studies, both *in vitro* and *in vivo*, have found age-related alterations (Altun et al., 2007; Dean III et al., 1981; Fahlström et al., 2011; Brooks & Faulkner, 1988; Muller-Delp et al., 2002). For example, age-related deficits appear in tasks like wire hanging and inclined screen (tests of muscle strength and fatigue) and rotarod (a test of fine motor coordination, balance, and fatigue) (Barreto et al., 2010;Shukitt-Hale et al., 1998). These findings are in agreement with a large base of literature from human studies that have found

decreased motor function as a function of age (for reviews, see Porter et al., 1995; Vandervoort, 2002). These deficits may be the result of loss of muscle tissue, namely reduction in the number, size, and functionality of type I and II muscle fibers (slow- and fast-twitch, respectively), with more atrophy of type II fibers (i.e., sarcopenia) (Evans & Lexell, 1995; Lexell, 1995; Vandervoort, 2000). However, evidence from human and animal studies suggests that these deficits may stem from cerebellar and cerebral cortex degeneration and dysregulation of nigrostriatal DA signaling (Dorce & Palerma-Neto, 1994; Forster et al., 1996).

Aged animals also exhibit increased RT (Burwell & Gallagher, 1993; Burwell et al., 1995; Gallagher & Burwell, 1989; Roux et al., 1994), a finding that mirrors human studies (Salthouse, 1993; Salthouse & Somberg, 1982), providing additional support for motor dysfunction in aging. It is important to note that age-related changes in RT may represent alterations in executive functions like information-processing speed and attention. For instance, RT deficits may be due to a dysfunction in attentional processing or an increase in attentional demand (Porsolt et al., 1995; Smith et al., 1999). In support of this notion, human imaging studies have found that older and aged adults rely on recruitment of additional cortical and subcortical brain regions than young adults, even when performing simple motor tasks like finger-tapping (Mattay et al., 2002). Moreover, dual tasks that require motor and cognitive control produce greater age-related deficits than either task performed alone and dual task performance is diminished relative to younger adults. These studies suggest that control of motor function may require additional recruitment of the prefrontal cortex (PFC) in aging, interfering with attentional control that results in performance decrements (for a review see Seidler et al., 2010).

Constructs of remembering show functional decline in aging and may reflect neurodegeneration in the hippocampus and projection pathways from the hippocampal formation to frontal cortices. Maze performance in rodents, the most common test used in animal models of aging, assesses spatial working memory (though many types of maze tasks can be used to test different facets of remembering). Across a variety of procedural variations,

maze performance in rodents shows a temporally-graded decline (Biegon et al., 1985; Bizon et al., 2009; Fiebre et al., 2006; Driscoll et al., 2006; Frick et al., 1995; Lindner, 1997; Gage et al., 1984, 1989; Gallagher et al., 1993, 2003; Muir et al., 1999; Rasmussen et al., 1996; Zyzak et al., 1995). In addition, procedures that tap domains of learning, flexibility, and remembering may be used in combination with a maze. For example, the Morris water maze (MWM) may be modified to include tests of delayed non-match to position (Markowska et al., 1996), repeated acquisition (Frick et al., 1995; van der Staay & de Jonge, 1993) and reversal learning (de Fiebre et al., 2006), all of which are also sensitive to age-related performance deficits. These additional tests, however, do not discretely test mnemonic function and likely tap several executive functions. It may also be the case that maze tasks rely on sensorimotor function, and concomitant deficits may degrade functional relations between maze performance and memorial processes.

In addition to maze performance, the avoidance learning paradigm has been useful in elucidating age-related changes in remembering of aversive stimuli and associated contextual stimuli. Aging animals, including mice and rats, display deficits in the acquisition of shock avoidance, measured by the amount of time spent in a no-shock context relative to a shock context (Dean III et al., 1981; Doyere et al., 2000). Similarly, in Pavlovian fear conditioning procedures, young rats freeze and show greater increases in heart rate to an auditory CS paired with shock during retention trials than older rats, suggesting an age-related impairment in remembering, or potentially differences in sensitivity to shock or perception of auditory stimuli (McEchron et al., 2003). In rodents, age-dependent deficits in fear conditioning are more robust in response to a trace-conditioned stimulus and contextual fear conditioning (Blank et al., 2003; McEchron et al., 2003, 2004; Villareal et al., 2004; Moyer & Brown, 2006; Oler & Markus, 1998), whereas delay-conditioned stimuli typically evoke normal responses in aged animals (Blank et al., 2003; McEchron et al., 2004; Moyer & Brown, 2006; Oler & Markus, 1998; Stoehr & Wenk, 1995; *cf*. Gould & Feiro, 2005).

Pavlovian conditioning-based tasks that do not use aversive conditioning also reliably produce age-dependent performance measures. A well-studied behavior in both humans and animal models is the conditioned nictitating membrane response or conditioned eye-blink (CEB), in which younger subjects perform better than aged counterparts. Notably, empirical evidence suggests that aging impairs acquisition of CEB during delay conditioning (Powell et al., 1981), trace conditioning (Graves & Solomon, 1985; Woodruff-Pak et al., 1987) and conditional discrimination procedures (Powell et al., 1984). In particular, lesion studies have shown that CEB is largely dependent upon cerebellar function, as bilateral (Daum et al., 1993; Topka et al., 1993) and unilateral (Woodruff-Pak et al., 1996) cerebellar lesions to regions ipsilateral to the conditioned eye impair conditioning. Additional support of a cerebellar role in CEB has been garnered via Purkinje cell knockout mice, which display impaired acquisition of conditioning (Chen et al., 1996). Avoidance, fear, and eye-blink conditioning procedures provides a relatively reliable and valid paradigm for investigating changes in the use of contextual information as a function of age. This literature supports the notion that normal aging is associated with specific impairments to conditioning processes that mediate remembering that are not universal (i.e., trace vs. delay conditioning), which likely relate to particular underlying neurobiological changes in the normally-aging organism.

The discrimination reversal task, a task aimed at measuring behavior flexibility and inhibition, is also used to study aging. The first session or first block of trials following a reversal in reinforcement contingencies is often taken as a measure of behavioral flexibility, i.e., inhibiting a previously learned response in favor of a novel response, and thought to be mediated by the orbitofrontal cortex (OFC) and striatum (Chudasama et al., 2003). Perseverative responding on a previously-reinforced alternative is indicative of an impairment in reversal learning and may reflect impulsivity. Recent reports suggest that perseveration is facilitated, in part, by 5-HT-mediated Glu (glutamate) signaling between the dorsal striatum and PFC (Carli et al., 2006; Agnoli & Carli, 2012). Generally, discrimination reversal research with

rodents has found that aging decreases performance, although some reports have found deficits in learning the initial discrimination while others have found only reversal learning deficits or both (Means & Holsten, 1992; Schoenbaum et al., 2002; Stevens et al., 1985). However, the fact that rodent and human research has consistently found that aging decreases sensitivity to delayed rewards (a reduction in impulsivity or risk aversion) may suggest this deficit is localized to motor impulsivity. In addition to the rodent literature, there is a breadth of non-human primate (Bartus et al., 1979; Lai et al., 1995; Rapp et al., 1990; Voytko, 1999) and canine research (Head et al., 1998; Milgram et al., 1994; Tapp et al., 2003) that support aging-induced differential decrements in discrimination and reversal learning. Moreover, human studies have found deficits in reversal learning in healthy aged adults relative to younger adults (Boutet et al., 2007; Weiler et al., 2008).

Time estimation and reproduction play an important role in a multitude of behavior. Timing is sensitive to pharmacological and experimental manipulations that are known to affect other processes, including attention and remembering, and it is likely that the interaction of these processes is necessary for accurate and precise timing (Buhusi & Meck, 2005). Indeed, evidence suggests that accurate and precise interval timing is necessary for fine and gross motor function, reflexes, speech recognition, decision-making, and divided attention among other processes (Balci et al., 2009; Krampe, 2002; Krampe et al., 2005; Meck et al., 2008; Vanneste & Pouthas, 1999). The timing paradigm has been particularly useful in characterizing neurobehavioral consequences of aging, both normal and neurodegenerative. In particular, impaired time perception appears to manifest during the course of normal aging as well as in patients with schizophrenia, PD and AD (for a review, see Balci et al., 2009; Lustig & Meck, 2005).

Typically, fixed interval (FI), peak interval (PI), temporal bisection, and differential reinforcement of low-rate (DRL) procedures are used to assess timing in rodents. Results from peak interval procedures suggest that aging accelerates clock speed (Meck, 2002; 2006), and

FI schedule performance suggests that aged rats' responding reflects distorted sensitivity to time (Campbell & Haroutunian, 1981; Lejeune, 1989; Lejeune & Jasselette, 1987; Lejeune et al., 1986). Aged animals also differ from younger animals on differential reinforcement of low-rate (DRL) procedures. For instance, Soffie & Lejeune (1991) found that old rats met a pre-set performance criterion more slowly than the young adults, requiring many more sessions to reach that criterion than the younger animals, similar to the findings of Lejeune (1989). In general, the animal literature supports findings from human studies (Coelho et al., 2004; Krampe et al., 2005; Vennesta & Pouthas, 1999; Lustig and Meck, 2005).

Early clinical research suggested that the cerebellum played a key role in functional timing (Ivy & Keele, 1989) and more recent neuroimaging studies (fMRI and PET) have also elucidated roles for the frontal cortex and basal ganglia (Bengtsson et al., 2004; Dreher & Grafman, 2002; Coull et al., 2004). These findings have been bolstered by the study of timing deficits in individuals with PD, AD, schizophrenia, and attention-deficit hyperactivity disorder (ADHD) that suggest, in part, large-scale oscillatory networks that originate from cortico-striatal DAergic neurons are integral for timing (i.e., the striatal beat frequency (SBF) model) (see Matell & Meck, 2004; Meck et al., 2008). These are regions known to be sensitive to both aging and neurotoxicant exposure. They also support the assumption that proper time estimation requires recruitment of other processes, including attention and remembering (Krampe et al., 2005; Lustig & Meck, 2005; Meck, 1991). That timing underlies so many complex cognitive and motor functions is not necessarily surprising given the signaling networks involved in time estimation, as many of the aforementioned processes rely on executive functions thought to be mediated, in large part, by frontal cortical signaling.

Behavioral consequences of Methylmercury exposure

The clinical signs observed following MeHg exposure depend on the developmental period, dose, and duration of exposure. Both human epidemiological studies and experimental animal models provide evidence that prenatal MeHg exposure produces diffuse central nervous

system (CNS) damage and that neurotoxicity occurs at lower exposure levels than adult-onset exposures (Burbacher et al., 1990; Cox et al., 1989; Farina et al., 2011; Rice & Barone, 2000). Often, prenatal exposure produces long-term cognitive and sensorimotor dysfunctions that are irreversible. Conversely, adult-onset exposure produces relatively focal damage. Pathological lesions typically occur in the occipital lobes near the calcarine fissure, primary somatosensory and motor cortices, and the cerebellum (Castoldi et al., 2000; Eto & Takeuchi, 1978; Merigan, 1986, Takeuchi et al., 1962), and deficits manifest as visual disturbances and sensorimotor dysfunction. In the peripheral system, MeHg affects dorsal root ganglia resulting in sensory dysfunction (Hunter & Russell, 1954; Itoh et al., 2001). Chronic, low-level MeHg exposure often produces deficits in behavior that manifest in a delayed fashion, i.e., delayed neurotoxicity, and appearance of symptoms may be dependent upon another catalyst, such as aging. This interaction suggests that MeHg toxicity and normal aging processes act upon similar neural substrates (Newland et al., 2015; Wiess & Reuhl, 1994; Weiss et al., 2002). In particular, the combination of CNS insult by MeHg and aging may produce a form of accelerated or degenerated aging in which behavior impairments associated with aging manifest prematurely (Heath et al., 2010; Newland & Rasmussen, 2000; Trasande & Landrigan, 2005; Weiss et al., 2002).

Many studies have focused on the effects of gestational exposure to MeHg and found disruptions of cognitive and motor function that, in some cases, only became apparent after significant aging (Newland & Rasmussen, 2000; Reed et al., 2008). Fewer have studied the effects of adult-onset exposure, and, in general, they have found that MeHg exposure produced motor-based decrements in gait, balance, wheel-running, rotarod performance, and locomotor activity in weanlings and both young and normal-aged adult rodents (Bellum et al., 2007; Dietrich et al., 2005; Franco et al., 2006; Heath et al., 2010; Hoffman & Newland, 2016). These motor deficits may stem from disruption of DAegic signaling via Ca^{2+}-mediated processes and lesioning of the cerebellar cortex. MeHg is known to affect hippocampal Ca^{2+} signaling and may

disrupt connection networks with the PFC that are important for integrating spatial memory and executive function (Kane & Engle, 2002; Seamans et al., 1998).

In a majority of prenatal MeHg exposure studies, behavior deficits may be linked to disturbances in reinforcement processes, perhaps linked to disruption of DAergic neurotransmission (Newland, 2015). Lower-dose MeHg exposure during prenatal development increases perseveration on reversal-based tasks (Reed et al., 2006) and decrease sensitivity to reinforcement (or augment reinforcer efficacy) on operant conditioning procedures (Newland et al., 2004; Paletz et al., 2006; Reed et al., 2008). Perseveration in reversal learning and tasks like the 5-choice serial reaction time (5-CSRTT) is thought to be mediated by the orbitofrontal cortex (OFC) and striatum (Chudasama et al., 2003). In particular, reinforcement processes such as delayed and probabilistic reinforcement, reinforcer preference, and behavior mediated by appetitive reinforcement involve PFC, OFC, and striatal signaling (Cardinal, 2006; Schultz et al., 2000). Indeed, these are areas likely damaged by MeHg. Again, however, there is paucity in the literature about cognitive effects of adult-onset MeHg exposure.

Most recently, Bailey et al. (2013) and Hoffman & Newland (2016) studied chronic MeHg exposure in adults and found that 2.6 mg/kg/day MeHg in adult BALB/c mice produced deficits in an incremental repeated acquisition (IRA) procedure (Bailey et al., 2013) and wheel-running and rotarod performance (Hoffman & Newland, 2016). In Bailey et al. (2013), MeHg disrupted responding of performance (well-learned) and learning (relatively novel) behavior chains. However, performance measures of IRA decreased with concurrent decreases in measures of motor function; separating motor and cognitive or motivational deficits was difficult. Using wheel-running and a bout analysis, Hoffman & Newland (2016) was able to delineate between the two components of behavior and found that adult-onset MeHg exposure produced motor deficits (speed of running) that were apparent, while the motivation to run remained unchanged. Combined, these reports support the notion that adult-onset MeHg exposure produces relatively specific motor dysfunction while sparing executive or cognitive functions. Still, there has been

little parametric analysis of the interaction between aging on chronic MeHg exposure. Moreover, there is little data regarding differential effects of MeHg as a function stage of adult development (i.e., early, middle, late adulthood, and senescence). The following sections examine potential interrelated neurobiological pathways, i.e., those that regulate Ca^{2+} homeostasis, which potentially mediate behavior change observed in normal aging and following MeHg exposure.

Neurobiological consequences of normal aging

There are numerous theories of aging that often contain overlapping notions and supporting evidence for the etiology of age-associated behavior deficits. Many older, seemingly simple theories that often involving single processes have lost favor as aging has come to be recognized as a constellation of processes. For example, an early theory hypothesized that functional declines during aging arose from widespread neuronal loss, or neurodegeneration (Brody, 1955). That is, aging was viewed as being directly related to the overall number of neurons in the brain, with greater loss resulting in greater functional decline. More recent evidence from human and non-human animal studies suggests that, in a region-specific manner, there is relatively little change in the number of neurons during normal aging, although distinct functional and morphological changes do occur (Hof & Morrison, 2004; Huang et al., 1984; Pannese, 2011; Terry et al., 1987). Newer theories of aging include the metabolic stability (Brink et al., 2009), telomere theory (Olovnikov, 1973, 1996), epigenetic oxidative redox shift (Brewer, 2010), mitochondrial theory (Loeb et al., 2005), mitochondrial hormesis (Ristow & Zarse, 2010), and the target of rapamycin theory (Blagosklonny, 2008; see also Rollo, 2014), among others. Many of these theories have foundations rooted in or linked to two distinct and well-supported older models that continuously garner considerable empirical support; the oxidative stress and calcium homeostasis theories of aging (Coyle & Puttfarcken, 1993; Ermak & Davies, 2002; Finkel & Holbrook, 2000; Harman, 1956; Khachaturian, 1984, 1989; Sohal & Weindruch, 1996; Toescu & Vreugdenhill, 2010). These two theories are discussed in more detail.

Free radicals and oxidative stress. The original free-radical theory of aging, proposed by Harman (1956), arose from studies of Hiroshima victims, following the discovery that radiation damage produces devastating effects that are primarily mediated by free-radical damage to macromolecules such as DNA, lipids, and proteins (Bokov et al., 2004). Free-radicals (e.g., hydroxyl, hydrogen peroxide, and hydroperoxyl) are byproducts of aerobic metabolism via oxidative phosphorylation; electrons derived from the citric acid cycle (tricarboxylic acid cycle or TCA) pass along cytochrome proteins on the inner mitochondrial membrane and react with free oxygen, leading to the production of an oxygen radical (O^{-}) (Cadenas, 2004). These free-radicals also react to form additional reactive oxygen and nitrogen species (ROS and RNS, respectively), both of which can cause cellular damage but also function as part of normal signaling pathways (Afanas'ev, 2010). Thus, the original theory was amended to the more general oxidative stress theory that includes damage from free-radicals and ROS/RNS (e.g., hydrogen peroxide and peroxynitrite) (Beckman & Ames, 1998). Subsequent experimentation over the last 50 years has sought to link macromolecular damage associated with aging and oxidative stress.

A strict interpretation of the oxidative stress theory predicts that a reduction in oxidative stress, either by a reduction in the pro-oxidant load or by an increase in antioxidant defense, or a combination of both, should increase lifespan (for in-depth reviews on the oxidative stress theory, see Beckman & Ames, 1998; Bokov et al., 2004; Cadenas & Davies, 2000; Finkel & Holbrook, 2000; Harman, 2006; Salmon et al., 2010; *c.f.* Speakman & Selman, 2011). That is, increased lifespan is inversely correlated with a reduction in or increased resistance to oxidative stress. Many animal models achieved extended lifespan by manipulating oxidative stress through environmental intervention, e.g., caloric restriction, and/or genetic mutation (Masoro, 2005; Sohal & Weindruch, 1996).

The oxidative stress theory is not without opposition, and opponents suggest that oxidative stress may not be the sole mediator of the aging process (Speakman & Selman,

2011). For example, Brand (2000) postulated the “uncoupling to survive” theory and presented evidence showing little correlation between increased metabolism and energy output (leading to excess ROS) and shortened lifespan (see Mookerjee et al., 2010; Rose et al., 2011; Speakman et al., 2004; Stier et al., 2014). Criticism of the oxidative stress theory has also arisen from studies of long-lived animals like the naked mole rat in which empirical evidence has shown almost no link between longevity of age and levels of ROS or biomarkers of ROS damage (Andziak et al., 2005; Buffenstein, 2005; Miwa et al., 2004).

Calcium homeostasis. The Ca^{2+} hypothesis of aging, proposed by Khachaturian (1984, 1989), originally posited that increased intracellular free Ca^{2+} or $[Ca^{2+}]i$ results in cell death and manifests as subtle deficits in behavior. Formed as a way of gaining insight into the etiology of AD, early forms of Khachaturian’s theory (1984, 1987) contained six elements (for reviews, see Toescu & Vreugdenhil, 2010; Verkhratsky & Toescu, 1998). First, the theory posited that perturbations to cellular mechanisms that control Ca^{2+} homeostasis produce normal and neurodegenerative aging. Second, normal and neurodegenerative aging fall on a continuum of development and neuroadaptation that occurs throughout life. Third, the plasticity of the nervous system is a balance between regenerative and degenerative processes, and the concentration of $[Ca^{2+}]i$ is integral to these processes, e.g., AD may be an imbalance in these regenerative and degenerative processes. Fourth, the functional product of the perturbation in $[Ca^{2+}]i$ homeostasis and the passage of time is a constant. This is a form of “Haber’s rule,” which holds that concentration (here, excess Ca^{2+} calcium concentration) X time is a constant. In this formulation, acute but large insults are functionally similar to chronic but small insults. Fifth, increased $[Ca^{2+}]i$ can result in cell death, implicating Ca^{2+}-mediated processes as a key factor in the final common pathway that leads to neuronal dysfunction and cell death. Finally, a wide variety of factors, acting alone or in combination, simultaneously or sequentially, over a long period of time, act to initiate changes associated with both normal aging and AD.

Accumulated evidence since the articulation of Khachaturian's hypothesis have led to adjustments of his original Ca^{2+} theory. Recent evidence supports the notion that impaired neuronal functional, instead of neuronal loss, accounts for behavior deficits associated with normal aging (Toescu & Vreugdenhil, 2010; Verkhratsky , 2004; Verkhratsky & Toescu, 1998). Even in early stages of neurodegenerative disease neuronal loss may be accompanied by little to no overt clinical manifestations. Thus, necessary modifications to the theory relative to normal aging are likely not necessary to account for neurodegenerative disorders like AD and PD. Evidence from *in vitro* and *in vivo* studies suggest that more subtle and nuanced Ca^{2+}-related changes that occur during normal aging result from increased Ca^{2+} release from intracellular stores via inositol(1,4,5) triphosphate (IP3) and ryanodine (Ry) receptors on endoplasmic reticulum, increased Ca^{2+} influx through L-type VGCCs, increased amplitude and duration of the Ca^{2+}-dependent, K^{+}-mediated afterhyperpolarization (AHP) (i.e., a shift from shorter to longer AHPs during which actions potentials cannot fire) which are mediated by L-type Ca2+ channels, reduced NMDA receptors-mediated Ca^{2+} influx, and reduced calcium buffering capacity (Toescu & Vreugdenhil, 2010). Given the involvement of Ca^{2+} in numerous molecular mechanisms that underlie learning, remembering, and motor function (discussed in later sections), these disruptions result in overt signs of aging.

Note that the oxidative stress theory and the calcium homeostasis theory are not completely at odds. Within the oxidative stress theory, it is entirely possible that ROS drives dysregulation of Ca^{2+} homeostasis, leading to signs of aging (perhaps producing a feed-forward loop). Indeed, evidence strongly suggests that oxidative damage via ROS disrupts Ca^{2+}-mediated processes (Davidson & Duchen, 2006; Ermak & Davies, 2002), in particular $[Ca^{2+}]i$ regulation which causes an influx of Ca^{2+} into the cytoplasm. This increase in $[Ca^{2+}]i$ leads to Ca^{2+} influx into mitochondria, which can result in cell dysfunction and death by disrupting the charge balance across the mitochondrial membrane (Ermak & Davies, 2002). Conversely, the

Ca^{2+} homeostasis theory presents a more mechanistic theory of the perturbations at the cellular level that result in the signs of normal aging.

Calcium

The Ca^{2+} ion is abundantly distributed throughout the mammalian body and its concentration is regulated with precision (for reviews, see Catterall, 2000; Rizzuto & Pozzan, 2006). There exists a steep electrochemical gradient across the plasma membrane of neurons in the central and peripheral nervous systems (CNS and PNS, respectively); $[Ca^{2+}]i$ can reach approximately 100 nM while extracellular levels $[Ca^{2+}]e$ generally approximate 1-2 mM or more (for a review, see Berridge et al., 2003). To maintain this gradient, plasma membrane Ca^{2+}-ATPases (PMCAs) and Na^{+}/Ca^{2+} exchangers extrude Ca^{2+} out of the cytoplasm against the gradient. In addition, intracellular mechanisms that are capable of sequestering or storing excess Ca^{2+} also aid in maintaining the steep Ca^{2+} gradient. A variety of well-characterized Ca^{2+} channels reside within the plasma membrane and these include relatively homogenous groups of either voltage-gated (VGCC) or ligand-gated (LGCC) channels. Some channels activate as the result of conformational changes and second-messenger proteins, i.e., the transient receptor potential family.

Calcium is a ubiquitous messenger and $[Ca^{2+}]i$ regulates many aspects of neuronal function so changes in its concentration produce both acute and long-term effects (Clapham, 2007; Berridge et al., 2000; Burgoyne, 2007). Ca^{2+} influences gene expression (Bading et al., 1997; West et al., 2001), neuronal growth, differentiation, and development (Hennings et al., 1980; Kater et al., 1988), neurotransmitter release and the associated mechanism of long-term potentiation (LTP) (Catterall, 2000; Catterall & Few, 2008; Neher & Sakaba, 2008), and apoptotic and necrotic cell death processes (Orrenius et al., 2003; Pinton et al., 2008; Zong & Thompson, 2006). While this list is not exhaustive, it represents some of the most fundamental processes necessary for normal structural and functional development in humans and non-human animals.

Calcium channels. The two main groups of Ca^{2+} channels are VGCC and LGCC. As their name implies, VGCCs are Ca^{2+}-permeable channels that are sensitive to changes in voltage that occur during the generation of an action potential (AP). In a quiescent state, VGCCs remain closed and block the influx of Ca^{2+} from the extracellular milieu. As the plasma membrane depolarizes during an AP, VGCCs open and allow entry of Ca^{2+} into the cell, causing a signaling cascade. This signaling cascade can have a number of different outcomes based upon the function of the neuron or cell. For example, in smooth muscle cells the influx of Ca^{2+} causes muscle contraction, while in CA1 hippocampal neurons the influx of Ca^{2+} is thought to facilitate LTP via calcium-binding proteins.

VGCCs are classified into high-voltage channels that include P/Q, N, R, and L-type channels and low-voltage channels represented by T-type channels. This classification is based on the strong depolarization necessary to activate high-voltage channels and relatively lower depolarization (20-30 mV more negative) necessary to active low-voltage channels (Catterall, 2000). This review and subsequent sections focus only on high-voltage channels, and particular attention is given to the L-type channel. High-voltage channels are heteromultimers comprising different conformations of five subunits located within the plasma membrane; the α_1, α_2, β, γ and δ subunits with the α_2 and δ subunits forming a $\alpha_2\delta$ complex (Tanabe et al., 1987; Takahashi et al., 1987). Figure 1 (Catterall, 2011) shows the five subunits and hypothetical consequences of Ca^{2+} influx (cartoon rendering, does not denote 3D protein structure). Importantly, the α1 subunit (light green shading) was shown to bind 1,4-dihydropyridines (DHPs), or calcium channel blockers (CCBs), and thus it was identified as the calcium-conducting pore (Catterall, 2011). The remaining four subunits were identified as auxiliary subunits that modulate pore function during Ca^{2+} influx (Arikkath & Campbell, 2003). Importantly, L-type Ca^{2+} channels (LTCCs) appear integral for LTP and as such they are thought to have a direct impact on the re-modeling that supports learning and remembering (Bauer et al., 2002; Thibault et al., 2001; Veng et al., 2003; Weisskopf et al., 1999). In general, LTCCs produce long-lasting Ca^{2+} currents

with an approximate rate of inactivation of 500 msec (T, N, P/Q, and R-type currents range from 20-80 msec) (Yamakage & Namiki, 2002). LTCCs are expressed ubiquitously in neuronal, endocrine, cardiac, smooth, and skeletal muscle, as well as in fibroblasts and kidney cells, but not in platelets. The functions of LTCCs are wide-ranging and include generation of APs and signal transduction that mediate auditory, visual, cardiac, smooth muscle, and endocrine function (for a review, see Catterall, 2011).

In contrast to VGCCs, LGCCs open or close in response to the binding of a ligand, or a chemical messenger (e.g., neurotransmitters), and are found in the plasma membrane and membranes of various intracellular organelles. The LGCCs associated with the plasma membrane are referred to as store-operated channels or Ca^{2+}-release activated Ca^{2+} channels, which are highly Ca^{2+}-selective ion channels that activate upon depletion of $[Ca^{2+}]i$ stores (Parekh & Putney Jr., 2005; Parekh, 2010). Calcium is released from intracellular stores in order to maintain prolonged elevations in $[Ca^{2+}]i$ and refilling these stores is dependent upon activation of Ca^{2+}-release activated Ca^{2+} (Clapham, 1995). Indeed, Ca^{2+}-release activated Ca^{2+} channels are important components that help drive exocytosis, stimulate mitochondrial metabolism, activate gene expression, and promote cell growth and proliferation (Parekh, 2010). The signaling of LGCCs is often relatively complex and supports fundamental processes of learning. For example, the glutamatergic *N*-methyl *D*-aspartate (NMDA) receptor is Ca^{2+}-sensitive and joint activation via ligand binding (glutamate) and depolarization results in an influx of Ca^{2+}, a process thought to mediate synaptic plasticity (Burnashev, 1998). While NMDA receptors are located within the plasma membrane, some LGCC are located within the intracellular membranes of organelles. Ryanodine and IP_3 receptors are located within membranes of the endoplasmic reticulum and the mitochondrial calcium uniporter (MCU) and Ry receptors are located within membranes of the mitochondria. The former LGCCs of the ER are responsible for the initial rise in cytoplasmic calcium that occurs after cell stimulation (Berridge, 1991; Sorrentino, 1995; Clapham, 1995) and the latter LGCCs of the mitochondria

help maintain proper levels of Ca^{2+} in cytosol and within mitochondria (Ryu et al., 2010; Hoppe, 2010).

Calcium signaling and homeostasis. Calcium is a versatile intracellular signaling ion that regulates a multitude of cellular functions over multiple time-courses ranging from msec to hours. Calcium homeostasis, or basal intracellular, organelle, and extracellular Ca^{2+} levels, is determined by reactions that introduce and remove Ca^{2+} from the cytoplasm of cells (see Fig. 2 for a graphic illustration of basic intracellular Ca^{2+} signaling). Under normal signaling conditions, Ca^{2+} that enters intracellular cytoplasm originates from the extracellular milieu via VGCCs and LGCCs and internal stores including ER, mitochondria, and calcium-binding proteins. Influx of [Ca2+]e via Ca^{2+} channels following stimulation is mostly protein-bound to buffers, including, calbindin, calretinin, and parvalbumin, though a small proportion binds to effectors such as calmodulin, troponin C, and synaptotagmin, that activate various cellular processes that operate over a wide temporal spectrum (Berridge et al., 2003). While calmodulin, troponin C, and synaptotagmin act to stimulate downstream systems directly, some signaling may be accomplished indirectly. For example, recruitment of Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), calcineurin, myosin light chain kinases (MLCKs), and phosphorylase kinase is accomplished through indirect mechanisms. In some cases, Ca^{2+} signals result in the formation of second messenger proteins that release Ca^{2+} stored within the SER (the primary internal pool of Ca^{2+}). Concurrently, Ca^{2+} itself may trigger a Ca^{2+}-induced Ca^{2+} release (CICR) from the SER that increases [Ca^{2+}]i. Calcium-induced Ca^{2+} release occurs when the influx of Ca^{2+} stimulates IP_3 and/or Ry receptors (Berridge, 1998; Berridge et al., 2000). These two channels are sensitive to Ca^{2+}, and this CICR process contributes to the rapid increase of Ca^{2+} during signaling and the development of regenerative Ca^{2+} waves.

Calcium channel blockers. In general, CCBs are a heterogeneous group of drugs commonly divided into four classes; dihyrdopyridine, phenylalkyamines, benzothiazapines, and diflourinated piparazines. These drugs block a variety of Ca^{2+} channels, including LTCCs

located on neurons, cerebral and peripheral vasculature, and cardiac smooth muscle (Bellemann et al., 1983; Cohen et al., 1987; Haws et al., 1983; Kazda et al., 1982). These effects produce vasodilation and, as such, they have wide clinical application for the treatment of cardiovascular and neurological conditions including stroke (cerebral ischemia and subarachnoid hemorrhage), angina pectoris, hypertension, cerebrovascular disease, and migraine (Allen et al., 1983; Gelmers, 1983, 1984). While drugs from all four classes pass the BBB to some extent and block L-type Ca^{2+} channels, nimodipine, a 1,4-dihydropyridine CCB, is among the most frequently used in human clinical and experimental animal research. As many CNS insults appear to produce increases in $[Ca^{2+}]i$ which disrupt cell function and viability, blockade of Ca^{2+} channels may alleviate neurobiological and behavior impairments associated with dysregulation of Ca^{2+} homeostasis by reducing $[Ca^{2+}]i$. The following section elucidates neurobiological consequences of Ca^{2+} dysregulation.

Dysregulation of calcium homeostasis

Cell death is mediated, in large part, by Ca^{2+} signaling and thus dysregulation of Ca^{2+} homeostasis may have detrimental consequences on cell viability. The presence of high, non-physiological concentrations of intracellular Ca^{2+} can produce neurodegeneration and cell death by activating biochemical cascades that result in either necrotic or apoptotic processes (Orrenius et al., 2003). These Ca^{2+}-mediated signaling cascades may include the activation of degradation enzymes, such as phospholipases, proteases and endonucleases, perturbation of cytoskeletal organization, and mitochondrial dysfunction. Therefore, unusually high $[Ca^{2+}]i$ often serves as a biomarker for numerous pathological processes (Schanne et al., 1979). However, many CNS insults mediated by Ca^{2+} do not manifest in the same manner or with similar temporal trajectories. For example, evidence strongly suggests that normal aging, neurodegenerative disorders (e.g., Alzheimer's disease), and exposure to toxicants like lead and mercury all disrupt Ca^{2+}-mediated processes. Yet none produces the same constellation or

time-course of symptoms. It is important, then, to delineate the molecular and neurochemical as well as behavioral consequences of the different phenotypes of calcium dysregulation.

Normal aging. Normal brain aging in the absence of specific pathology is correlated with dysregulation of Ca^{2+} homeostasis (for reviews, see Buchholz et al., 2007; Foster, 2007; Foster & Kumar, 2002; Gareri et al., 1995; Toescu & Verkhratsky, 2007; Toescu & Vreugdenhill, 2010; Verkhratsky & Toescu, 1998). Yet, normal aging does not appear to be correlated with gross neuronal loss but instead appears to reflect neuronal impairment stemming from loss of synapses and dendrites (Morrison & Hof, 1997). This has led some to suggest that normal aging is better represented as a shift in the homeostatic reserve of neurons in the aging brain (see Toescu, 2005). As such, aging decreases the capacity to oppose the damaging effects of strong, excessive stimulation despite the presence of functionality in the absence of excessive stimulation (i.e., quiescence). For example, aged neurons show a delayed recovery to resting $[Ca^{2+}]i$ following large stimulation-evoked Ca^{2+} signals (Toescu & Verkhratsky 2000; Xiong et al. 2002). The reduced clearance rate and return to normal $[Ca^{2+}]i$ may be the result of functional or biological changes in the properties of the Ca^{2+} removal systems. Evidence suggests that age-dependent alterations may exist in the function of the PMCA (Michaelis et al., 1996) that involve changes in phosphorylation properties or calmodulin-binding properties (Zaidi et al. 1998). When cytosolic Ca^{2+} is reduced by decreasing the level of stimulation, the rate of recovery in aged neurons is significantly improved and nears recovery observed in the young neurons (Toescu & Xiong, 2004). Similarly, a difference in the number of non-viable neurons becomes apparent only after prolonged exposure to *in vitro* conditions (no difference at 2-3 h, difference appears after 5 h), likely reflecting an increased susceptibility of aged neurons (Xiong et al., 2002). It is important to note that decreased homeostatic reserve does not imply altered resting state $[Ca^{2+}]i$. Reviewed by Verkhratsky & Toescu (1998) and Toescu (2005), evidence supports the notion that, during the resting state, aged neurons do not show elevated $[Ca^{2+}]i$. While recent evidence showing elevated $[Ca^{2+}]i$ in aged neurons may conflict with this theory, it is

possible that the delayed recovery discussed above is the source of the reported higher [Ca^{2+}]i (i.e., the elevation is transient, not sustained).

There is also increased Ca^{2+} influx via VGCCs in aging neurons, and primary neurons of the hippocampus appear more susceptible to this increase (Hajieva et al., 2009; Landfield et al., 1984; Raza et al., 2007; Thibault et al., 1996). Aging results in increased Ca^{2+} release from the SER stores mediated by both IP_3 and Ry receptors (Thibault et al., 2007). In addition, mitochondria are more depolarized, which affects both the energy balance and mitochondrial Ca^{2+} uptake. This change in mitochondrial equilibrium results in an increase in the latency to recover following stimulation (i.e., reduction in Ca^{2+} waves). While the SER may contain a larger internal pool of Ca^{2+}, a preponderance of research suggests that dysfunction of mitochondria underlies the decrease in homeostatic reserve noted in aged neurons (see Thibault et al., 2007; Toescu & Vreugdenhill, 2010).

Neurodegenerative aging. Neurodegenerative disorders, including Alzheimer's (AD) and Parkinson's (PD), comprise some disorders of aging that may be viewed as pathological deviations from the trajectory of normal aging. Normal age-related deficits are compounded by this pathology and lead to the manifestation of clinical symptoms that increase with severity over time. This has led many researchers to hypothesize and provide support for models of neurodegenerative aging that have shared mechanisms of action with normal aging.

Alzheimer's disease is a progressive and irreversible degeneration of learning and cognitive processes that leads to behavioral deficits across a wide range of domains. In most cases, advanced age and the e4 allele of the polymorphic apolipoprotein E gene are major risk factors for AD pathophysiology (Corder et al., 1993), which is characterized by the presence of plaques composed of β-amyloid (Aβ) peptide and neurofibrillary tangles associated with the tau-protein (Hardy & Selkoe, 2002). As mentioned earlier, Khachaturian originally proposed dysregulation of Ca^{2+} homeostasis as a model of AD, suggesting AD may share common mechanisms with normal aging. Animal models provide some support for a role of Ca^{2+}-

mediated processes in the development of AD, and transgenic mutants (e.g., presenilin 1 and amyloid precursor protein mutants) show increased aberrant Ca^{2+} regulation as a function of age before the development of Aβ toxicity. Moreover, evidence supports a primary role of Ca^{2+} dysregulation in the hyperphosphorylation of tau protein that leads to neurofibrillary tangle development (for in-depth reviews on the relation of Ca^{2+} and AD, see Bezprovanny & Mattson, 2008; La Ferla, 2002; Thibault et al., 2007). In the former, dysfunction of Ca^{2+} regulation within the SER may adversely affect amyloid precursor protein, which is involved in the regulation of cytosolic Ca^{2+} levels (via CICR) among other roles, leading to increased Aβ production (Mattson et al., 1993). In the latter, presenilin 1 disruption may affect IP_3 receptors of the SER causing CICR, which may, in part, be mediated by overloading of intracellular Ca^{2+} stores via Ry receptors (Guo et al., 1996). However, there is evidence that Aβ formation and toxicity precedes and leads to increased [Ca2+]i and destabilization of Ca^{2+} homeostasis, although this finding would still suggest that Ca^{2+} dysregulation plays an early and prominent role in the pathophysiology of AD and provides potential targets for therapeutic intervention.

The role of Ca^{2+} is not restricted to AD, and dysregulation of Ca^{2+} homeostasis may also play a prominent role in the pathophysiology of PD (Surmeier et al., 2007; 2010). Parkinson's disease, the second most common neurodegenerative disorder after AD, is also strongly associated with aging and it appears to have little genetic basis (Shulman et al., 2011). The most common pathophysiological marker of PD, degeneration and loss of DAergic neurons in the substantia nigra pars compacta (SNc), produces bradykinesia, rigidity, and tremor. Importantly, SNc DAergic neurons rely on LTCC that have a specific pore-forming Cav1.3 subunit (a specific α subunit) necessary for characteristic pacemaking (i.e., automatic and regular generation of APs) pattern of firing APs. Dysregulation of these LTCCs may perturb an array of behavioral domains related to SNc DAergic signaling, including motor function and time estimation. Evidence from rodent models has found that these perturbations indeed lead to deficits in motor function and timing, both of which are deficits observed in human patients with

PD. Constantly activated neurons (i.e., pacemaking) impose increased oxidative phosphorylation demands, which produce ROS, and these demands may preferentially accelerate the aging of SNc DAergic neurons. Indeed, evidence supports the notion that SNc DAergic neurons age faster relative to other types of neurons (Surmeier et al., 2010). Moreover, studies with isradipine, an L-type CCB that more selectively blocks LTCCs with a Cav1.3 subunit, provides neuroprotection against MPTP-based animal models of PD (Ilijic et al., 2011; Meredith et al., 2008).

The study of neurodegenerative diseases suggests that Ca^{2+} homeostasis plays a large role in the pathophysiology and symptomology of the two most common diseases of aging. The literature strongly suggests that these neurodegenerative forms of aging share common mechanisms with normal aging. Thus, in the study of normal and neurodegenerative aging, it may be beneficial to use animal models of aging that involve perturbed Ca^{2+} homeostasis. The study of environmental contaminants, in particular MeHg, may yield a relatively novel way in which to investigate molecular, cellular, and behavioral deficits associated with aging.

Environmental contaminants. Exposure to some environmental toxicants adversely impacts Ca^{2+} signaling and homeostasis, and the ability of the aging brain to buffer increased [Ca2+]i may amplify these effects. For example, the environmental neurotoxicants lead (Pb) and methylmercury (MeHg) both produce neurotoxicity that appears to be mediated by Ca^{2+} (Atchison & Hare, 1994; Bressler et al., 1999; Ceccatelli et al., 2010; Toscano & Guilarte, 2005). The following section focuses on MeHg-induced changes in Ca^{2+} signaling and homeostasis.

Methylmercury is a potent neurotoxicant that causes motor dysfunction, sensory disturbances, and learning deficits in humans and non-human primates and animals. Once ingested, MeHg is almost exclusively absorbed through the gastrointestinal (GI) tract (approximately 95%) (Clarkson, 1972). Most MeHg in blood is bound to red blood cells (RBCs) via cysteine residues in hemoglobin (Ceccatelli et al., 2010). It is thought that MeHg forms a MeHg-cysteine complex that closely resembles the large neutral amino acid methionine

(Aschner & Aschner, 1990; Kerper et al., 1992). The MeHg-cysteine complex is then transported into endothelial cells of the blood-brain barrier (BBB) by the large neutral amino acid carrier (a universal transporter for amino acids) and passes the BBB, gaining access to most areas of the brain (Aschner & Aschner, 1990). Methylmercury in brain tissue undergoes slow demethylation and forms selenium-based inert complexes that appear semi-permanent; these deposits have been found in diseased persons 10 yrs. post-MeHg exposure (Vahter et al., 1995). In addition to the BBB, MeHg crosses the placental barrier causing concentrations approximately 5 times higher in the fetal brain than in maternal blood. The rate of excretion of MeHg is proportional to the simultaneous body burden, which varies among species, and thus the half-life of MeHg varies among species. For instance, the half-life of MeHg is approximately 16 days in the rat, 8 days in the mouse (Clarkson, 1972), and 70-80 days in humans (Kershaw et al., 1980).

Methylmercury disrupts multiple aspects of pre- and post-synaptic neurotransmission, including receptor function, reuptake, and second-messenger systems in large part because of its affinity for sulfhydryl (SH) groups (Atchison, 2005; Castoldi et al., 2001). MeHg affects Glu, γ-aminobutyric acid (GABA), and dopamine (DA) neurotransmitter systems. MeHg causes an efflux of Glu and DA, and to lesser extent GABA; each has a different effect on neuronal functioning. Increases in extracellular Glu lead to excitotoxic damage from over-activation of glutamatergic NMDA receptors. MeHg also blocks the reuptake of Glu from the synapse by inhibition of excitatory amino acid transporters on astrocytes adjacent to the synapse. Astrocyte function is discussed in more detail below. GABA (inhibitory neurotransmission) appears more sensitive to the effects of MeHg than Glu (excitatory neurotransmission). MeHg produces a block of hippocampal GABAergic neurotransmission at lower doses relative to Glu neurotransmission. The effects on GABA receptors are mostly localized at $GABA_A$ receptors and MeHg acts to downregulate mRNA levels for this receptor. This is consistent with the finding that MeHg decreases levels of glutamic acid decarboxylase (GAD), the enzyme

responsible for the synthesis of GABA from Glu (O'Kusky & McGeer, 1985; O'Kusky et al., 1988). MeHg indirectly affects GABAergic signaling via second messenger G-proteins and receptor subunits (Fitsanakis & Aschner, 2005).

Similar to MeHg-induced Glu release, MeHg induces the spontaneous release of DA, increasing extracellular DA concentration. In particular, Faro and colleagues (1997, 2001, 2003, and 2007) conducted a series of *in vitro* and *in vivo* studies determining that MeHg causes a concentration-dependent block of the DA transporter (DAT). The reduced reuptake from the synapse increases extracellular DA. These effects have been localized to the striatum. Based on the affinity of MeHg for SH-containing thiols, Faro et al. (2005) predicted that the co-administration of SH-containing compounds with MeHg may interfere with MeHg's block of DAT. Indeed, glutathione sulfhydryl (GSH) and cysteine (a GSH precursor) both decrease MeHg-induced increases in extracellular DA by reducing the amount of MeHg available to disrupt DAT. GSH, a free-radical scavenger, functions to maintain reduction oxidation (redox) homeostasis. Faro and colleagues also provided evidence that the disruption of DAT was Ca^{2+}-independent. Newer evidence suggests that, while DAT disruption may be Ca^{2+}-independent, not all MeHg-induced increases in extracellular DA are the result of DAT dysfunction (Tiernen et al., 2013, 2015).

Methylmercury also affects astrocytes and this may contribute to the excitotoxicity caused by increasing extracellular Glu (see Ni et al., 2012). Notably, astrocytes provide the main route for reuptake of Glu from synapses via excitatory amino acid transporters. They also help regulate glutamatergic synapses and play a pivotal role in the biosynthesis of Glu. To accomplish these functions, astrocytes contain large stores of Glu that form a steep concentration gradient. MeHg induces an efflux of Glu from astrocytes and blocks astrocytic reuptake of Glu that leads to prolonged activation and depolarization at glutamatergic synapses and ultimately to excitotoxic damage and cell death (Aschner et al., 1993). In support of this, Park et al. (1995) and more recently Ramanathan & Atchison (2011) showed *in vitro* that the

NMDA receptor antagonist MK-801 (dizocilpine) blocks the MeHg-induced astrocyte-mediated increase in activation of glutamatergic NMDA receptors (a net result of reducing excitotoxic damage). MeHg also blocks the synthesis of GSH, likely increasing the sensitivity of astrocytes to ROS damage. In particular, MeHg reduces the phosphorylation of cystine and cysteine, the precursors of GSH reducing GSH in astrocytes (a factor in astrocyte sensitivity to MeHg relative to other glial cells like microglia) (Ni et al., 2012).

Unlike normal aging processes, MeHg causes widespread and dose-dependent cell death, with lower doses inducing apoptosis and higher doses inducing necrosis (Ceccetelli et al., 2010). *In vitro* models provide evidence that MeHg disrupts Ca^{2+} homeostasis in two distinct phases (see Atchison, 2005; Atchison & Hare, 1994; Hare & Atchison, 1995b; Marty & Atchison, 1997). In phase one, MeHg causes a release of intracellular Ca^{2+} from internal organelle stores. IP_3 receptor and to a lesser extent Ry receptor stimulation on the plasma membrane of SERCA induces Ca^{2+} release into the cytoplasm (Limke et al., 2004). This release of Ca^{2+} by the SERCA may be mediated by MeHg binding to muscarinic acetylcholine (ACh) receptors (M3 in particular), causing an upregulation of IP_3 and subsequent binding to IP_3 receptors, releasing Ca^{2+} (Atchison, 2005; Marty & Atchison, 1997; Tiernan et al., 2013). Mitochondria are generally positioned such that the Ca^{2+} released from the SER is spatially nearby, and mitochondria uptake excess Ca^{2+} in an attempt to buffer $[Ca^{2+}]i$. The increase in internal mitochondrial levels of Ca^{2+} disrupts the mitochondrial plasma membrane and produces an efflux of Ca^{2+} back into the cytoplasm. In phase two, extracellular Ca^{2+} enters the neuron, although it is less clear the mechanisms that control this influx. Initial *in vitro* studies implicated N, Q, and L-type channels, but it appears that all present types of Ca^{2+} channels contribute to this influx. The influx of extracellular Ca^{2+} is thought to cause the spontaneous release of norepinephrine (NE) and possibly DA (Tiernan et al., 2013). Much less is known about the function of the second phase, but it is important nonetheless as blockade of this phase can antagonize MeHg neurotoxicity (Marty & Atchison, 1998).

In combination, these two phases disrupt Ca^{2+} homeostasis, affecting cerebellar and striatal granule, hippocampal, and glial cells, and associated neurotransmitter systems. In particular, the granule layer of the cerebellum appears sensitive to MeHg toxicity relative to other regions including neighboring Purkinje cells. This differential sensitivity is not fully understood, although evidence points to the localization of specific muscarinic acetylcholine (M3) and $GABA_A$ (α6-subtype) receptors in cerebellar granule cells that mediate this exacerbated toxicity. Prolonged $[Ca^{2+}]i$ levels may also adversely affect nuclear processes and signaling. For example, the nuclear envelope that connects the cytoplasm to the nucleus contains a Ca^{2+} store, though relatively small, that is gated by IP_3 and Ry receptors, which may result in disruptions of nuclear Ca^{2+} signaling. Most recently, Tiernan et al. (2013) provided *in vitro* evidence that disruption of Ca^{2+} homeostasis via MeHg stimulates tyrosine hydroxylase (TH), the rate-limiting step in DA synthesis, thereby increasing phosphorylation of DA and loading vesicular stores with excess DA that is then released into the synapse (Tiernen et al., 2013). The alteration of DA synthesis may be crucial to understanding the behavioral effects of low-dose MeHg exposure, as perturbed DA signaling is thought to underlie some of the behavioral deficits observed following gestational and postnatal exposure.

The neurotoxicity of MeHg is linked to Ca^{2+} homeostasis but this is not the sole cause. Other mechanisms appear important, due in large part to MeHg's high affinity for -SH groups. MeHg exposure increases levels of ROS and free-radical production, e.g., levels of superoxide, hydrogen peroxide and lipid peroxidation, as well as levels of peroxynitrite (an oxidant caused by the pairing of superoxide and nitric oxide). In a feedback loop, MeHg depletes GSH and reduces superoxide dismutase (SOD), both of which are antioxidants and ROS scavengers. The resulting decrease in antioxidant defense results in free-radical mediated damage, which in turn may produce more ROS. It is important to note, however, that the increased level of ROS induced by MeHg is also likely to be related to mitochondrial dysfunction because MeHg-induced Ca^{2+} dysregulation disrupts mitochondrial respiration, alters the electron transport

chain, and perturbs the mitochondrial membrane potential. As briefly mentioned earlier, CCBs reduce [Ca^{2+}]i by blocking Ca^{2+} channels, among other effects. A pharmacological challenge with a CCB may provide insight into neurobiological correlates of behavior mediated by Ca^{2+} signaling. Then, neuroprotection afforded by CCBs against consequences of aging and contaminant exposure would provide support for the notion that dysregulation of Ca^{2+} homeostasis via increased [Ca^{2+}]i mediates the onset of behavior dysfunction.

Nimodipine, an L-type calcium channel blocker

The CCB nimodipine exhibits relatively high lipophilicity and thus it easily crosses the blood brain barrier (BBB) and pools in high concentrations in cerebrospinal fluid (CSF). Nimodipine blocks Ca^{2+} currents through voltage-gated L-type Ca^{2+} channels, reducing neuronal excitability, neurotransmitter release, axonal transport, and the activity of Ca^{2+}-dependent enzymes (Towart et al., 1979). Also, nimodipine, like many CCBs, is a potent vasodilator that can increase cerebral flood flow at doses that do not increase peripheral blood flow (Bork et al., 2015; Haws et al., 1983; Kazda et al., 1981). These properties may be responsible for the beneficial effects of nimodipine following CNS insult and potentially advantageous effects in healthy subjects.

Nimodipine has numerous clinical uses (for a review, see Tomassoni et al., 2008), primarily for the treatment of hypertension, stroke (cerebral ischemia and subarachnoid hemorrhage) and vascular dementia (Allen et al., 1983; Betz et al., 1985; Feigin et al., 1998; Scriabine et al., 1989). For example, reduced tissue contraction via CCB protects against hypertension, but inhibiting Ca^{2+} overload which increases cell viability combats the effects of a stroke (if treatment is rendered within approximately 12 hours of the ischemic episode) (Tomassoni et al., 2008). In addition to the variety of clinical applications, researchers continue to use nimodipine (and other CCBs) in two distinct arenas of preclinical research: 1) CCB's for the treatment of CNS insult (e.g., protecting against cognitive signs of aging or toxicant

exposure) and 2) CCB's as nootropic drugs (e.g., increasing cognitive performance in healthy animals).

Experimental animal models (*in vivo* and *in vitro*) have found that nimodipine, as well as some other CCBs (e.g., nifedipine and amlodipine), is protective against a number of CNS insults including glutamate toxicity, cerebral ischemia and hypoxia, hydrocephalus, and hippocampal lesions (Bork et al., 2015; de Jong et al., 1990; Haile et al., 2012; Iimuro et al., 1996;; Li et al., 2009; Solomon et al., 1995; Thompson et al., 1990; Veng et al., 2003; Zhang et al., 2012). For example, *in vitro* work by Bork et al. (2015) showed that pretreatment with nimodipine reduced cytotoxicity induced via alcohol (200 mM EtOH) and osmotic stress (450 mOsmol/L) in PC12-cells.

In models of aging, studies have shown that nimodipine can ameliorate age-related deficits in conditioning, remembering, and maze performance. In particular, Deyo et al. (1989) found that infusions of nimodipine aided aging rabbits in the acquisition of CEB by reducing trials to criterion such that there was no difference between aged and young rabbits. These findings were subsequently replicated and several lines of research have shown that nimodipine, at doses that facilitate CEB in rabbits, reduces AHPs thereby increasing neuronal firing rate (Thompson et al., 1990; Disterhoft, Moyer, & Thompson, 1994; Disterhoft et al., 1995). In addition to respondent-based procedures, nimodipine appears to facilitate maze learning and performance in senescent animals, which was correlated with increased intracellular buffering of Ca^{2+} by Ca^{2+}-binding proteins (Batuecas et al., 1998). Together, these data suggest that blockade of L-type Ca^{2+} channels facilitates remembering by enhancing neuronal excitability in aging animals.

In models of MeHg toxicity, *In vitro* (Limke et al., 2004;) and *in vivo* (Bailey et al., 2013; Hoffman & Newland, 2016; Sakamoto et al., 1996) studies support the notion that nimodipine affords neuroprotection by blocking LTCCs. For example, Sakamoto et al. (1996) showed that a variety of CCBs other than nimodipine protect again MeHg insult (5.0 mg/kg/day for 12 days).

Bailey et al. (2013) and Hoffman & Newland (2016) found that chronic nimodipine (2-20 mg/kg/day) afforded dose-dependent neuroprotection to adult BALB/c mice chronically exposed to approximately 2.6 mg/kg/day MeHg. Nimodipine attenuated or blocked deficits in an incremental repeated acquisition (IRA) procedure (Bailey et al., 2013) and wheel-running and rotarod performance (Hoffman & Newland, 2016). It is worth noting that under the IRA procedure MeHg reduced overall response rates, and this effect was coincident with deficits in performance measures on the IRA; separating motor and cognitive or motivational deficits was difficult in that study but the authors suggested that the IRA deficits were secondary to motor deficits.

Using wheel-running and a bout analysis of running's microstructure, Hoffman & Newland (2016) were able to decouple motivational and motor effects of chronic MeHg exposure. They did this by partitioning wheel-running into bouts using a change-point algorithm from which estimates of motor function, or the rate of responding within a bout, and motivation, the inter-bout interval (inverse of the rate at which bouts are initiated) were derived. They found that MeHg produced motor deficits (within-bout running rate) were apparent while bout-initiation rate (a marker of motivation) remained unchanged. Taken together, studies of aging or MeHg with nimodipine suggest that nimodipine's L-type channel blockade may protect aged neurons from excess $[Ca^{2+}]i$ and the detrimental behavioral effects that result from dysregulation of Ca^{2+} homeostasis. The longitudinal study of MeHg exposure and the ability to offset MeHg's effects may provide insight into mechanisms of aging, both normal and neurodegenerative. Many studies of aging often use neuromolecular endpoints, e.g., tau pathology, loss of substantia nigra DAergic neurons, and reduction in dendritic spine density. The study of overt behavior may provide information about neuromolecular pathology as different components and classes of behavior may have different neural correlates, which themselves may be differentially sensitive to insult. The next section discusses one particular way in which to study behavior called bout analysis that may be highly sensitive to subtle changes in behavior.

Quantifying behavior change

A prominent feature of the impact of neurotoxicant exposure or the aging process may be a reduction in response rate. While response rate may adequately describe global behavioral dysfunction, it can be difficult to determine whether alterations in response rate are due to changes in motor function or the control over behavior by reinforcers, i.e., conditions related to motivation (Newland, 1995). While there is significant overlap between motor function and motivational variables that control behavior, the underlying neural substrates vary and CNS insults may differentially affect these components. In this instance, global or molar measures of performance may be substituted for more fine grain analyses that investigate molecular determinants of behavior. Bout analysis assumes that response rate is the conflation of two measures: the rate at which animals initiate a stint of reinforced target behavior and the rate at which target behavior is emitted. The different types of responses that comprise these two rate measures may be differentially controlled (Blough, 1963; Nevin & Baum, 1980; Shull, 1991; Shull et al., 2001). Analyses that measure change in these response distributions may help characterize the onset and progression of pathology and aid in understanding neural correlates of a pathological phenotype. Bout analysis and in particular log-survivor bout analysis, provides a useful analytical means for describing motor and motivational contributions to behavior that occur in bouts.

Bout analysis. It is possible to analyze the microstructure of bouts and this generally involves estimating the periodicity and duration of bouts (Andrew, 1963, 1964; Machlis, 1977; Shull, 2001). Behavior under intermittent reinforcement often produces two response distributions: responses separated by relatively long intervals and responses separated by relatively short intervals. For example, under a VI 60” schedule of reinforcement, a rat may initiate short bursts of responses that produces long and short interresponse times (IRTs), e.g., a broken-stick model of IRTs (Shull, 2001). Responses that terminate short or long IRTs may be viewed as separate classes of behavior that are differentially sensitive to manipulation.

Quantitative analysis of IRT distributions has generally found that responses that initiate a bout (bout-initiation responses) are relatively sensitive to motivational manipulations such as satiation, deprivation, and reinforcer devaluation (Shull et al., 2001, 2003, 2004). Conversely, within-bout responses appear sensitive to motoric variables, such as response cost (Shull et al., 2004; Shull & Grimes, 2003; Brackney et al., 2011). Previous studies suggest that adult-onset MeHg exposure produces selective motor dysfunction while sparing "cognitive" function (mediated by motivation). Aging may act on both motor function and motivation, and the interaction of MeHg and aging may blur motor and motivational effects of the toxicant. High-rate behavior, which taxes motor function, may be ideal for studying the effects of methylmercury and a bout analysis of the IRT distributions may accurately estimate the contribution of motor or motivational variables to decrements in overall response rate.

Note that the contingencies that produce high-rate responding usually link the response rate to the reinforcement rate. Thus, it is possible that as impairment progresses following toxicant exposure decreases in response rate will lead to decreases in reinforcer rate and this could, in turn, further reduce response rates, confounding measurement of toxicant effects (a positive feedback loop). In the study of aging or toxicant exposure, it is predictable that, at some point, animals' motor function may deteriorate and thus some high-rate schedules may confound the separation of a behavior from the contingencies that maintain it. Procedural manipulations to high-rate schedules may still allow for the independence of motor and motivational contributions to behavior. In particular, the percentile schedule of reinforcement has been a useful tool in not only generating high-rate behavior but also for decoupling response and reinforcer rate.

A percentile (PCNT) schedule of reinforcement is designed to generate high-rate operant behavior while adjusting the response criterion in real time according to an animal's response rate. In this way, the schedule can adjust a response criterion dynamically if, for example, motor impairment diminishes the ability to respond (Galbicka, Johnson et al., 2009).

Under a PCNT schedule, each response terminates an IRT that is then compared with previous IRTs through a look-back window. Responses that terminate IRTs shorter than a given percentage of the previous IRTs meet criterion for reinforcement. Thus, even in the face of impairment, a subjects' target responding may still contact reinforcement, disentangling the effect of reinforcer loss on response rate with CNS insult-induced decreases in responding. This schedule stands in contrast to other high-rate schedules like a differential reinforcement of high-rate (DRH) schedule in which the response criterion is fixed and unchanging. Impairment under the DRH schedule, which manifests as reduced responding, leads to a direct decrease in reinforcer delivery. Thus, while initially producing high-rate behavior, DRH schedule performance during impairment may reflect the conflation of motor and motivational factors effects that disrupt behavior.

It is also valuable to study high-rate behavior across different sources of reinforcement. That is, behavior under the control of external schedules of reinforcement versus behavior under the control of intrinsic or automatic reinforcement processes (Catania, 1991). Both extrinsically and intrinsically reinforced high-rate behavior may produce similar IRT distributions, but the two may be mediated by different neurobiological correlates and differentially sensitive to MeHg exposure, nimodipine, and/or aging. For example, Johnson et al. (2011) studied the high-rate nose-poking and wheel-running of two different strains of mice using a bout analysis approach. Wheel-running obviously contains a large motor component, i.e., the ability to turn the wheel, but the motivation to run also plays a large role in maintenance of the behavior and wheel-running is a highly salient, relative spontaneous, and intrinsically reinforced behavior in rodents. Thus, wheel-running is capable of producing response distributions amenable for bout analysis to separate motoric from motivational contributions to behavior. Johnson et al. (2011) found that these two types of behavior were differentially sensitive to the GABA agonist pentobarbital. Thus, the use of high-rate behavior controlled by different sources of

reinforcement coupled with a bout analysis approach may ultimately help to characterize neural correlates of MeHg toxicity and aging and rescue by nimodipine.

The current research

The aims of the current study were first to study the interaction between aging and chronic MeHg exposure, second to study the efficacy of the L-type CCB nimodipine to antagonize MeHg effects, and third to study the efficacy of a bout analysis approach to differentiate between motor and motivational deficits induced by MeHg. To that end, two age cohorts of mice were exposed to MeHg and/or nimodipine which produced a 2 (age group) X 2 (MeHg) X 2 (nimodipine) full factorial design. In Experiment 1, high-rate nose-poking maintained under a percentile schedule of reinforcement was analyzed using a bout analysis that analyzes motor and motivation components that comprise behavior. In Experiment 2, the performance of the same animals from Experiment 1 was measured using wheel-running and rotarod performance. Wheel-running data was also analyzed using a bout-analysis approach. Experiments 1 and 2 were carried out simultaneously and funded by a grant from the National Institute of Health [ES003299].

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Figure Captions

Figure 1. A cartoon illustration of the subunit structure of an L-type voltage-gated calcium channel (LTCC). The structure contains α1, α2, β, γ and δ subunits with the α2 and δ subunits forming a α2δ complex. The α1 subunit (light green shading) is the calcium-conducting pore. The arrows show hypothetical consequences of calcium entry via membrane depolarization: muscle contraction, synaptic neurotransmission, protein phosphorylation and enzymatic regulation that signal nuclear transcription factors and ultimately lead to changes in gene transcription.

Figure 2. A steep concentration gradient requires tight control over intracellular [Ca2+]i. Under normal signalling conditions, [Ca2+]i originates from the extracellular milieu via VGCCs and LGCCs and from internal stores including ER and mitochondria, Influx of [Ca2+]e via Ca2+ channels following stimulation is mostly protein-bound to buffers, including, calbindin, calretinin, and parvalbumin, though a small proportion binds to effectors such as calmodulin, troponin C, and synaptotagmin. In some cases, Ca2+ signals result in the formation of second messenger proteins that release Ca2+ stored within the ER. Concurrently, Ca2+ itself may trigger a Ca2+-induced Ca2+ release from the ER when the influx of Ca2+ stimulates IP3 and/or Ry receptors.

Figure 1.1

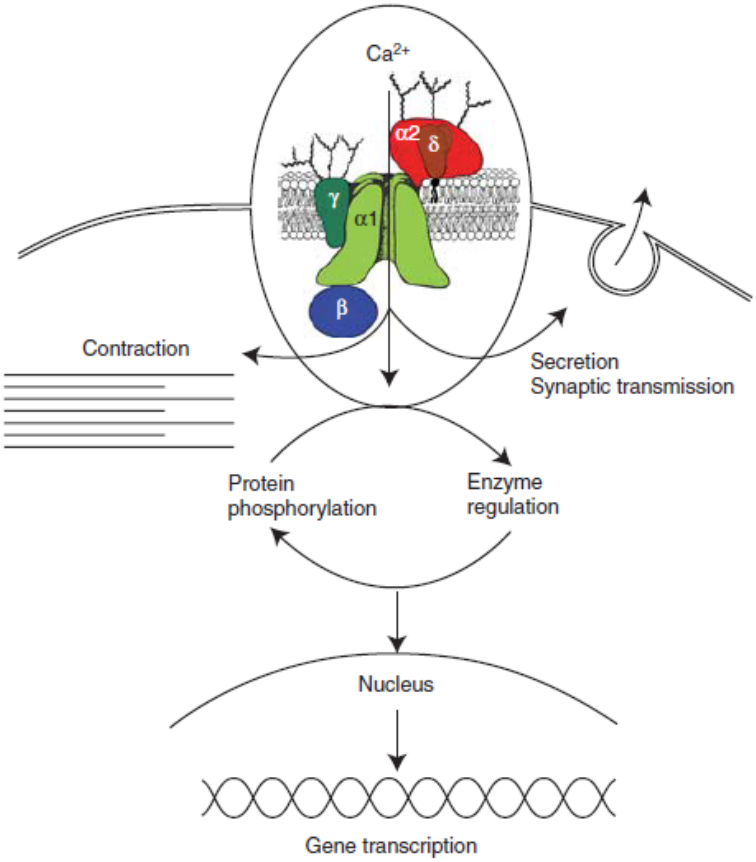
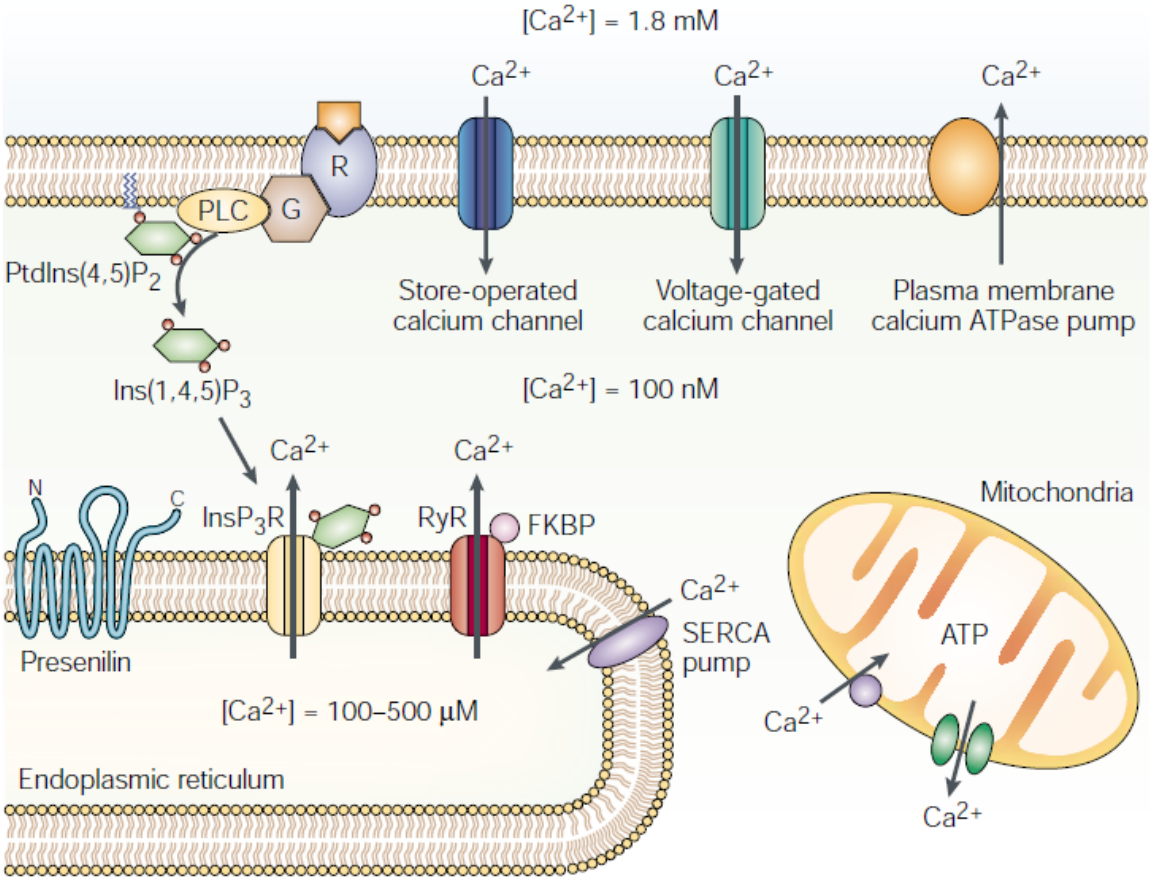

Ca2+
α2
δ
γ
α1
β
Contraction
Secretion
Synaptic transmission
Protein
phosphorylation
Enzyme
regulation
Nucleus
Gene transcription

Figure 1.2


[Ca2+] = 1.8 mM
Ca2+
Ca2+
Ca2+
R
PLC
G
PtdIns(4,5)P2
Store-operated calcium channel
Voltage-gated calcium channel
Plasma membrane calcium ATPase pump
Ins(1,4,5)P3
[Ca2+] = 100 nM
Ca2+
Ca2+
N
C
InsP3R
RyR
FKBP
Mitochondria
Ca2+
SERCA pump
ATP
Presenilin
[Ca2+] = 100–500 µM
Ca2+
Endoplasmic reticulum
Ca2+

Chapter 2

A bout analysis reveals age-related methylmercury neurotoxicity and nimodipine neuroprotection

Abstract

Age-related deficits in motor and cognitive functioning may be driven by perturbations in calcium (Ca2+) homeostasis in nerve terminals, mechanisms that are also thought to mediate the neurotoxicity of methylmercury (MeHg). Calcium-channel blockers (CCBs) protect against MeHg toxicity in adult mice, but little is known about their efficacy in other age groups. Two age groups of BALB/c mice were exposed to 0 or 1.2 mg/kg/day MeHg and 0 or 20 mg/kg/day of the CCB nimodipine for approximately 8.5 months. Adults began exposure on postnatal day (PND) 72 and the retired breeders on PND 296. High-rate operant behavior was maintained under a percentile schedule, which helped to decouple response rate from reinforcer rate. Responding was analyzed using a log-survivor bout analysis approach that partitioned behavior into high-rate bouts separated by pauses. MeHg-induced mortality did not depend on age but nimodipine neuroprotection was age-dependent, with poorer protection occurring in older mice. Within-bout response rate (a marker of sensorimotor function) was more sensitive to MeHg toxicity than bout-initiation rate (a marker of motivation). Within-bout rate declined almost 2 months prior to overt signs of toxicity for the MeHg-only retired breeders but not adults, suggesting greater delay to toxicity in younger animals. Motor-based decrements also appeared in relatively healthy adult MeHg + NIM animals. Aging appeared to alter the processes underlying Ca2+ homeostasis thereby diminishing protection by nimodipine, even in mice that have not reached senescence. The study of MeHg exposure presents an experimental model by which to study potential mechanisms of aging. [Funding provided by NIEHS grant R01 003299].

1. Introduction

Methylmercury (MeHg) is a global pollutant and the primary concerns about its health effects are due to its neurotoxicity [1]. Prenatal exposures produce diffuse central nervous system (CNS) damage and cognitive dysfunction [2,3,4] whereas adult-onset exposures produce relatively focal damage that appears in the primary motor cortex, sensory regions of the cerebral cortex, cerebellar granule cells, and dorsal root ganglion and results in motor dysfunction [5,6,7]. Methylmercury-induced disruptions of intracellular signaling and cell death have been linked, at least in part, to dysregulation of Ca^{2+} homeostasis inside nerve terminals [8,9]. Similarly, neuronal degeneration during aging is thought to be mediated by changes in the level of intracellular Ca^{2+} [10,11,12]. Chronically elevated levels of intracellular Ca^{2+} in neurons and reduced ability to buffer Ca^{2+} levels during normal aging provoke subtle age-associated declines and mild impairment [12,13,14,15,16]. Chronic MeHg exposure, acting to disrupt Ca^{2+} homeostasis, may exacerbate age-related declines in motor or cognitive functioning and accelerate normal or neurodegenerative aging, as has been noted with MeHg [17].

The excess intracellular Ca^{2+} produced by MeHg and aging suggests that preventing increased Ca^{2+} influx into intracellular cytosol could be neuroprotective. Calcium channel blockers reduce intracellular Ca^{2+} by blocking Ca^{2+} channels located in neuronal cell membranes, cerebral and peripheral vasculature, and cardiac smooth muscle [18,19,20,21]. Nimodipine, a 1,4-dihyrdopyridine CCB, is an L-type Ca^{2+} blocker with excellent selectivity for the CNS [22], and is an ideal candidate to protect against Ca^{2+}-mediated CNS insults like MeHg exposure. *In vitro* [23,24] and *in vivo* [24,25,26] studies support this notion. For example, Bailey et al. [25] and Hoffman & Newland [26] found that chronic nimodipine (2-20 mg/kg/day) afforded dose-dependent neuroprotection in adult BALB/c mice chronically exposed to 2.6 mg/kg/day MeHg. Nimodipine attenuated or blocked deficits in an incremental repeated acquisition (IRA) procedure [25], wheel-running and rotarod performance, and mortality [26]. CCBs, including

nimodipine, also attenuate or block selective signs of normal aging [27,28,29,30,31] and other CNS insults [32,33,34,35,36,37; *c.f.* 38].

It is difficult to separate motor from motivational components of behavior in models of neurotoxicant-induced motor deficits because the behavior is closely coupled to the motivation to engage in it [39]. Procedures that produce high-rate responding, such as fixed-ratio, variable-ratio, and differential reinforcement of high rate schedules (DRH) inherently link reinforcement rate to response rate. Thus, impairment may produce a positive feedback loop wherein response rate decrements drive reductions in reinforcer rate which could, in turn, further reduce response rate, confounding motor deficits with the consequences of reinforcer loss. In the current study, we separated motor and motivational influences first by manipulating the contingency linking responding to the delivery of reinforcers and second by using an analytical approach capable of differentially estimating the contribution of motoric and motivational components of behavior may be advantageous.

We used both percentile (PCNT) and differential reinforcement of high rate (DRH) schedules to maintain high-rate nose-poking. The PCNT schedule is particularly appealing because it titrates the response criterion in real-time according to the subject's recent performance [40,41]. As response rate declines, a PCNT schedule relaxes the response criterion, making it easier to obtain reinforcers. This allows behavior to contact reinforcement even in the face of impairment, disentangling the effect of reinforcer loss on response rate with MeHg- or aging-induced decreases in responding. In contrast, response rate decrements under the DRH schedule generally lead to a direct decrease in reinforcer delivery. To separate further reinforcer rate from motor deficits, criterion responses were reinforced under a random interval 30s (RI 30s) schedule of reinforcement, which randomly reinforced criterion response patterns at an average rate of two reinforcers per min.

The analytical approach to separating influences over behavior was based on the observation that high-rate behavior typically occurs as bouts of response bursts separated by

intervals during which the animal is disengaged from the target behavior, is to use a dynamic analysis that breaks a response epoch second-by-second into bouts. [42-47]. The key response unit on which this analysis is based is the interresponse time (IRT). A bout comprises a run of short IRTs, while the initiation of a new bout typically terminates a long IRT. We used a log-survivor analysis, described in detail by Shull and colleagues [42-44], to partition these IRTs into two distinct distributions. The short IRTs produced by response bursts, or within-bout responses, serve as an index of motor function. In contrast, the long IRTs that represent inter-bout intervals, the inverse of which is bout-initiation rate, serve as an index of the motivation to engage in the target behavior. These interpretations are supported empirically by studies that show that changes in motivating operations like food deprivation selectively affect bout-initiation rate [41,47], whereas manipulations that makes responding more difficult [45] or compounds like MeHg [26] and pentobarbital [46] with known motoric effects preferentially affect within-bout rate. This analysis assumes that responding can be described as three orthogonal components, within-bout rate, bout-initiation rate, and bout length [42-44], which is supported by Hoffman & Newland's [26] reconstruction of overall response rate in control and MeHg-exposed mice by a linear combination of these three terms derived from a change-point analysis.

The present study used a log-survivor bout analysis approach to disentangle motoric from motivational deficits in high-rate nose-poking induced by chronic MeHg exposure and neuroprotection by nimodipine in two age cohorts of male BALB/c mice.

2. Material and methods

2.1 Subjects

Adult and retired breeder male BALB/c mice (*N*=112) were purchased from Harlan Laboratories (Indianapolis, IN) and housed in an Optimice® rack system in an AAALAC-accredited temperature- and humidity-controlled vivarium that was maintained on a 12-hour light-dark cycle (lights on at 6:00am). Two age cohorts, two MeHg water concentrations, and

two nimodipine diets produced a 2 (age) X 2 (MeHg) X 2 (nimodipine) full factorial design with 12-16 mice per exposure group by age.

2.1.1 Adults

The adult cohort (*n*=51) arrived at 49 days of age. Upon arrival, mice were housed in pairs in clear polycarbonate cages, separated by a clear Plexiglas© divider that prevented physical contact, but allowed visual, olfactory, and auditory interaction. Due to the aggressiveness of adult male BALB/c mice [48], animals remained separated for the duration of the study. Their weight was maintained at approximately 24 g by feeding approximately 2.5 g standard rodent chow per animal per day, adjusted according to their body mass, with free access to water except during experimental sessions. After 4 months, adults transitioned to a final target weight of approximately 26 g by feeding approximately 3.0 g rodent chow per day.

2.1.2 Retired breeders

The retired breeder age cohort (*n*=63) arrived at 273 days of age and were housed in the same manner as the adults. Upon arrival, they weighed 26-30g, which was reduced and maintained at a final target weight of approximately 26 g by feeding approximately 3.0g standard rodent chow per animal per day with free access to water except during experimental sessions.

2.2 Methylmercury and nimodipine exposure

Methyl mercuric chloride (CH_3HgCl) was procured from Alfa-Aesar (Ward Hill, MA, USA) and dissolved into water to produce the water solutions. Nimodipine was procured from Sigma-Aldrich (St. Louis, MO) and mixed into standard rodent chow manufactured by Purina TestDiets and based on a 5LL2 laboratory chow diet. Based on measurements of water and food consumption and weight (data not shown), MeHg exposure corresponded to approximately 0 and 1.2 mg/kg/day of Hg and nimodipine exposure corresponded to approximately 0 and 20.0 mg/kg/day. Exposures began at 72 and 296 days of age for adults and retired breeders,

respectively, and continued for 253 days until animals were 325 and 549 days of age. Experimental procedures for both age groups began on the first day of exposure.

2.3 Apparatus

Experiments were conducted in standard Med Associates Inc. modular operant conditioning chambers (St. Albans, VT, product #ENV-007). Each chamber measured 30.5 cm L x 24.1 cm W x 29.2 cm H and contained two stainless steel front and back walls and two Plexiglas© side walls. Mounted on the front wall were two nose-poke holes (Left and Right), separated by a pellet dispenser. Above each nose-poke hole was a yellow LED. Interrupting an infrared beam in the nose-poke hole registered a response. The pellet dispenser delivered 20mg sucrose pellets. Chambers had two Sonalert™ tones (2900 and 4500 Hz, nominally) calibrated to an amplitude of 70 dB for presentation of auditory stimuli. Located near the ceiling of the chamber on the back wall was a single 2.8-W house light. Sound-attenuating cabinets enclosed operant chambers with a fan to circulate air for ventilation.

2.4 Procedure

Experimental sessions were conducted in the operant chambers described above. With the exception of autoshaping, all sessions lasted 32.5 min and occurred 4 days per week. In addition to nose-poking, wheel-running and rotarod tests were conducted Fri – Sun; these data are described in a separate manuscript (see Shen et al., under review [48]).

2.4.1 Autoshaping

Nose-poking at two spatially distinct locations (left and right) was autoshaped for both age cohorts. The autoshaping procedure has been described in detail previously [see 49 and 50] and was implemented without modification for both age groups. Autoshaping sessions ended when animals met a specific response criterion or after 4hr whichever occurred first.

2.4.2 Percentile (PCNT)

The PCNT schedule of reinforcement used in this study was designed to generate high-rate operant behavior while adjusting the response criterion according to an animal's ability to

respond. Each left nose-poke terminated an IRT that then was compared with the 10 previous IRTs (i.e., a look-back window of 10 IRTs or 11 responses). Responses that terminated IRTs shorter than 50% of the previous 10 met the high-rate criterion (10:0.5) and were paired with a 0.2 s tone. These criterion responses were reinforced under a random interval (RI) 30 s schedule; a schedule that produces reinforcers unpredictably but at relatively constant overall rate of approximately 2/min. Criterion nose-pokes, including those followed by reinforcement, were paired with the same tone.

2.4.3 Differential reinforcement of high rate (DRH)

Under the DRH schedule, bursts of responses to the right nose-poke hole were reinforced. Criterion bursts eligible for reinforcement consisted of a 9-response burst that started and ended within 4 s (9:4). Criterion bursts of responses were paired with a 0.2 s tone and reinforced under an RI 30 s schedule of reinforcement as described in section 2.4.2.

2.4.4 Training

Initially, only the PCNT schedule was available for animals to respond under, which was counterbalanced across left and right nose-pokes. Sessions began with the illumination of the nose-poke hole and the corresponding LED light. Reinforcement initially followed criterion IRTs using a dense schedule that was slowly thinned as response rates increased (see Table 1). Each session consisted of six 5 min components during which the PCNT schedule was active. Between components, subjects experienced a blackout lasting 30 s during which nose-poke holes and LED lights were not illuminated and there were no programmed consequences for nose-pokes.

The DRH schedule was added after response rates increased under the PCNT schedule to form a multiple schedule of reinforcement. Components within a single session alternated between PCNT and DRH schedules to produce six 5 min components (3x each schedule), each separated by a 30 s blackout. The first component of each session alternated between PCNT and DRH schedules across days. Similar to the PCNT, the initial RI schedule used during DRH

components was shorter, producing a dense schedule of reinforcement. The DRH criteria for reinforcement were more rigid relative to the PCNT schedule, and thus the response criterion slowly incremented to its full value. Table 1 shows the progression of the two schedules from training until final values.

2.5 Humane endpoints

Mice were inspected daily and their body weight measured before every experimental session. Any mice that appeared ill or that displayed overt signs of MeHg toxicity (weight loss, limb clasping, severe motor dysfunction) were placed under 24hr observation in a heated cage with access to food and the attending veterinarian was consulted. Every effort was undertaken to keep mice alive, provided it did not prolong distress. Animals that met predefined criteria were euthanized according to procedures approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

2.6 Brain mercury (Hg) concentration

Brains were taken when an animal was euthanized either due to MeHg neurotoxicity or at the end of the study. Cold-vapor atomic absorption was performed by the Michigan State University Diagnostic Center for Population and Animal Health (DCPAH) to determine whole-brain Hg concentration (total Hg). Brains from following exposure groups were analyzed: adult MeHg-only (n=12), retired breeder MeHg-only (n=12), adult MeHg + NIM (n=12), retired breeder MeHg + NIM (n=12), adult control (n=4), retired breeder control (n=4), and finally adult NIM-only (n=4) and retired breeder NIM-only (n=4).

2.7 Data analysis

2.7.1 Survival Analysis

Mantel-Cox survival analysis was used to determine differences in mortality between age and exposure groups. Multiple comparison tests were made by applying the Holm-Sidak correction (shown in parentheses).

2.7.2 Bout Analysis

The microstructure of responding during PCNT and DRH schedules was assessed using log-survivor bout analysis. IRTs were collected separately for the PCNT and DRH schedules, aggregated within-session from three components for each schedule, and sorted from shortest to longest. Physical constraints prevented IRTs shorter than 0.02” but an unbiased log-survivor analysis requires a Y intercept at the shortest IRT so the entire distribution was shifted to the left by 0.02” by subtracting each IRT by that amount. The resulting IRT distributions were fitted to the bi-exponential model described in Eq. 1.

$$Y(t) = (1 - p)e^{-wt} + pe^{-bt} \quad (1)$$

Based on the findings of Johnson et al. [46], Eq. 1 was log-transformed to provide a better fit of the data and is described by Eq. 2.

$$\log_{10} Y(t) = \log_{10}((1 - p)e^{-wt} + pe^{-bt}) \quad (2)$$

Here, $Y(t)$ represents the proportion of IRTs > t sec; p is the proportion of all IRTs that occur between bouts; $(1 - p)$ is the proportion of all IRTs that occur within bouts. Using nonlinear least squares, estimates for the parameters bout-initiation rate (b), within-bout response rate (w), and bout length ($1/p$) were obtained for each individual subject after each session. The model requires at least 50 responses to produce reliable bout parameters estimates, and this occurred for all subjects after 18 sessions under the PCNT schedule and 25 sessions under the DRH schedule. Following training, differences in parameter estimates between schedules (PCNT and DRH) were minor and thus, for brevity, only data from the PCNT schedule are reported. Also, bout length was either as sensitive or less sensitive to MeHg exposure as bout-initiation rate, and always less sensitive than within-bout rate so, for brevity, it is not discussed.

2.7.3 Event analysis

Response patterns changed with experience under the schedules, but age-related differences between unexposed control mice remained for a majority of the study. To accommodate these differences, analyses of exposure-related effects were performed on a session-by-session basis. The performance of individual mice was compared with the

performance of their age-matched unexposed control group. After each session, raw parameter estimates from individual subjects were standardized using the mean and SD of the age-matched control group from that session to produce Z-score units. The threshold for impairment was designated as a Z-score at or below -1.0 (i.e., at least one SD unit below the control mean for that day). We chose impairment as 1 SD below the mean because performance of healthy-aging adults that is 1.0-1.5 SD below the mean of adults on memory and learning tasks generally meets criteria for age-associated mild cognitive impairment [51,52,53,54] (for a review see Jekel et al., 2015 [55]). Impairment was defined as a Z-score of less than -1.0, and the latency to impairment was determined as the time at which a Z-score dropped below -1.0 for at least 75% of the remaining sessions.

Latency to impairment was obtained for each animal on each dependent measure and submitted to Mantel-Cox analysis (Holm-Sidak correction). Animals that survived until the end of the study were censored. This is the same approach used for survival analyses but termed "event" analysis to avoid confusion. For individual animals, the latency to impairment for each dependent measure was also compared with latency to mortality, using a difference score, to identify which measures, if any, served as early and reliable predictors of MeHg toxicity and nimodipine neuroprotection. These data were analyzed using an analysis of variance (ANOVA). Statistical analyses were conducted using RS/1 software (Brooks Automation, Chelmsford, MA), Systat v.13, and SigmaPlot for Windows v.12.5. Graphs were created using SigmaPlot for Windows v.12.5 and tables were created using Microsoft Excel v.14.

3. Results

3.1 Mortality

Animals were tracked for 262 days (nose-poking was tracked for 253 days). Figure 1 shows mortality for all exposure groups. The Mantel-Cox test found a statistically significant difference among the eight curves [χ^2 (7) = 89.45, p<0.01]; p-values from multiple comparison tests are shown in parentheses.

For adults, the MeHg-only group (p<0.01) but not MeHg + NIM (p=0.52) or NIM-only (p=0.85) groups differed from control. The adult MeHg-only group also differed from the MeHg + NIM group (p<0.01). For retired breeders, both the MeHg-only and the MeHg + NIM groups differed from control (p<0.01 and p=0.04, respectively), but they did not differ from each other (p=0.87). Comparing between ages, adult and retired breeder MeHg-only groups were not different (p=0.99), but MeHg + NIM groups were significantly different (p=0.03), with adults surviving longer than retired breeders. Median mortality in exposure days (25^{th} and 75^{th} percentiles in parentheses) could be determined for three groups: adult MeHg-only, 111 days (102-138); retired breeder MeHg-only, 107 days (90-143); retired breeder MeHg + NIM, 138 days (108-232)

3.2 Age differences in behavior

Figure 2A-D shows session averages of within-bout rate (A), bout-initiation rate (B), response rate (C), and reinforcer rate (D) for unexposed control animals. Lines represent the best fit of a LOESS smoothing algorithm. There were large, systematic differences between control adult and retired-breeders from the outset that persisted throughout the study. Retired breeders had higher overall response rates than adults (C), a result of higher within-bout (A) and bout-initiation rates (B). Despite the wide range of response-rate differences across sessions, the RI 30” schedule of reinforcement in both the PCNT and DRH (not shown, as explained in section 2.7.2) components produced a relatively constant reinforcer rate across sessions (D), although it was slightly lower for adults early in training. A decline in overall response rates (C) in the retired breeders was associated with a decline in the rate at which bouts were initiated (B), not within-bout rate (A). To reveal specific influences of age on responding, Figure 3A-D shows these same dependent measures plotted as a function of chronological age, starting at 100 days of age for the adults and 324 days of age for the retired breeders. Interestingly, following acquisition, the curve for the retired breeders seems to be a

nearly seamless extension of the curve for the adults for overall response rate (C) and within-bout rate (A) and nearly so for the bout-initiation rate (B).

3.3 Methylmercury toxicity and nimodipine neuroprotection

Figure 4 shows within-bout rates for adults and exemplifies the event analysis. Each line within a plot corresponds to an individual subject. The left column presents raw parameter estimates of within-bout rate for control, NIM-only, MeHg-only, and MeHg + NIM exposure groups (from top to bottom). The right column shows these rates normalized as Z-score units calculated from the mean and standard deviation of the control group. This approach allows each exposure group to be compared with age- and experience-matched control mice. Vertical lines along the abscissa represent the time at which an animal met criteria for impairment. These latencies to impairment were analyzed using a Mantel-Cox analysis, which are shown in Fig. 5 and 6.

Figure 5 show the results of the event analyses for bout-initiation and within-bout rates and Fig. 6 shows the results for overall response- and reinforcer rates. The median latency to impairment is marked on the abscissa for key exposure groups and the difference between latencies between MeHg and MeHg + NIM groups, in days, is also shown on the graphs. Note that median latencies could not be calculated for adult bout-initiation rate (Fig. 5) and response rate (Fig. 6) for adult MeHg-only and MeHg + NIM groups since so few subjects showed deficits on these measures. Multiple comparisons tests revealed a number of significant differences among exposure groups, catalogued in detail in Table 2. While every dependent measure was eventually affected by MeHg, the time-course of disruption was not the same for each measure. For brevity and clarity the specific statistics are omitted in the narrative below, but all effects described are associated with an omnibus *p*-value of less than 0.01.

First, control and NIM-only groups did not differ in either age group. Second, for adults, MeHg-only mice differed from both control and NIM-only groups on all dependent measures but MeHg + NIM did not differ from control or NIM-only groups on any measure and MeHg-only and

MeHg + NIM groups differed on all dependent measures. That is, for the adults the MeHg + NIM mice resembled controls, not MeHg-exposed mice.

Third, for retired breeders, MeHg-only and MeHg + NIM mice differed from both control and NIM-only groups on all dependent measures with the exception that control and MeHg + NIM mice did not differ on response rate. Fourth, MeHg-only and MeHg + NIM did not differ on any measure. That is, for the retired breeders, MeHg + NIM mice largely resembled MeHg-exposed mice and differed from controls. Finally, between ages, MeHg-only groups did not differ on any measure except within-bout rate and the MeHg + NIM groups differed on all dependent measures.

3.4 Predicting impairment

The measure with the shortest latency to impairment, i.e., the most sensitive to MeHg, was within-bout rate for all exposure groups (Fig. 5, 6). For adult and retired breeder MeHg-only groups, the median latency to impairment was 102 and 49 days, respectively, and for adult and retired breeder MeHg + NIM groups, the median latency to impairment was 209 and 90 days, respectively. To test the hypothesis that within-bout rate is the earliest reliable indicator of MeHg-induced behavior impairment and predictor of MeHg-induced toxicity, we compared latency to impairment and latency to mortality for all animals that died; a long latency is associated with an early marker of impairment. This generated a quantitative measure of prediction for each dependent measure.

Figure 7 shows the latency from impairment to death for adult and retired breeder MeHg-only groups and the retired breeder MeHg + NIM for bout-initiation rate, reinforcer rate, response rate, and within-bout rate. Adult MeHg + NIM animals were omitted because so few died (see Fig. 1). A mixed ANOVA revealed a *Dependent Measure* X *Group* interaction [$F(6,108) = 6.35$, $p<0.01$]. Post-hoc tests revealed that for within-bout rate and response rate, the latency from impairment to death was shorter for the adult MeHg-only group than the retired breeder MeHg-only ($p<0.01$) and MeHg + NIM groups ($p<0.01$).

3.5 Brain Hg concentrations

Control and NIM-only samples had undetectable brain Hg concentrations (< 0.1 ppm). Adult MeHg-only animals had average brain concentrations of 24.5 ± 1.39 ppm (± SEM) when they were euthanized due to MeHg toxicity. Adult MeHg + NIM animals had average brain Hg concentrations of 17.3 ± 1.27 ppm; most of these were taken at the end of the study and the mice showed few or no overt signs of toxicity. Retired breeder MeHg and MeHg + NIM animals had average brain Hg concentrations of 22.2 ± 0.67 and 20.0 ± 1.7 ppm, respectively, when they were euthanized due to MeHg toxicity. Because brain Hg concentrations for the MeHg + NIM adults were obtained at the end of the study, these Hg levels were not compared to those taken earlier in the study when animals were euthanized due to MeHg toxicity. Two-tailed t-tests revealed no significant difference in brain Hg levels between MeHg-only adult and retired breeders [$t(22)=1.432$, $p=0.17$] or between MeHg-only and MeHg + NIM retired breeders [$t(22)=1.205$, $p<0.24$].

4. Discussion

The present study was designed to characterize the role of age in determining sensitivity to the neurotoxic effects of chronic MeHg exposure and protection by nimodipine. The effects of low- and high exposure levels are qualitatively similar but quantitatively different [56]. Higher exposure levels produce short latencies to the onset of neurotoxicity and greater spread between relatively sensitive and insensitive endpoints. The exposure regimen used here, which was relatively high, could model lower exposure levels but more rapidly. With low exposure levels the delay to toxicity is longer, but the signs of exposure are the same [56]. The current study found that: 1) chronic adult-onset exposure to 1.2 mg/kg/day MeHg produced *age-independent* mortality in male BALB/c mice, 2) the latency to MeHg-induced motor deficits was *age-dependent* with earlier impairment occurring in older animals 3) 20 mg/kg of nimodipine afforded *age-dependent* neuroprotection from MeHg insult with greater protection in younger animals, but the drug had no effect when administered alone, 4) a log-survivor bout analysis

divorced motor and motivational aspects of high-rate nose-poking, and 5) decrements in within-bout rate, a putative measure of motor function, were most sensitive to MeHg and served as an early predictor of neurotoxicity.

4.1 Age differences

Throughout the study the retired breeders responded more than adults, although this discrepancy dissipated as the study progressed. The adults may have been less engaged in nose-poking for sucrose, reflecting differences in motivation or reinforcer efficacy or engagement in behavior incompatible with nose-poking. In support of this notion, the higher response rate seen in the retired breeders (see Fig. 2 and 3) was driven entirely by a higher bout-initiation rate, which is especially sensitive to motivational manipulations such as satiation/deprivation and response cost [40,41]. Within-bout rate between the two age groups was indistinguishable Adults eventually reached the same response and bout-initiation rates as the retired breeders when they reached comparable ages, as revealed by Fig. 3, providing support for the idea that the motivational variables affect behavior in an age-dependent manner. Response rate declined as retired breeders aged and this was due to a decrease in bout-initiation rate while reinforcer rate and within-bout rate remained constant. This suggests that aged retired breeders paused longer between bouts and were less likely to initiate a high-rate response bout that was reinforced by sucrose (Figs. 2-3).

4.2 Chronic nimodipine

There were no detectable effects of nimodipine on mortality or high-rate nose-poking within adult and retired breeder groups, consistent with previous studies from our laboratory [25, 26]. Nimodipine and similar L-type CCBs produce measureable vascular [28], electrophysiological [57], and neurochemical changes [58,59]. Evidence suggests nimodipine may facilitate select forms of learning [59,60,61,62,63] in healthy adult animals, although other studies with nimodipine contradict these results [64,65]. Nimodipine does reliably attenuate learning deficits associated with normal aging [27,29,30,36,66,67] and protects against CNS

insult in experimental models of Parkinson's disease [68,69,70,71]. Here, over the course of 8.5 months nimodipine did not affect any dependent measure in animals that aged from 2.5-10 months (adults) and 9.5-18 months (retired breeders).

4.3 Motor dysfunction

Within-bout rate, a measure of motor function derived from the log-survivor bout analysis, was the most sensitive measure to MeHg exposure (Fig. 5-7; Table 2). Between age groups, deficits appeared 53 days earlier in retired breeders than adults in the MeHg-only group and 119 days earlier in retired breeders than adults in the MeHg + NIM group. Within age, nimodipine significantly delayed MeHg's effect on within-bout rate by 107 days for the adult MeHg + NIM mice compared to only 53 days for the retired breeders.

In general, these findings agree with previous studies [26,53,72,73,74,75] and support the notion that adult-onset MeHg produces primary and early degradation of motor function followed by secondary cognitive deficits. Importantly, the motivation to respond was not affected even when the ability to respond was impaired. In past studies, e.g., Bailey et al. [25], declines in performance-based measures were coupled with commensurate motor deficits, suggesting that impaired motor function influences the motivation to engage in active behavior. Recently, Hoffman & Newland [26], using a bout analysis approach to parse motor and motivational contributions to wheel-running, noted that MeHg diminished the speed of wheel-running, indicative of a diminished motor function, but it did not diminish the motivation to engage in wheel-running even as animals met criteria for euthanasia.

Our report (Fig. 5, 6, 7) supports the conclusions of Hoffman & Newland [26], as log-survivor estimates of within-bout rate and bout-initiation rate of nose-poking were differentially sensitive to the effects of MeHg. Further support is derived from the observation that median latency to reduced reinforcer rate, a motivation-based measure that is not included in the log-survivor model, was at or near the median latency for bout-initiation rate. Also, nimodipine delayed MeHg effects on nose-poking in a similar manner to Hoffman & Newland's [26] finding

that nimodipine delayed MeHg effects on wheel-running and rotarod performance. Running is a natural act for which its reinforcement is inextricably embedded in the act itself, referred to as automatic reinforcement [76]. In contrast, nose-poking is a relatively contrived behavior and the reinforcement of nose-poking is easily divorced from the act. Taken together, motor deficits do not appear to affect the influence of a reinforcer (intrinsic or extrinsic), or motivation. They also suggest that nimodipine's protection does not depend on the function or topography of the motor act, which stands in contrast to some drug effects (see Johnson et al. [46]).

This longitudinal study of chronic MeHg exposure provided direct evidence that MeHg-induced behavior deficits are age-dependent. To illustrate this point, Figure 7 shows that, on average, latency from within-bout rate and response rate impairment to death was 43 and 33 days longer, respectively, for retired breeders than adults. Together, these findings suggest an exaggerated delay to neurotoxicity in younger mice and supports the hypothesis that the delayed neurotoxicity of MeHg [77,78,79] is linked to the cumulative impact of cellular dysfunction that results from disruptions in Ca^{2+} homeostasis. Both Bailey et al. [25] and Hoffman & Newland [26] reported a complete block of MeHg's effects by 20 mg/kg/day nimodipine after 160 days of exposure. The present study ran longer and showed that MeHg's neurotoxicity eventually appeared. Thus, nimodipine delayed but did not prevent MeHg neurotoxicity even in the adult mice.

4.4 Methylmercury-induced mortality and nimodipine neuroprotection

Methylmercury-induced mortality was age-independent whereas nimodipine neuroprotection was age-dependent, with greater protection afforded to adults than retired breeders (Fig. 1). The fact that MeHg-induced mortality was age-independent suggests that the mechanisms underlying MeHg's disruption of behavior may differ from those related to its mortality. Nimodipine's protection from MeHg-induced mortality supports the hypothesis that chronic MeHg toxicity is due, at least in part, to disruption of Ca^{2+} homeostasis. The observation that it was age-dependent provides support for the notion that age-induced perturbation of Ca^{2+}

signaling mitigated nimodipine's effects. Together with Hoffman & Newland [26], in which adult-onset chronic MeHg (2.6 mg/kg/day) induced mortality after a median of approximately 97 days, these results show that survival is dose-related.

There was no difference in brain Hg concentration among animals euthanized due to severe MeHg toxicity: adult MeHg-only and retired breeder MeHg-only and MeHg + NIM mice. For these three groups, these brain Hg levels represent a lethal concentration. In contrast, adult MeHg + NIM animals had, on average, brain Hg concentrations that were 7.15 ppm lower than adult MeHg-only animals, although no statistical test was performed because the conditions under which the brains were taken were different between the two groups. A similar result was reported by Bailey et al. [25], using adult male BALB/c mice chronically exposed to 2.6 mg/kg/day MeHg and 20 mg/kg/day nimodipine. In both studies, brain samples from adult MeHg + NIM animals were collected at the end of the study from relatively healthy mice and do not represent a lethal dose. In most cases, this meant that adult MeHg + NIM animals were exposed to MeHg longer than MeHg-only animals but acquired less Hg in their brains. Nonetheless, in both studies the MeHg + NIM mice had lower brain Hg concentrations.

Together, these findings suggest that nimodipine acts to reduce the bioavailability of MeHg, an effect that may be mediated by age. For example, CCBs could affect the uptake of MeHg in the gut, its elimination, its passage across the blood-brain barrier, or its retention in the brain. The mechanism(s) of action by which nimodipine lowers brain Hg is not clear at this time but it does indicate that nimodipine acts, at least in part, by altering the toxicokinetics of MeHg.

4.5 Mechanisms of aging and methylmercury neurotoxicity

MeHg neurotoxicity is thought to elevate Ca^{2+} concentration in nerve terminals in two distinct temporal phases, the first phase a result of Ca^{2+} release from intracellular stores (i.e., mitochondria and smooth endoplasmic reticulum) and the second a result of an influx of extracellular Ca^{2+} via voltage-gated Ca^{2+} channels [80,81]. These distinct phases may, in part, contribute to the delayed neurotoxicity of MeHg. The more subtle Ca^{2+}-related changes that

occur during normal aging, which do not directly lead to cell death, likely include increased Ca^{2+} release from intracellular stores via inositol(1,4,5) triphosphate (IP_3) receptors and ryanodine (Ry) receptors, increased Ca^{2+} influx through L-type Ca^{2+} channels, increased amplitude and duration of the Ca^{2+}-dependent, K^+-mediated afterhyperpolarization (i.e., a shift from shorter to longer afterhyperpolarizations during which actions potentials cannot be produced), reduced NMDA receptor-mediated Ca^{2+} influx, and reduced Ca^{2+} buffering capacity [16,82,83]. Thus, reduced protection by nimodipine in older animals may be due chronic MeHg exposure taxing an already perturbed Ca^{2+} system, which could accelerate signs of aging or neurodegeneration. This supposition is supported by our finding of motor impairment in relatively healthy MeHg + NIM adults after a lengthy exposure regimen (253 days). Theoretically, a higher dose of nimodipine would be needed to offset chronic MeHg exposure in aging animals and completely block deficits in younger animals.

5. Conclusion

The findings reported here showed that MeHg produced significant behavior deficits and mortality in two age groups of mice and nimodipine attenuated these deficits in an age-dependent manner. Log-survivor bout analysis of high-rate operant behavior, which parses responding into motoric and motivational components, identified components of the molecular structure of behavior differentially sensitive to chronic MeHg exposure and nimodipine neuroprotection. Chronic MeHg exposure produced relatively similar mortality between the two age groups. Relative to mortality, motor deficits were the earliest signs to appear and, even in the face of significant decreases in response speed, mice continued to initiate bouts of nose-poking, which suggests that MeHg did not diminish the motivation to respond. The younger adults experienced a longer delay to toxicity than older retired breeders. Nimodipine attenuated MeHg-induced mortality and behavior deficits, but protection was substantially diminished in older animals. One methodological contribution is the use of an event analysis to quantify behavior changes in a situation in which attrition diminishes sample size over the course of a

study. The finding of age-dependent nimodipine neuroprotection provides evidence that MeHg exposure in aging animals may tax and already-perturbed Ca^{2+} signaling system.

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Table 2.1

Multiple schedule training

Percentile (PCNT)		**Differential reinforcement (DRH)**		
Look-back window	*RI value*	*Response-burst requirement*	*RI value*	*Sessions*
10 (50%)	5”	N/A	N/A	12
10 (50%)	10”	N/A	N/A	1
10 (50%)	20”	N/A	N/A	4
10 (50%)	20”	2 (1”)	5”	2
10 (50%)	30”	4 (2”)	5”	2
10 (50%)	30”	6 (3”)	5”	4
10 (50%)	30”	9 (4”)	5”	2
10 (50%)	30”	9 (4”)	10”	2
10 (50%)	30”	9 (4”)	20”	2
10 (50%)	30”	9 (4”)	30”	Final values

Table 2.1. Training progression for the PCNT and DRH components of the high-rate multiple schedule. The zigzag line represents when both schedules reached their final values.

Table 2.2

Event analysis: Multiple pairwise comparisons

Age	Group 1	Group 2	Response rate	Reinforcer rate	Bout-initiation rate	Within-bout rate	Bout length
Adult	Control	NIM	0.78	0.51	0.93	0.77	0.85
	Control	MeHg	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	Control	MeHg + NIM	0.28	0.07	0.94	0.06	0.48
	NIM	MeHg	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	NIM	MeHg + NIM	0.60	0.78	0.91	0.52	0.85
	MeHg	MeHg + NIM	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Retired breeder	Control	NIM	0.88	0.95	0.97	0.97	0.99
	Control	MeHg	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	Control	MeHg + NIM	0.07	***<0.01***	***0.03***	***0.01***	***0.02***
	NIM	MeHg	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	NIM	MeHg + NIM	***0.04***	***<0.01***	***0.02***	***<0.01***	***<0.01***
	MeHg	MeHg + NIM	0.18	0.92	0.69	0.18	0.71
Adult vs. Retired breeder	MeHg	MeHg	0.41	0.97	0.98	***<0.01***	0.85
	MeHg + NIM	MeHg + NIM	***0.01***	***0.01***	***0.02***	***0.01***	***<0.01***

Table 2.2. Multiple pairwise comparison tests from the event analysis (Mantel-Cox). Bold and italicized *p*-values represent a significant difference between Group 1 and Group 2.

Figure Captions

Figure 2.1. Survival analysis plots (e.g., mortality) are shown for adult (solid lines) and retired breeder (dashed lines) age cohorts. Each colored line represents a different exposure group; black = control, green = NIM-only, red = MeHg-only, and blue = MeHg + NIM. Asterisks denote groups that were significantly different from age-matched control and NIM-only groups (p<0.01).

Figure 2.2. Mean within-bout rate (A), bout-initiation rate (B), response rate (C), and reinforcer rate (D) as a function of *exposure day* under the PCNT schedule. Open circles and filled triangles represent adult and retired breeder age cohorts, respectively. Lines represent the best fit of LOESS smoothing algorithm.

Figure 2.3. Mean within-bout rate (A), bout-initiation rate (B), response rate (C), and reinforcer rate (D) as a function of *chronological age* under the PCNT schedule. Open circles and filled triangles represent adult and retired breeder age cohorts, respectively. Lines represent the best fit of LOESS smoothing algorithm.

Figure 2.4. An example of the statistical methods used in the event analysis. The left column shows raw parameter estimates of within-bout rate as a function of exposure day for individual subjects (each line represents one subject) from the adult cohort (top to bottom: control, NIM-only, MeHg-only, and MeHg + NIM). On a session-by-session basis, individual animal performance was standardized using the mean and SD of the control group to produce Z-scores. These Z-scores are shown in the right column for the same exposure groups with dashed lines demarcating ±1 SD. For standardized plots (right side), vertical lines on the abscissa represent the latency to impairment for an individual animal.

Figure 2.5. Event analyses (survival analysis) for bout-initiation rate (top panel) and within-bout rate (bottom panel), separated by age (left and right). Shown near the abscissa of each Kaplan-Meier plot are the median latencies to impairment for the MeHg-only and MeHg + NIM exposure groups, as well as the difference between the two groups. Exposure groups: black = control, green = NIM-only, red = MeHg-only, and blue = MeHg + NIM.

Figure 2.6. Event analyses (survival analysis) for response rate (top panel) and reinforcer rate (bottom panel), separated by age (left and right). The format is the same as Fig. 2.5. Note that for response rate, too few adult MeHg + NIM animals reached impairment to determine median latency to impairment. Exposure groups: black = control, green = NIM-only, red = MeHg-only, and blue = MeHg + NIM.

Figure 2.7. The bar chart show the average latency (days) from impairment to death for the following measures: within-bout rate, response rate, bout-initiation rate, and reinforcer rate (±SEM). Note that the adult MeHg + NIM group is excluded because so few animals died from MeHg toxicity (see text for details). The latency from impairment to death was longest for within-bout rate (*, all *p's*<0.001). For within-bout rate and response rate, the average latency for adult MeHg-only animals was significantly shorter compared to retired breeder MeHg-only and MeHg + NIM animals (#, all *p*'s<0.001)

Figure 2.1

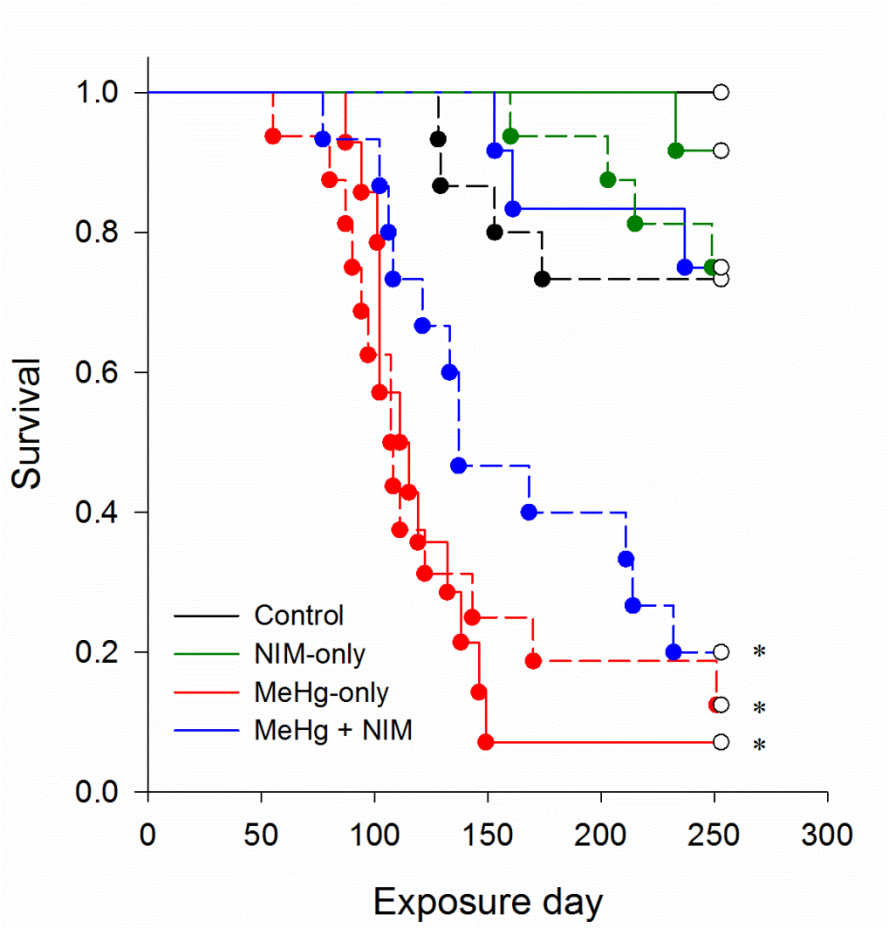

1.0
0.8
0.6
0.4
0.2
0.0
Survival
0
50
100
150
200
250
300
Exposure day
Control
NIM-only
MeHg-only
MeHg + NIM
*
*
*

Figure 2.2

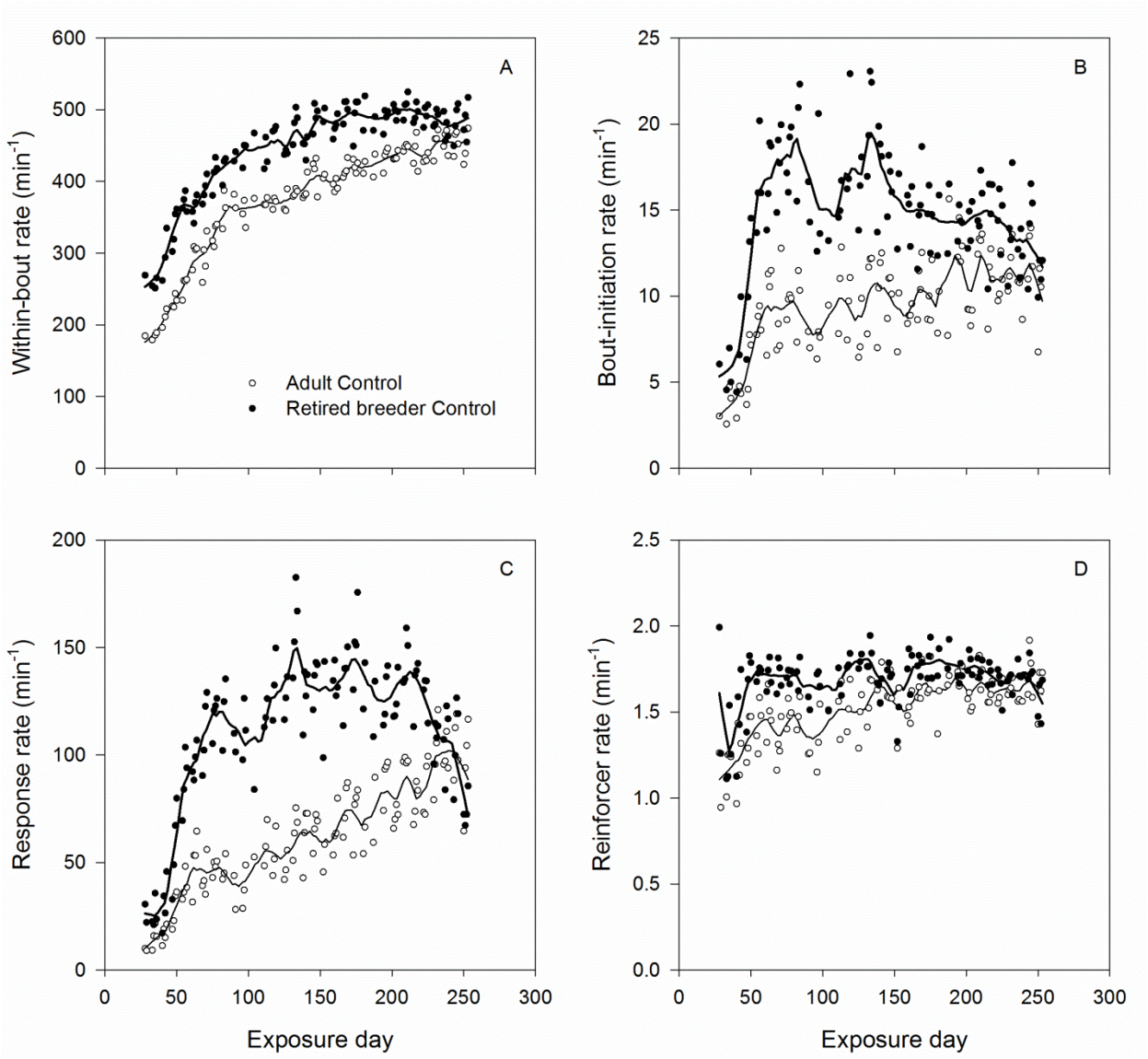

A
B
C
D
Within-bout rate (min-1)
Bout-initiation rate (min-1)
Response rate (min-1)
Reinforcer rate (min-1)
Adult Control
Retired breeder Control
Exposure day
Exposure day

Figure 2.3

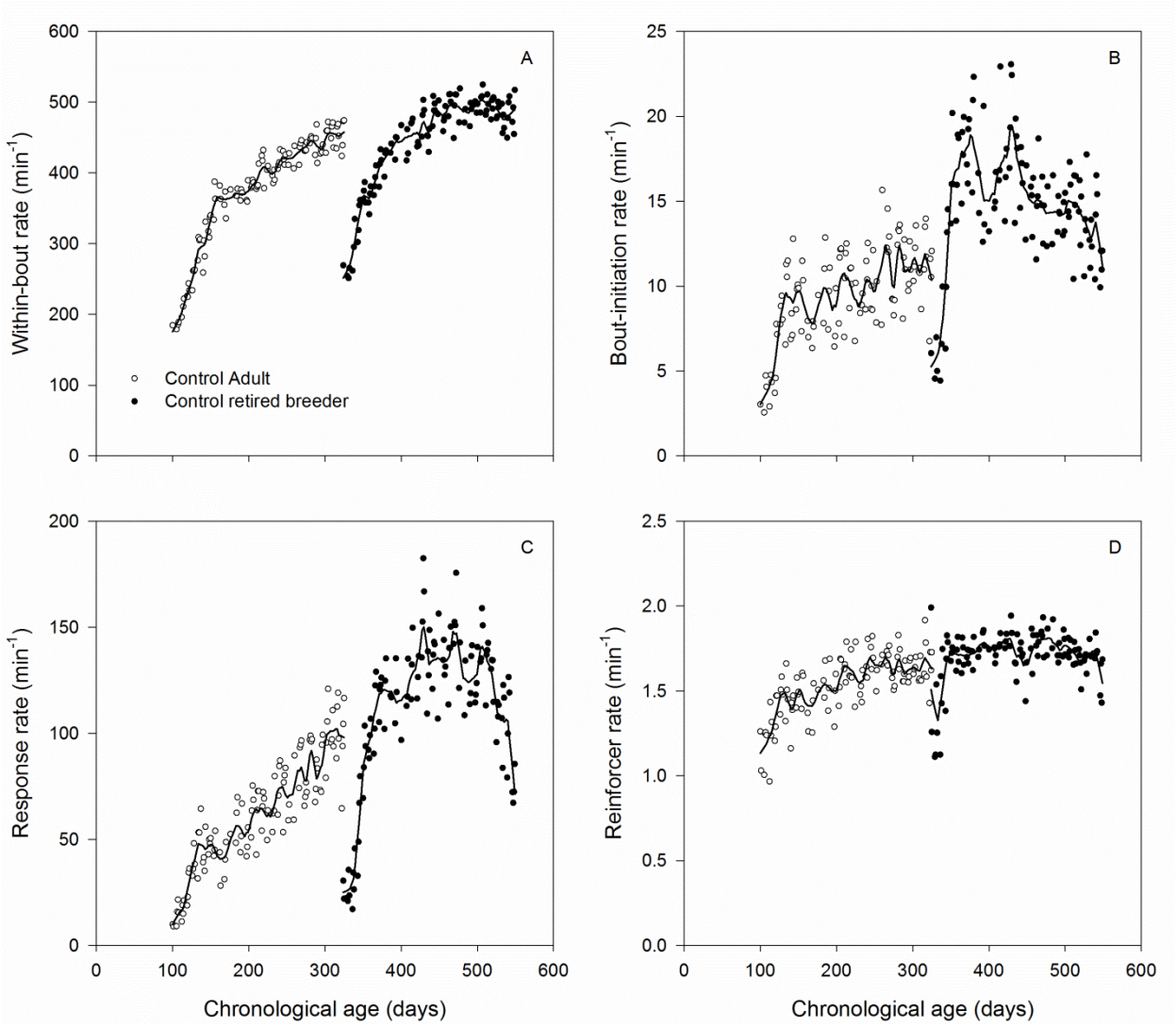

A
B
C
D
Within-bout rate (min-1)
Bout-initiation rate (min-1)
Response rate (min-1)
Reinforcer rate (min-1)
Control Adult
Control retired breeder
Chronological age (days)
Chronological age (days)

Figure 2.4

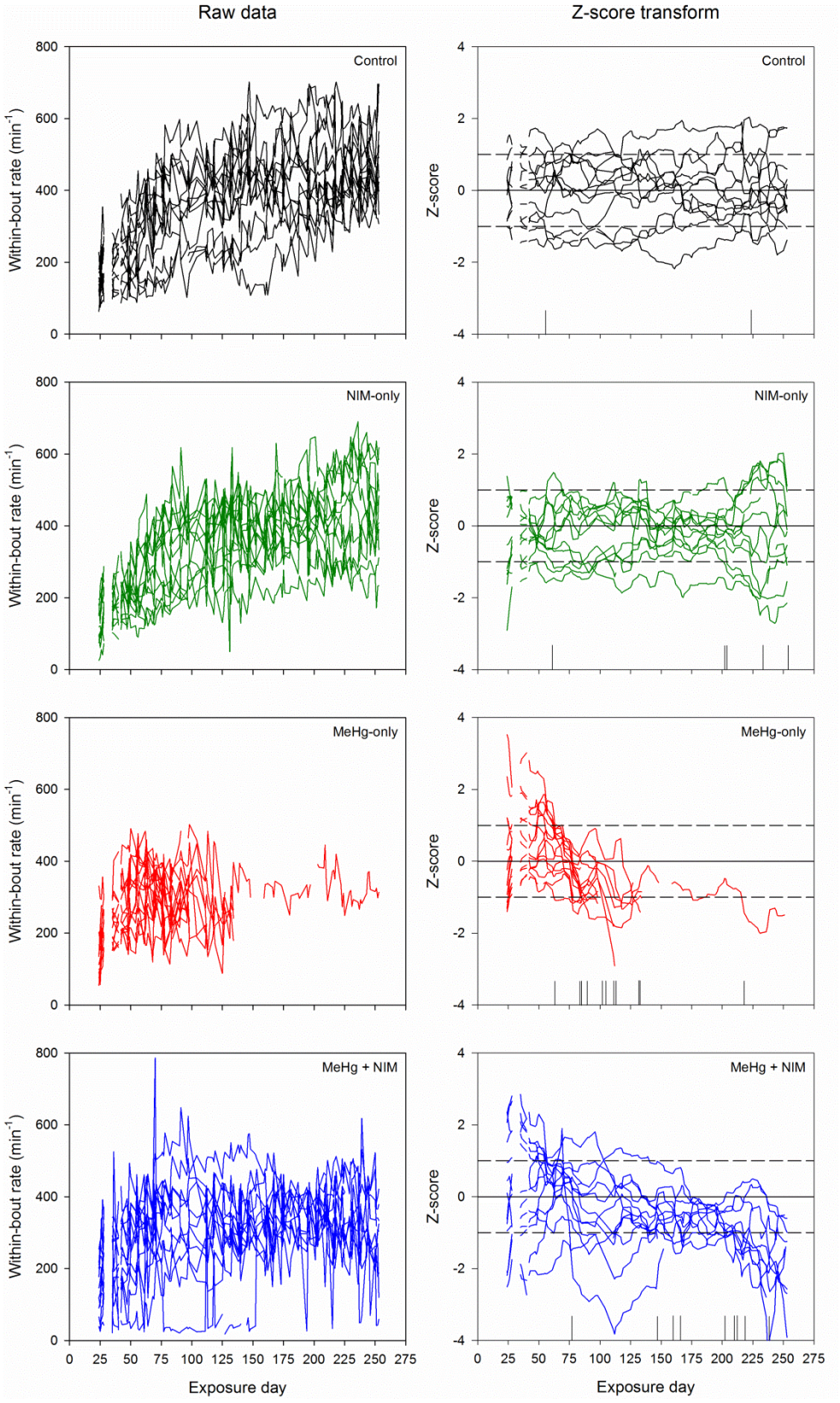

Raw data
Z-score transform
Control
NIM-only
MeHg-only
MeHg + NIM
Within-bout rate (min-1)
Z-score
Exposure day
0
200
400
600
800
-4
-2
2
4
0 25 50 75 100 125 150 175 200 225 250 275

Figure 2.5

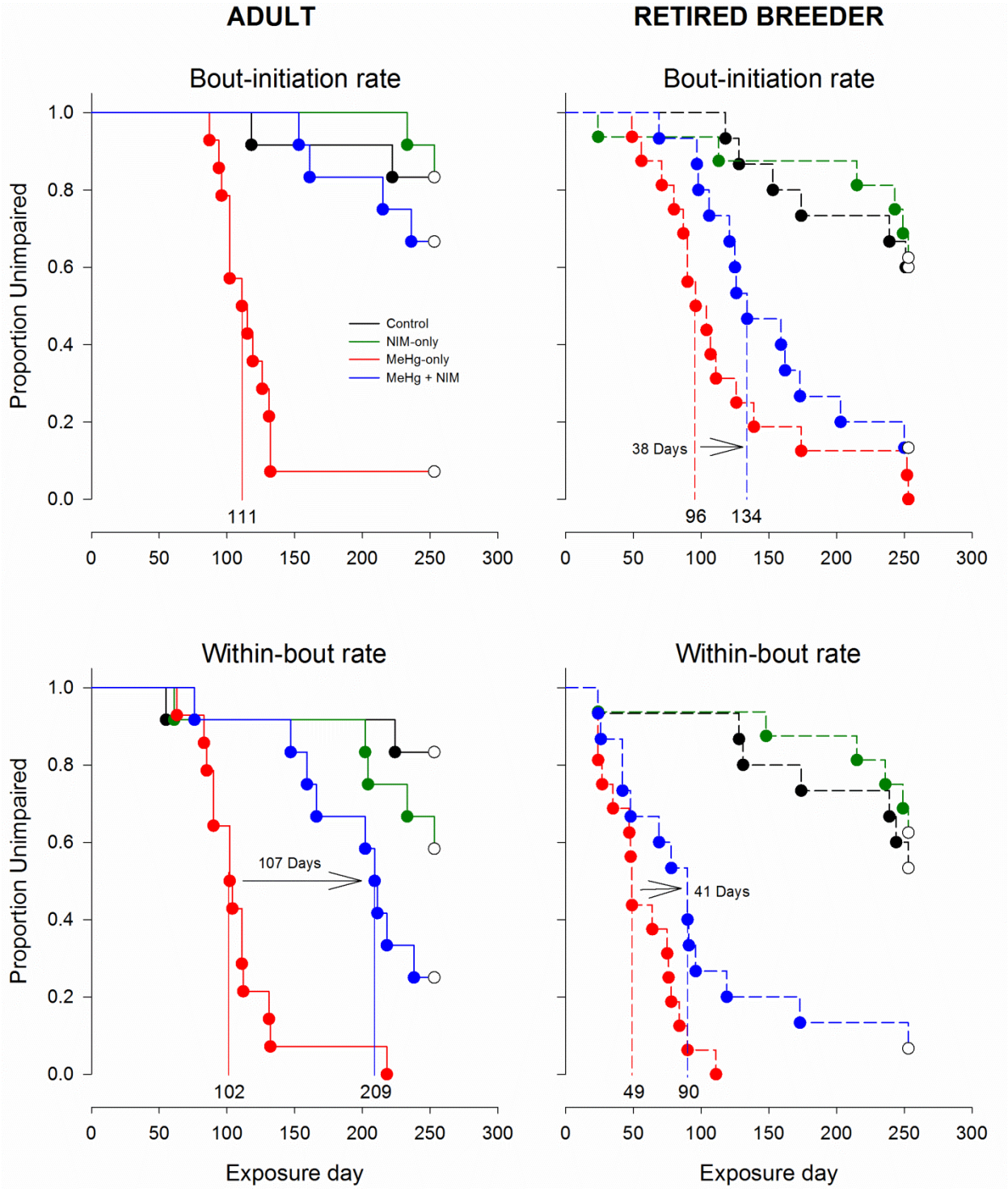

ADULT
RETIRED BREEDER
Bout-initiation rate
Bout-initiation rate
Proportion Unimpaired
Control
NIM-only
MeHg-only
MeHg + NIM
111
38 Days
96
134
Within-bout rate
Within-bout rate
107 Days
41 Days
102
209
49
90
Exposure day
Exposure day

Figure 2.6

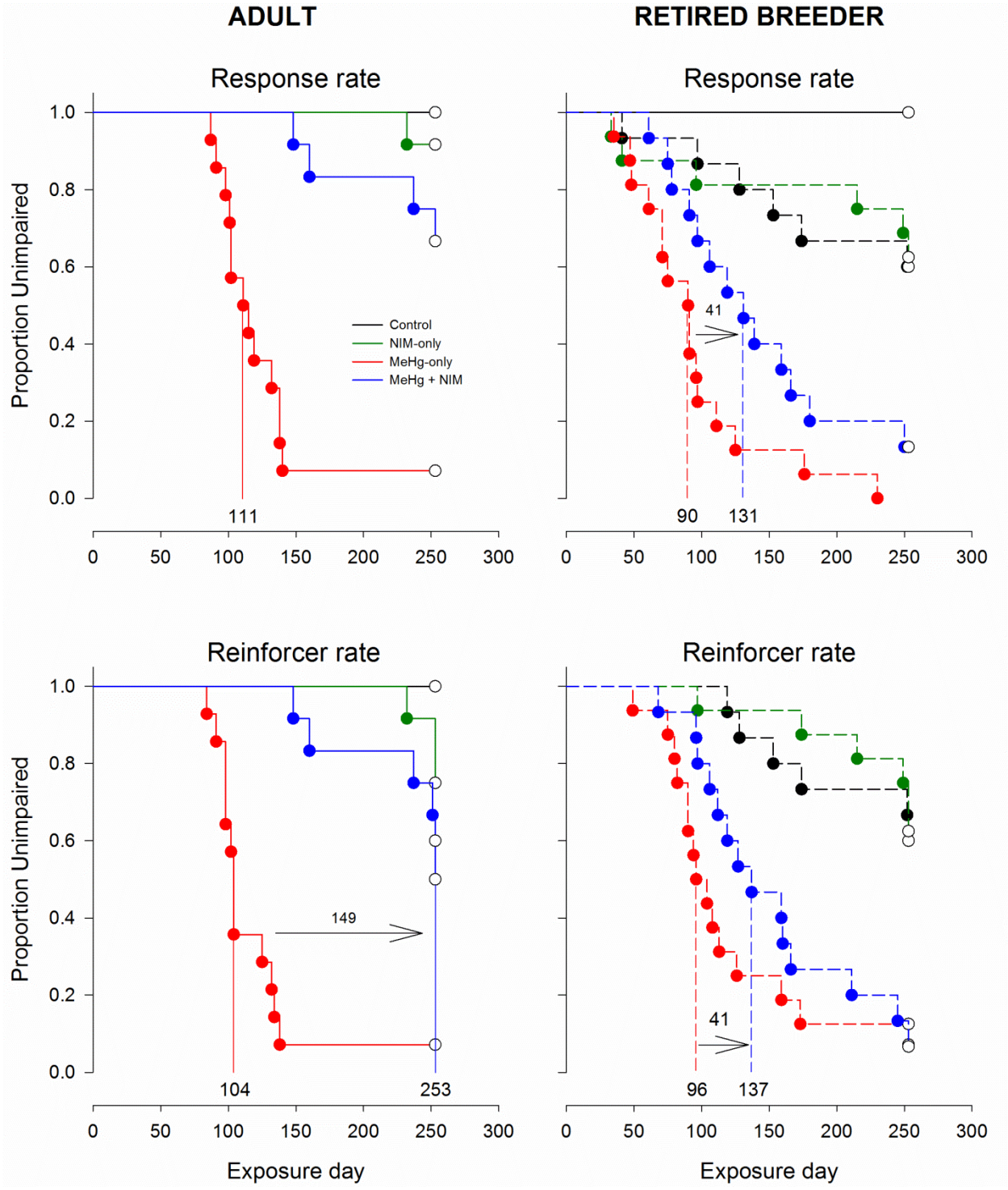
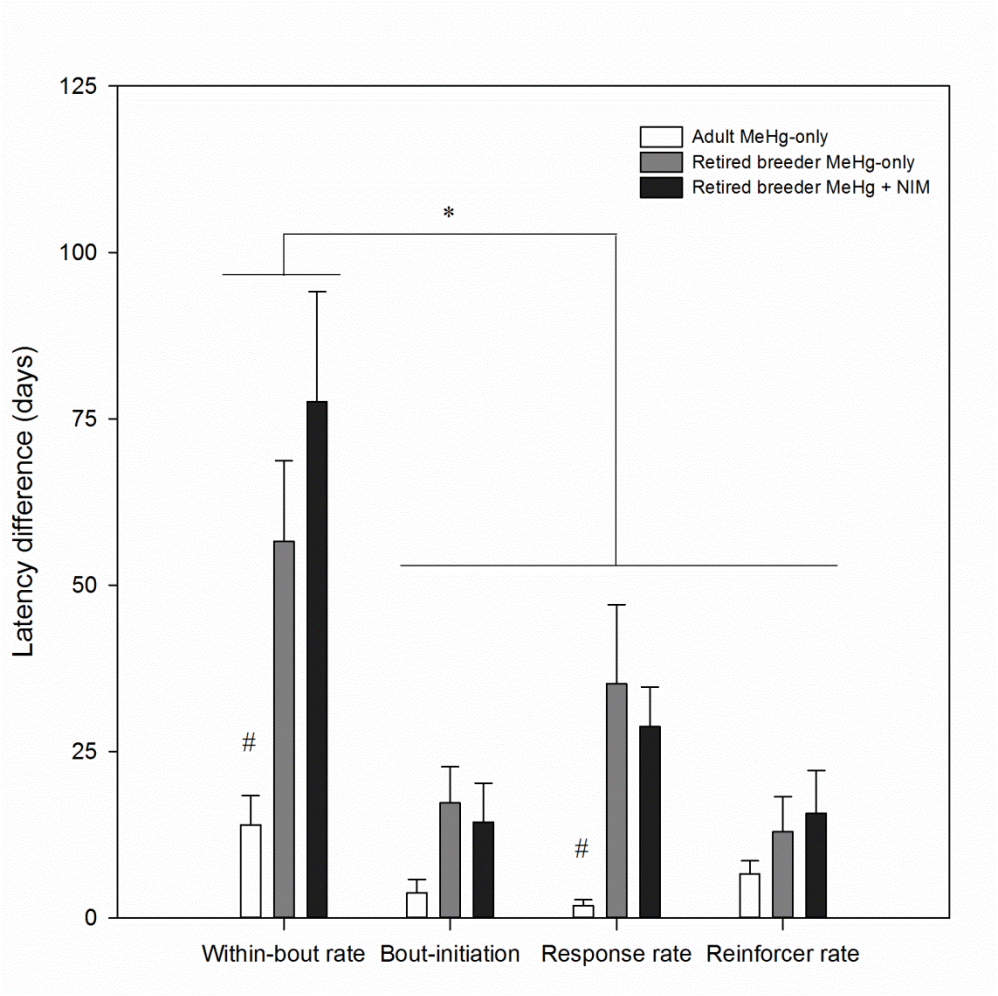

ADULT
RETIRED BREEDER
Response rate
Response rate
Reinforcer rate
Reinforcer rate
Proportion Unimpaired
Proportion Unimpaired
Control
NIM-only
MeHg-only
MeHg + NIM
111
41
90
131
149
104
253
41
96
137
0
50
100
150
200
250
300
0.0
0.2
0.4
0.6
0.8
1.0
Exposure day
Exposure day

Figure 2.7


125
100
75
50
25
0
Latency difference (days)
Adult MeHg-only
Retired breeder MeHg-only
Retired breeder MeHg + NIM
*
#
#
Within-bout rate
Bout-initiation
Response rate
Reinforcer rate

Chapter 3

Chronic methylmercury exposure: neurotoxicity and age-dependent nimodipine neuroprotection of wheel-running and rotarod performance in BALB/c mice

Abstract

Methylmercury (MeHg) neurotoxicity is thought to be mediated, in part, by dysregulation of calcium (Ca^{2+}) homeostasis, a mechanism that may also slowly and progressively degrade neuronal function during normal aging. Longitudinal studies of MeHg exposure provide a powerful approach to the study of neurobehavioral mechanisms of both MeHg toxicity and aging. Wheel-running and rotarod performance were assessed in two age groups of BALB/c mice chronically exposed to 0 or 1.2 mg/kg/day MeHg and 0 or 20 mg/kg/day nimodipine, a 1,4-dihyrdopyridine L-type calcium channel blocker (CCB), for approximately 8.5 months. Adults began exposure on postnatal day (PND) 72 and retired breeders on PND 296. Wheel-running was analyzed using a log-survivor bout analysis that partitioned behavior into bouts. MeHg produced relatively age-independent deficits in wheel-running and rotarod performance, whereas nimodipine afforded greater protection to adult mice than to retired breeders. Rotarod and within-bout response rate (markers of sensorimotor function) were more sensitive to and reliable predictors of MeHg toxicity than bout-initiation rate, a marker of motivation to run. The motivation to run appeared to be least affected by MeHg exposure. While chronic MeHg exposure produced functionally similar behavior deficits between age groups, the age-dependent neuroprotection by nimodipine supports the notion that underlying neurobiological systems mediated, in part, by Ca^{2+} signaling, are differentially affected in older adults.

1. Introduction

Methylmercury, an established neurotoxicant and global pollutant, produces central nervous system (CNS) damage, which is more diffuse following prenatal exposures [1]. Signs and symptoms of MeHg toxicity may not manifest until interaction with a secondary event or catalyst, i.e., delayed neurotoxicity (Rice, 1995; Weiss et al., 2002) [2,3]. Similar to MeHg toxicity (see Atchison, 2005; Atchison & Hare, 1994) [4,5], the normal aging process is thought to be mediated, at least in part, by chronically elevated levels of intracellular Ca^{2+} or a dysregulation of Ca^{2+} homeostasis [6-8], but only MeHg results in pervasive cell death. Thus, chronic MeHg exposure during aging may further perturb intracellular Ca^{2+} function and exacerbate age-related declines in motor or cognitive functioning and accelerate normal or neurodegenerative aging [3,9]. However, there is paucity in the literature regarding differential consequences of early and late adult-onset MeHg exposure [10,11].

Chronic adult-onset higher-dose MeHg exposure has been known to produce “glove and stocking” sensory disturbances, weakness, cerebellar ataxia, and visual and auditory dysfunction [12-14]. Exposure to chronic lower levels of MeHg has been reported to produce mild sensorimotor dysfunction [15-17] and tremor [18-20]. Accordingly, motor dysfunction may be a primary behavioral marker of protracted adult-onset MeHg exposure. Unknown pre-adult exposure levels in participants of epidemiological studies and potential comorbidity with other diseases make interpretation of these findings studies less straightforward.

In rodent models, postnatal MeHg exposure also produces sensorimotor dysfunction, including impaired gait and balance, reduced open-field activity and locomotion, and hind-limb flexion [10,21-25]. Many of these reports, i.e., Bellum et al., 2007, 2013, Deitrich et al., 2005, and Wakabayashi et al., 1995 [10,21,22,25], followed sub-chronic and/or high-dose exposure regimens. More recently, Hoffman & Newland [26] found that chronic MeHg exposure (2.6 mg/kg/day MeHg) disrupted wheel-running and rotarod in adult mice and Shen et al. (under review) [27] found that chronic MeHg exposure (1.2 mg/kg/day MeHg) produced deficits in high-

rate nose-poking. Both studies used bout analysis models to estimate the microstructure of behavior, yielding measures of motor function and motivation to run or nose-poke. In both studies, MeHg impaired motor function, as reflected in within-bout response rates, while having a delayed effect on the control over nose-poking by an extrinsic reinforcer or over running by its intrinsic reinforcement, as reflected by the rate at which bouts are initiated.

Preventing increased Ca^{2+} influx into intracellular cytosol, returning intracellular Ca^{2+} to homeostatic or basal levels, may be neuroprotective against MeHg and/or aging insults. Nimodipine, a 1,4-dihyrdopyridine L-type CCB, has excellent selectivity for the CNS [28] and may shield neurons against CNS insult. *In vitro* [29,30] and recent *in vivo* studies [26,27,30,31] have shown that nimodipine and similar CCBs protect against MeHg toxicity. For example, in Hoffman & Newland's [26] study, chronic dietary nimodipine (2 or 20 mg/kg/day) dose-dependently blocked MeHg-induced deficits (2.6 mg/kg/day), with 20 mg/kg/day wholly blocking MeHg-induced deficits MeHg-induced deficits (2.6 mg/kg/day). Shen et al. [27] extended these findings and showed that nimodipine neuroprotection (20 mg/kg/day) against MeHg-induced behavioral dysfunction (1.2 mg/kg/day) was age-dependent. They also raised the possibility that nimodipine's protection may have been partially due to toxicokinetic considerations because nimodipine reduced the brain concentration of MeHg [27,31]. Nimodipine and other CCBs have also been shown to attenuate or block selective signs of normal aging in animal models [32-37], but a full characterization of the interaction between MeHg exposure and aging is still lacking.

The current study was designed to assess neurobehavioral effects of chronic MeHg exposure in two age cohorts and characterize potential age-related nimodipine neuroprotection. Wheel-running and rotarod performance measured in two age cohorts of male BALB/c mice chronically exposed to either 0 or 10.0 ppm MeHg via daily drinking water and either 0 or 200 ppm nimodipine via daily chow. A log-survivor bout analysis approach (Shull et al., 2001) [38] was used to parse wheel-running data into bout of running separated by pausing, which provided parameter estimates of the rate at which bouts occurred (bout-initiation rate), the

speed of running within a bout (within-bout rate), and the length of a given bout (bout length). To account for attrition due to MeHg toxicity and age-related differences in behavior, individual raw data from wheel-running and rotarod was compared against the mean of the age-matched control group on a session-by-session basis (standardized Z-score units).

2. Methods

2.1 Subjects

Adult and retired breeder male BALB/c mice (*N*=112) were purchased from Harlan Laboratories (Indianapolis, IN) and housed in an Optimice® rack system in an AAALAC-accredited temperature- and humidity-controlled vivarium that was maintained on a 12-hour light-dark cycle (lights on at 6:00am). Two age cohorts, two MeHg water concentrations, and two nimodipine diets produced a 2 (age) X 2 (MeHg) X 2 (nimodipine) full factorial design with 12-16 mice per exposure group by age. Prior to exposure, animals' motor function was tested by assessing wheel-running and rotarod performance (one session each) to ensure preexisting differences did not exist among groups within each age group. Then they were randomly assigned to exposure groups with the constraint that the groups were similar on pre-exposure wheel-running and rotarod performance.

The adult cohort (*n*=51) arrived at 49 days of age and were housed in pairs in clear polycarbonate cages, separated by a clear Plexiglas© divider that prevented physical contact, but allowed visual, olfactory, and auditory interaction. Adult BALB/c mice are aggressive and group housing may result in serious injury or death [39]. Their weight was maintained at approximately 24-25 g and after 4 months transitioned to a final target weight of approximately 26-27 g. The retired breeder age cohort (*n*=63) arrived at 273 days of age and were housed in the same manner as the adults. Upon arrival, they weighed 26-30g, which was reduced and maintained at a final target weight of approximately 26-27 g.

2.2 Methylmercury and Nimodipine Exposure

Methylmercuric chloride (CH_3HgCl) was procured from Alfa-Aesar (Ward Hill, MA, USA) and dissolved into water to produce the water solutions. Nimodipine was procured from Sigma-Aldrich (St. Louis, MO) and 200 ppm nimodipine was mixed into standard rodent chow manufactured by Purina TestDiets and based on a 5LL2 laboratory chow diet. Based on measurements of water and food consumption and weight (data not shown here), MeHg exposure corresponded to approximately 0 and 1.2 mg/kg/day of Hg and nimodipine exposure corresponded to approximately 0 and 20.0 mg/kg/day. Exposures began when adults and retired breeders were 72 and 296 days old, respectively, and lasted 262 days until the groups were 334 and 558 days old, respectively.

2.3 Apparatus

Wheel-running sessions were conducted in 16 standard Med Associates Inc. operant conditioning chambers (St. Albans, VT, product #ENV-007). Each chamber measured 30.5 cm L x 24.1 cm W x 29.2 cm H and contained an in-chamber activity wheel (product #ENV-043A). Responses were recorded as quarter-wheel revolutions (approximately 13.5 cm) with centisecond resolution in the measurement of the time between each quarter-wheel displacement. Operant chambers were enclosed in sound-attenuating cabinets with a fan to circulate air for ventilation. Rotarod sessions were conducted using a standard 5-station Med-Associates© Rotarod for mice (product #ENV-575M) during which the speed of the rotating cylinder accelerated from 4 to 40 rpm over 5-min at a constant acceleration of 0.12 revolutions per sec. An infrared beam below the rotating rod detected when an animal fell.

2.4 Procedure

Animals received one wheel running session per week, which occurred in the operant chamber described above, and one rotarod test per week. Wheel-running and rotarod sessions did not occur on the same day. Also, animals nose-poked for sucrose pellets under a high-rate

operant schedule of reinforcement, and these data are reported elsewhere (see Shen et al., [27]).

Mice received one wheel-running session per week (conducted on Sat or Sun) that lasted 135 min. There were no experimenter-controlled contingencies in place during wheel-running sessions. A clear plastic divider prevented access to the chamber's food tray or other response manipulanda that were used in other procedural manipulations. The first two sessions were conducted on exposure days 30 and 44, after which time sessions were conducted on a weekly basis. Mice also received one rotarod session per week (conducted on Fri) and one trial per session. An individual subject's trial ended upon falling from the rod and breaking the infrared beam below. All trials were conducted such that the rod's direction of rotation was counter-clockwise relative to the subject's body.

2.5 Data Analysis

2.5.1 Bout analysis

The microstructure of wheel-running was assessed using log-survivor analysis [38,40, 41], a method for separating behavior into bouts of responding. Thus, interresponse times (IRTs), or the time between quarter-wheel revolutions, were collected and sorted from shortest to longest. The analysis was applied to each session for each individual subject. To avoid excessive influence by a few very long interresponse times, 1% of the longest interresponse times (IRTs) were trimmed. For each session, IRTs were rank ordered from shortest to longest and IRT distributions were fitted to the bi-exponential model proposed by Shull et al. (2001, 2003) and Shull & Grimes (2003) [38,40,41] using non-linear least-squares regression, which is described in Eq. 1.

$$Y(t) = (1-p)e^{-wt} + pe^{-bt} \quad (1)$$

Based on the findings of Johnson et al. (2011, 2009) [42,43], Eq. 1 was log-transformed to provide a better fit of the data, and this is described by Eq. 2.

$$\log_{10} Y(t) = \log_{10}((1-p)e^{-wt} + pe^{-bt}) \quad (2)$$

Here, $Y(t)$ represents the proportion of IRTs > t sec, p is the proportion of all IRTs that occur between bouts, $(1 - p)$ is the proportion of all IRTs that occur within bouts, and t is the duration of a given IRT. Using nonlinear least squares, estimates for the parameters bout-initiation rate (b), within-bout response rate (w), and bout length ($1/p$) were obtained for each individual subject after each session.

2.5.2 Event analysis

Here and in Shen et al. [27], response patterns changed with experience but age-related differences between unexposed control mice remained for a majority of the study. To accommodate these differences and the relatively high attrition due to MeHg toxicity, individual analysis of subjects' performance was performed on a session-by-session basis.

Daily estimates were evaluated as a time series, but to deemphasize session-by-session variations the data were smoothed using a nine-point LOESS algorithm. In the current study, the algorithm employed a moving window that emphasized the "current" session's data and then applied a weighting function that progressively diminished the influence from four sessions prior to and after the current session; the window slides to the next session and repeats the weighting function [44]. The smoothed performance of each mouse was compared with the performance of their age-matched control on the same day. Raw parameter estimates from individual subjects were standardized using the mean and SD of the age-matched control group to produce Z-score units. For the purposes of this study, the threshold for impairment was designated as a Z-score at or below -1.0 (i.e., at least one SD unit below the control mean for that day).

The exposure day on which a response measure for a given subject passed the one-SD threshold and did not recover for at least 75% of remaining sessions was designated as the latency to impairment and used as an event for subsequent event analyses. We designated impairment as 1 SD below the mean because performance of healthy-aging adults that is 1.0-

1.5 SD below the mean of adults on memory and learning tasks generally meets criteria for age-associated mild cognitive impairment [45,46] (for a review see Jekel et al., 2015 [47]).

Latency to impairment was obtained for each animal on each dependent measure and submitted to Mantel-Cox analysis followed by multiple comparison tests using the Holm-Sidak correction. For individual animals, the latency to impairment for each dependent measure was also compared with latency to mortality, using a difference score, to identify which measures, if any, served as early and reliable predictors of MeHg toxicity and nimodipine neuroprotection. These data were analyzed using linear mixed effects (LME) with age and exposure as fixed effects and subject and measure as random effects. Raw wheel-running and rotarod performance of the control age groups was compared using repeated measures analysis of variance (ANOVA) as there was little attrition in these groups.

Log-survivor fits were conducted using RS/1 software (Brooks Automation, Chelmsford, MA), statistical analyses were conducted using Systat v.13 and SigmaPlot for Windows v.12.5. Graphs were created using SigmaPlot for Windows v.12.5 and tables were created using Microsoft Excel v.14.

3. Results

3.1 Mortality

The study continued for 262 days. Fifty of 112 mice (44.6%) met humane endpoints and were euthanized: for adults, 1 NIM-only, 13 MeHg-only, and 3 MeHg + NIM mice were euthanized and for retired breeders 4 control, 3 NIM-only, 15 MeHg-only, and 12 MeHg + NIM were euthanized. These data as well as brain hg concentrations are discussed in detail in Shen et al. [27].

3.2 Wheel-running

3.2.1 Age differences

Figures 3.1 show average total distance, bout-initiation rate, within-bout rate, and bout length as a function of session and chronological age, respectively. The repeated measures

ANOVA revealed main effects of *Age* [F(1,25) = 26.66, $p<0.001$] and *Session* [F(31,685) = 3.21, $p<0.001$] on total distance, but no interaction. There were main effects of *Age* [F(1,25) = 10.67, $p=0.003$] and *Session* [F(31,685) = 1.841, $p=0.004$] on bout-initiation rate, but no interaction. There was a main effect of *Session* on within-bout rate [F(31,685) = 8.51, $p < 0.001$], but no main effect of *Age* and no interaction. Finally, there was a main effect of *Age* [F(1,25) = 5.064, $p=0.03$] on bout length, but no main effect of *Session* and no interaction.

The majority of age-related differences in wheel-running became significantly different on the 10th wheel-running session (i.e., exposure day 95 of the study) such that retired breeders ran farther than adults. Differences in bout-initiation rate were sporadic, with retired breeders showing similar or higher bout-initiation rates as adults. On days 95, 173, 186, 200, and 235 retired breeders had longer bout lengths than adults (Holm-Sidak). Within-bout rate increased across the first ten or so sessions and then plateaued for both adult and retired breeders; there was no age effect on this measure.

3.2.2 MeHg toxicity and nimodipine neuroprotection

Figure 3.2 shows adult total distance run (m) per session, which serves as an example to illustrate how the event analysis was conducted. The left column of Fig. 3.2 presents raw data of total distance for control, NIM-only, MeHg-only, and MeHg + NIM exposure groups (from top to bottom). Each line within a plot shows the smoothed time series for an individual subject. The right column of Fig. 3.2 presents standardized total distance data as Z-score units (taken from the control group) and, again, each line within a plot corresponds to an individual subject. Each vertical line near the abscissa marks the latency to impairment for an individual subject, or an event, which was determined as the time at which a Z-score dropped below -1.0 for at least 75% of the remaining sessions. These impairment latencies were plotted as event curves (“survival”) and analyzed using Mantel-Cox survival analysis (Fig. 3.3-3.4).

Figures 3.3-3.4 show the event analyses that were carried out for all dependent measures of wheel-running. The left columns show adult mice and the right columns show

retired breeders, while the top and bottom rows show a single dependent measure. Within each plot, drop lines shown the median latency to impairment for MeHg-only and MeHg + NIM exposure groups. The difference between these two groups is shown, which is denoted by the arrow between two exposure groups. Analyses revealed a statistically significant difference between survival curves of the eight exposure groups for each dependent measure. For brevity and clarity the specific statistics are omitted, but all effects described are associated with an omnibus *p*-value of less than 0.05. Multiple comparisons revealed a number of significant differences among exposure groups, which are catalogued in detail in Table 3.1. While each dependent measure was affected by MeHg, the time-course of disruption was not the same for each measure (see Table 3.1, Fig. 3.3-3.4).

First, control and NIM-only groups did not differ on any measure for adults or retired breeders. Second, for adults, MeHg-only mice differed from both control and NIM-only groups on all dependent measures , MeHg + NIM mice differed from both control and NIM-only groups on total distance and within-bout rate but not bout-initiation rate or bout length , and MeHg-only and MeHg + NIM groups differed on all dependent measures. Third, for retired breeders, MeHg-only and MeHg + NIM mice differed from both control and NIM-only groups on all dependent measures and MeHg-only and MeHg + NIM did not differ on any measure. Finally, between age groups, MeHg-only groups did not differ on any measure, whereas the MeHg + NIM groups differed on bout-initiation rate and bout length but not total distance or within-bout rate.

3.3 Rotarod

3.3.1 Age differences

Figure 3.5 shows the average maximum RPM for control adults and retired breeders. Repeated measures ANOVA revealed a main effect of *Age cohort* [$F(1,25)=5.77$, $p=0.02$] and *Session* [$F(27,674)=4.02$, $p<0.001$] but no *Age cohort* X *Session* interaction such that adults reached higher maximum speeds than the retired breeders.

3.3.2 MeHg toxicity and nimodipine neuroprotection

The same event analysis used for wheel-running, described above, was used for rotarod. Figure 3.6 shows latency to impairment for adults (left) and retired breeders (right). Again, analysis revealed a statistically significant difference between survival curves for maximum RPM and multiple comparisons are catalogued in Table 3.1.

First, control and NIM-only groups did not differ for adults or retired breeders. Second, for adults, the MeHg-only group differed from control and NIM-only groups, the MeHg + NIM group differed from control but not the NIM-only group, and the MeHg-only group differed from MeHg + NIM group. Third, for retired breeders, MeHg-only and MeHg + NIM groups differed from control and NIM-only and they also differed from each other. Finally, between age cohorts, there was no difference between MeHg-only groups but there was a difference between MeHg + NIM groups.

3.4 Predicting impairment.

Inspecting Fig. 3.3-3.4 and Fig. 3.6, rotarod performance and within-bout rate appeared to show the shortest latency to impairment for both adults and retired breeders. To test the hypothesis that measures of motor function, rotarod performance and within-bout rate, were the earliest reliable predictors of MeHg-induced impairment, we compared the latency from impairment to mortality for all animals that died. The adult MeHg + NIM group and all control and NIM-only groups were omitted because so few of these animals died and mortality did not vary among the groups.

Figure 3.7 shows average latency in days from impairment to death for adult and retired breeder MeHg-only groups and the retired breeder MeHg + NIM for rotarod, total distance, within-bout rate, bout-initiation rate, and bout length. The LME model revealed a main effect of *Measure* [$F(4,144) = 10.13$, $p<0.001$], but no main effect of *Age* or *Nimodipine* and no interaction. Tukey's HSD confirmed a longer latency between impairment and mortality for rotarod relative to total distance ($p<0.01$), bout-initiation rate ($p<0.01$), and bout length ($p<0.01$)

but not within-bout rate (p=0.08). Also there was a longer latency for within-bout rate relative to bout-initiation rate (p<0.01).

4. Discussion

The present study found the following: 1) there were age difference in wheel-running and rotarod performance and nimodipine alone had no effect on these measures, 2) chronic MeHg exposure produced relatively *age-independent* sensorimotor deficits in mice on both running and rotarod performance, 3) nimodipine neuroprotection from MeHg toxicity was *age-dependent* because there was greater protection for adults relative to retired breeders, and 4) rotarod performance and within-bout running rate, both measures of sensorimotor function, were the earliest reliable and reliable indicators of MeHg toxicity. The findings reported here support and extend previous studies on the effects of chronic, adult-onset MeHg exposure and neuroprotection by the L-type CCB nimodipine [26,27,31,48]

4.1 Age differences

Adult and retired breeder control groups (0 ppm MeHg, 0 ppm nimodipine) differed on wheel-running and to lesser extent on rotarod. Overall, the retired breeders ran further than the adults because they initiated bouts of running at a higher rate. This was due, in part, to a decrease and leveling-off of bout-initiation rate (Fig 3.1). Within-bout rate was indistinguishable between age groups, but speed increased rapidly with experience. As bout-initiation rate is a marker of motivation, wheel-running may have served as a more effective reinforcer for the retired breeders than adults, both of which were equally deprived of running otherwise. Although no other behavior was directly measured, the results suggest that adult mice engaged in more behavior that was incompatible with running, such as exploring or grooming.

In contrast, the adult mice outperformed the retired breeders on rotarod, indicating better sensorimotor coordination in the younger age group. This is consistent with the suggestion above that differences in bout-initiation rate were not driven by motor dysfunction. Interestingly, for retired breeders that aged from approximately 9.5 to18 months old over the course of the

study, there was no decline in the motivation to run as measured by bout-initiation rate. This contrasts with the recent report of Shen et al. [27] in which the motivation to nose-poke for sucrose under a high-rate percentile (PCNT) schedule declined as a function of age. Nose-poking is an arbitrary response that is maintained by an extrinsic reinforcer while the reinforcement for running is inextricably embedded in the act itself, which makes running an "automatically" or intrinsically reinforced behavior [49]. As these two activities are differentially sensitive to drug effects [42], they could also be differentially sensitive to aging, too.

4.2 Chronic nimodipine

The chronic administration of 20 mg/kg/day nimodipine did not produce decrements or benefits in the wheel-running or rotarod performance of adult or retired breeder mice. Dihyrdopyridine CCBs like nimodipine block a variety of Ca^{2+} channels, including L-type channels located on neurons, cerebral and peripheral vasculature, and cardiac smooth muscle [50-53]. Studies have found that nimodipine attenuates or blocks selective signs of normal aging [32-37], but effects in healthy animals are less clear. Recently, Bailey et al. [31] and Hoffman & Newland [26] found that chronic nimodipine had no effect on performance in an incremental repeated acquisition procedure, wheel-running or rotarod performance in adult mice. Shen et al. [27] also found no effect of chronic nimodipine on adult and retired breeder nose-poking under a high-rate schedule of reinforcement.

4.3 Motor dysfunction

4.3.1 Methylmercury neurotoxicity

Chronic exposure to 1.2 mg/kg/day MeHg produced age-independent deficits in wheel-running and rotarod performance (see Table 3.1). The median latency to impairment of motor function (i.e., rotarod, within-bout rate, and total distance) was shorter than median latency to impairment of motivation (bout-initiation rate) (Fig. 3.3-3.4 and Fig.3.6). For MeHg-only adults, the median latency to within-bout rate impairment was the shortest (82 days), followed closely by rotarod (86 days), total distance (88 days), bout length (89 days) and finally bout-initiation

rate (102 days). For MeHg-only retired breeders, the median latency to rotarod impairment was the shortest (51 days), followed by total distance (75 days), within-bout rate (81 days), bout-initiation rate (89 days), and finally bout length (96 days) (see Fig. 3.3-3.4 and Fig. 3.6).

Although the difference in median latency to rotarod impairment between MeHg-only adults and retired breeders was not statistically significant, it is important to look at the distribution of impairment latencies. For the retired breeders, 25% were impaired during the first rotarod session (exposure day 15) and never recovered. In comparison, the adult group did not reach 25% impairment until exposure day 51. Bellum and colleagues [10,21] found that 1.0 mg/kg/day MeHg for 5 days produced measureable deficits in middle-late adolescent and aged C57BL/6 mice, suggesting that this dose range of MeHg is capable of producing motor deficits relatively quickly. While these studies [10,21] found no age difference between exposure groups, the study was acute and exposure ceased during the testing period.

To determine which measures were early predictors of MeHg toxicity, the latency from impairment to mortality was determined for adult and retired breeder MeHg-only mice and for the retired breeders MeHg + NIM mice (Fig. 3.7). The adult MeHg + NIM group as well as all control and NIM-only groups were excluded because so few mice in these groups died and mortality did not differ among them (see Shen et al. [27]). Decrements in rotarod and within-bout rate were the most reliable predictors of toxicity, meanwhile decrements in bout-initiation rate occurred very close in time to mortality, i.e., on average 11-12 days prior to death for all three groups tested (Fig. 3.3-3.4 and Fig. 3.6).

These results extend previous findings of adult-onset MeHg exposure in rodents [22,26,27,48,54]. For example, Heath et al. [48] found that chronic adult-onset exposure to 0.4 mg/kg/day and 1.2 mg/kg/day MeHg in rats decreased overnight wheel-running. Unexposed animals fed both low and high selenium (Se) diets increased wheel-running as a function of session; 0.4 mg/kg/day MeHg blocked this increase and 1.2 mg/kg/day decreased wheel-running [48]. Similarly, Hoffman & Newland [26] found that chronic adult-onset exposure to 2.6

mg/kg/day MeHg in mice decreased the latency to rotarod and wheel-running impairment. As with the present study, MeHg decreased within-bout rate and total distance but had no effect on bout-initiation rate. Also, in Hoffman & Newland's [26] study, which used a higher dose, latency from rotarod impairment to death was approximately 21-28 days, which is approximately half that observed in the current study for the adults. Combined, the current study and Hoffman & Newland [26] provide evidence that adult-onset MeHg exposure produces primary motor dysfunction in a dose-related manner. These behavioral studies do not identify specific neural correlates, but it seems reasonable to assume that damage to dorsal root ganglion or cerebellum may underlie the deficits seen here because of their sensitivity to adult-onset MeHg exposure [55-57].

4.3.2 Nimodipine neuroprotection

Chronic nimodipine, 20 mg/kg/day, attenuated deficits in wheel-running and rotarod in an age-dependent manner (Fig. 3.3-3.4 and Fig. 3.6). For both MeHg + NIM adults and retired breeders, the median latency to rotarod impairment was the shortest (205 and 100 days, respectively), followed closely by within-bout rate (213 and 121 days, respectively). For MeHg + NIM adults, impairment of total distance (236 days), bout length (248 days), and bout-initiation rate (258 days) followed, whereas bout length (130 days), total distance (137 days), and bout-initiation rate (137 days) followed for MeHg + NIM retired breeders (see Fig. 3.3-3.4 and Fig. 3.6).

Measures of motor function, most prominently rotarod, were the earliest and most reliable indicators of MeHg toxicity for MeHg + NIM mice. For adults, relative to the MeHg-only group, nimodipine delayed impairment in rotarod, within-bout rate, total distance, bout-initiation rate, and bout length by 119, 131, 148, 159, and 156 days, respectively (i.e., difference in median latencies) (Fig. 3.6). For retired breeders, nimodipine delayed impairment in bout length, within-bout rate, bout-initiation rate, rotarod, and total distance by 34, 40, 48, 49, and 62 days, respectively (Fig. 3.3-3.4 and Fig. 3.6). Thus, nimodipine delayed the onset of MeHg

neurotoxicity to a greater extent in adults relative to retired breeders. While very few MeHg + NIM adult animals died, most MeHg + NIM retired breeders were euthanized due to MeHg toxicity. The average latency from impairment to death for the MeHg + NIM retired breeders was similar to that of the adult and retired breeder MeHg-only groups (Fig. 3.7). This finding reiterates the disparity in MeHg-induced disruption of motoric (rotarod and within-bout rate) and motivational (bout-initiation rate) components of behavior.

The findings with adult mice conform to previous studies on nimodipine neuroprotection from MeHg conducted in our laboratory [26,27,31] and others that have also used CCBs [30,58]. Importantly, Hoffman & Newland [26] reported a complete elimination of MeHg effects by 20 mg/kg/day nimodipine after 160 days of exposure, whereas the findings here show that this protection is dependent on age and duration of exposure. That is, 20 mg/kg/day nimodipine afforded muted protection against MeHg neurotoxicity in the aging retired breeders and, despite better protection, motor impairment was observed in the adults as they aged due to the longitudinal nature of the study (262 days of exposure).

Another important finding is that of the progression of MeHg toxicity among retired breeders. It became apparent that when MeHg-induced behavioral deficits appeared, the progression from impairment to mortality was relatively similar between MeHg-only and MeHg + NIM animals (Fig. 3.7). That the latency from impairment to death was not different for MeHg-only and MeHg + NIM animals is intriguing, as it suggests that there was a threshold for nimodipine protection after which point the drug's effect completely ceased. That is, nimodipine altered the time at which select impairments manifested, but once apparent nimodipine had little effect on the progression towards MeHg-induced mortality.

4.4 Mechanisms of MeHg toxicity and nimodipine neuroprotection

Dysregulation of Ca^{2+} homeostasis appears to play a prominent role in MeHg-induced disruption neuronal and glial function and cell death [59,60]. *In vitro* models provided evidence that MeHg disrupts Ca^{2+} homeostasis in two distinct phases (see [5,58.61]). MeHg preferentially

targets granule cells, especially those located within the cerebellum, while neighboring Purkinje cells are left relatively intact. The more subtle Ca^{2+}-related changes that occur during normal aging likely include increased Ca^{2+} release from intracellular stores through inositol(1,4,5) triphosphate (IP3) receptors and ryanodine (Ry) receptors, increased Ca^{2+} influx through L-type Ca^{2+} channels, and increased amplitude and duration of the Ca^{2+}-dependent, K+-mediated afterhyperpolarization (AHP) [62-64]. While some neuronal loss occurs in aging, age-related functional decline in normal aging may occur in the absence of widespread neuronal loss [65,66]. Calcium channel blockers like nimodipine delay MeHg-induced Ca^{2+} dysregulation (Marty & Atchison, 1997) and may facilitate Ca^{2+} buffering thereby decreasing long-term intracellular Ca^{2+} levels. In addition, CCBs may help to buffer chronically elevated levels of Ca^{2+} produced during the aging process, reducing cognitive and motor deficits seen in aged animals.

Note that both Bailey et al. [31] and Shen et al. [27] found that nimodipine appeared to lower brain Hg concentrations in adult mice. Brain Hg concentrations from MeHg-only animals were determined at the time of euthanasia and represent a lethal dose of MeHg. Conversely, the brain Hg levels of MeHg + NIM animals, in general, were determined at the ends of the studies. Thus, MeHg + NIM animals were often exposed to MeHg for much longer durations but still showed reduced accumulation of Hg in the brain. It is possible that nimodipine decreased the bioavailability of MeHg, perhaps by decreasing MeHg absorption in the gut, increasing excretion, or reducing passage across the blood brain barrier (BBB). These are speculations, and while the exact mechanism(s) by which nimodipine reduces brain Hg concentration is not currently understood, these studies support the notion that nimodipine augments the toxicokinetics of MeHg.

4.5 *Summary of findings*

The results from our laboratory [the current study, 26,27,31], characterize, *in vivo*, chronic adult-onset MeHg exposure and neuroprotection by nimodipine in mice. The following observations have been made. First, MeHg produced early and specific deficits in motor

function followed by reductions in motivation to engage in target behavior. Apparently, the reinforcer maintaining both wheel-running and nose-poking remains effective even in the face of motor impairment. Second, many deficits occurred earlier in older retired breeder mice that were not yet senescent than younger adult mice. It is possible that MeHg acted to accelerate aging processes mediated by Ca^{2+} signaling and hasten decline in motor function. Third, nimodipine delayed MeHg neurotoxicity in an age-dependent manner and protection was afforded across a range of phenotypes that appeared rooted in motor function. Fourth, long-term chronic dosing regimens showed that nimodipine's protection eventually waned, which suggests that the aging process mitigated nimodipine's ability to reduce intracellular Ca^{2+} levels or perhaps that animals developed tolerance to the drug. Fifth, chronic nimodipine administered alone has little to no effect on motor or cognitive function in younger or older adult BALB/c mice. Finally, a bout analysis approach provided quantitative measures of motoric and motivational components of behavior, which are often difficult to decouple. Motor function and motivation to engage in target behavior were differentially sensitive to MeHg, with MeHg preferentially affecting the former regardless of age. This approach coupled with the event analysis described here and in Shen et al. [27] contribute a potential methodology for quantifying behavior changes in a situation in which attrition diminishes sample size in a longitudinal study.

5. Conclusion

The present study characterized the effects of chronic adult-onset MeHg exposure and nimodipine neuroprotection in two age groups of mice. Generally, MeHg produced decrements in wheel-running and rotarod performance and nimodipine attenuated these deficits in an age-dependent manner. Nimodipine alone had no discernible effect on behavior. Log-survivor analyses of wheel-running independently estimated motor and motivational components, which were differentially sensitive to MeHg. Latency to decline in motor function (rotarod, within-bout rate, and total distance) was shorter than the latency to decline in the motivation to run (bout-initiation rate). This study supports models of adult-onset MeHg toxicity mediated by disruptions

in Ca^{2+} homeostasis that produces primary motor deficits followed by secondary cognitive deficits. The underlying neural substrates that mediate MeHg toxicity, intracellular Ca^{2+}-signaling, may also be perturbed during the normal aging process. The finding of age-dependent nimodipine neuroprotection provides evidence that MeHg exposure in aging animals may tax and already-perturbed Ca^{2+} signaling system. This study also provides support for the use of an event analysis to quantify behavior changes when sample size diminishes over the course of a study due to attrition.

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Table 3.1

Survival analysis: Pairwise multiple comparisons

Age	Group 1	Group 2	Total distance	Within-bout rate	Bout-initiation rate	Bout length	Rotarod
Adult	Control	NIM	0.90	0.51	0.82	0.90	0.72
	Control	MeHg	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	Control	MeHg + NIM	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	NIM	MeHg	***0.01***	***<0.01***	0.12	0.33	***<0.01***
	NIM	MeHg + NIM	***0.03***	***0.03***	0.79	0.79	0.08
	MeHg	MeHg + NIM	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Retired breeder	Control	NIM	0.71	0.74	0.97	0.93	0.97
	Control	MeHg	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	Control	MeHg + NIM	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	NIM	MeHg	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	NIM	MeHg + NIM	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
	MeHg	MeHg + NIM	0.53	0.07	0.66	0.18	***0.02***
Adult vs. Retired breeder	MeHg	MeHg	0.90	0.88	0.99	0.86	0.75
	MeHg + NIM	MeHg + NIM	0.07	0.14	***0.03***	***<0.01***	***0.02***

Table 3.1. Relevant pairwise comparisons from the event analysis (Mantel-Cox). Bold and italicized *p*-values represent a significant difference between Group 1 and Group 2.

Figures captions

Figure 3.1 Mean total distance (A), bout-initiation rate (B), within-bout rate (C), and bout length (D) as a function of day for wheel-running. Open and closed circles represent the control adult and control retired breeder age cohorts, respectively. Error bars represent ± standard error of the mean (SEM). a = main effect of Age, $p<0.05$; b = main effect of Session, $p<0.05$.

Figure 3.2. An example of the statistical tactics used to conduct the event analysis. Raw estimates (left) of total distance as a function of exposure day were converted to Z-scores (right). Each line within each plot represents an individual subject from the adult exposure groups (top to bottom: Control, NIM-only, MeHg-only, and MeHg + NIM). Each vertical line on the abscissa of the plots on the right side represents the latency to impairment for an individual animal. The dashed horizontal lines demarcate ±1.0 Z-score unit.

Figure 3.3. Event analyses (survival analysis) for bout-initiation rate (top panel) and within-bout rate (bottom panel), with adults on the left and retired breeders on the right. Shown near the abscissa of each Kaplan-Meier plot are the median latencies to impairment for the MeHg-only and MeHg + NIM exposure groups, as well as the difference between the two groups. Exposure groups: black = control, green = NIM-only, red = MeHg-only, and blue = MeHg + NIM.

Figure 3.4. Event analyses (survival analysis) for total distance (top panel) and bout length (bottom panel), with adults on the left and retired breeders on the right. The format is identical to Fig. 3.3 Exposure groups: black = control, green = NIM-only, red = MeHg-only, and blue = MeHg + NIM.

Figure 3.5. Mean rotarod performance (maximum speed in rpms) for adult (open circles) and retired breeder (filled circles) control groups as a function of day. Error bars represent ± standard error of the mean (SEM). a = main effect of Age, $p<0.05$; b = main effect of Session, $p<0.05$.

Figure 3.6. Event analyses (survival analysis) for rotarod performance (maximum RPM), with adults on the left and retired breeders on the right. The format of the panels is identical to that of Fig. 3.3-3.4. Exposure groups: black = control, green = NIM-only, red = MeHg-only, and blue = MeHg + NIM.

Figure 3.7. The bar chart show the average latency (days) from impairment to death for bout-initiation rate, bout length, total distance, within-bout rate, and rotarod (left to right). Note that the adult MeHg + NIM group is excluded because so few animals died from MeHg toxicity (see text for details). Error bars represent ± SEM. a = main effect of Dependent Measure, $p<0.05$.

Figure 3.1

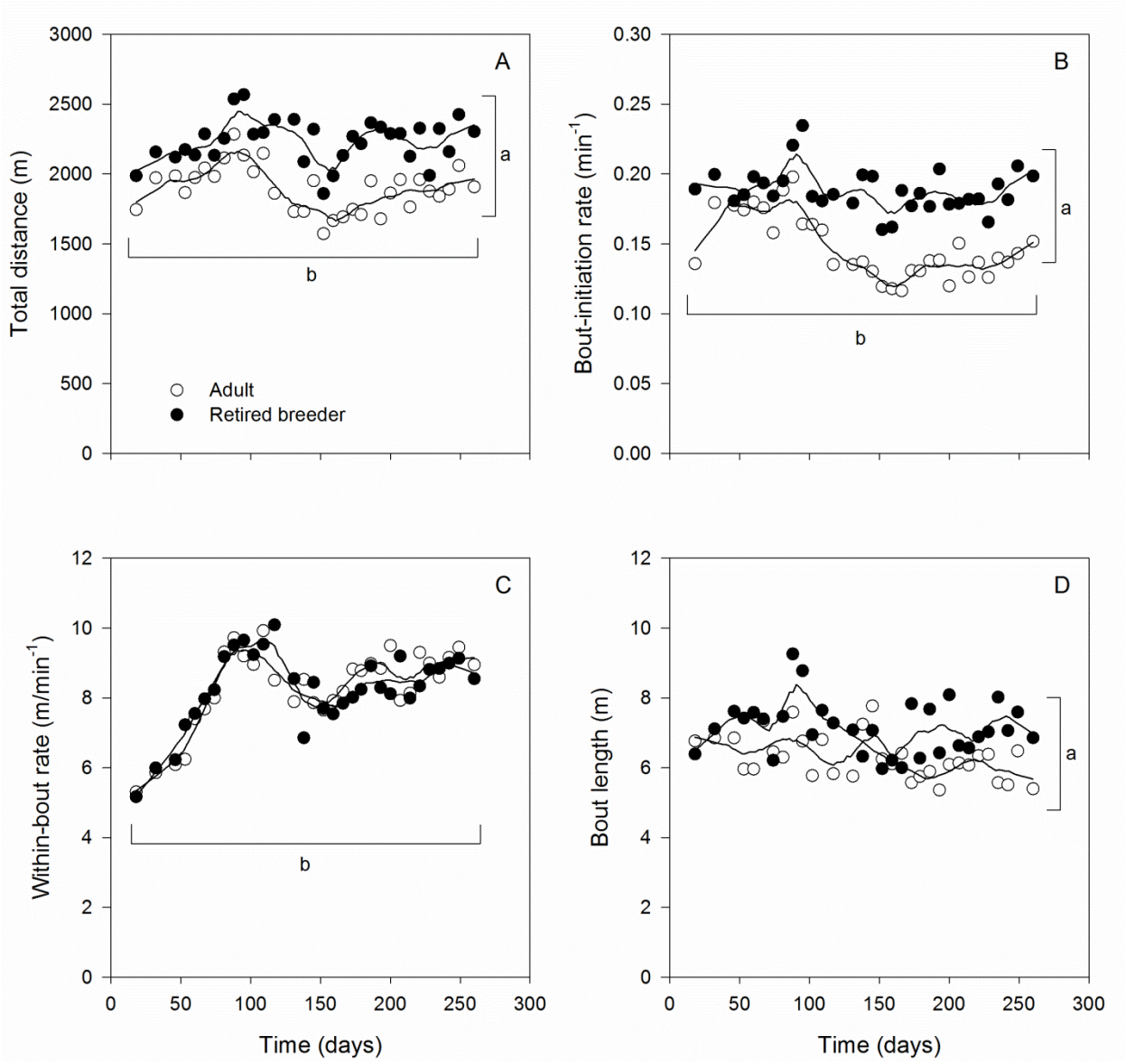

A
B
C
D
Total distance (m)
Bout-initiation rate (min^{-1})
Within-bout rate (m/min^{-1})
Bout length (m)
Time (days)
Time (days)
Adult
Retired breeder
a
b
a
b
b
a

Figure 3.2

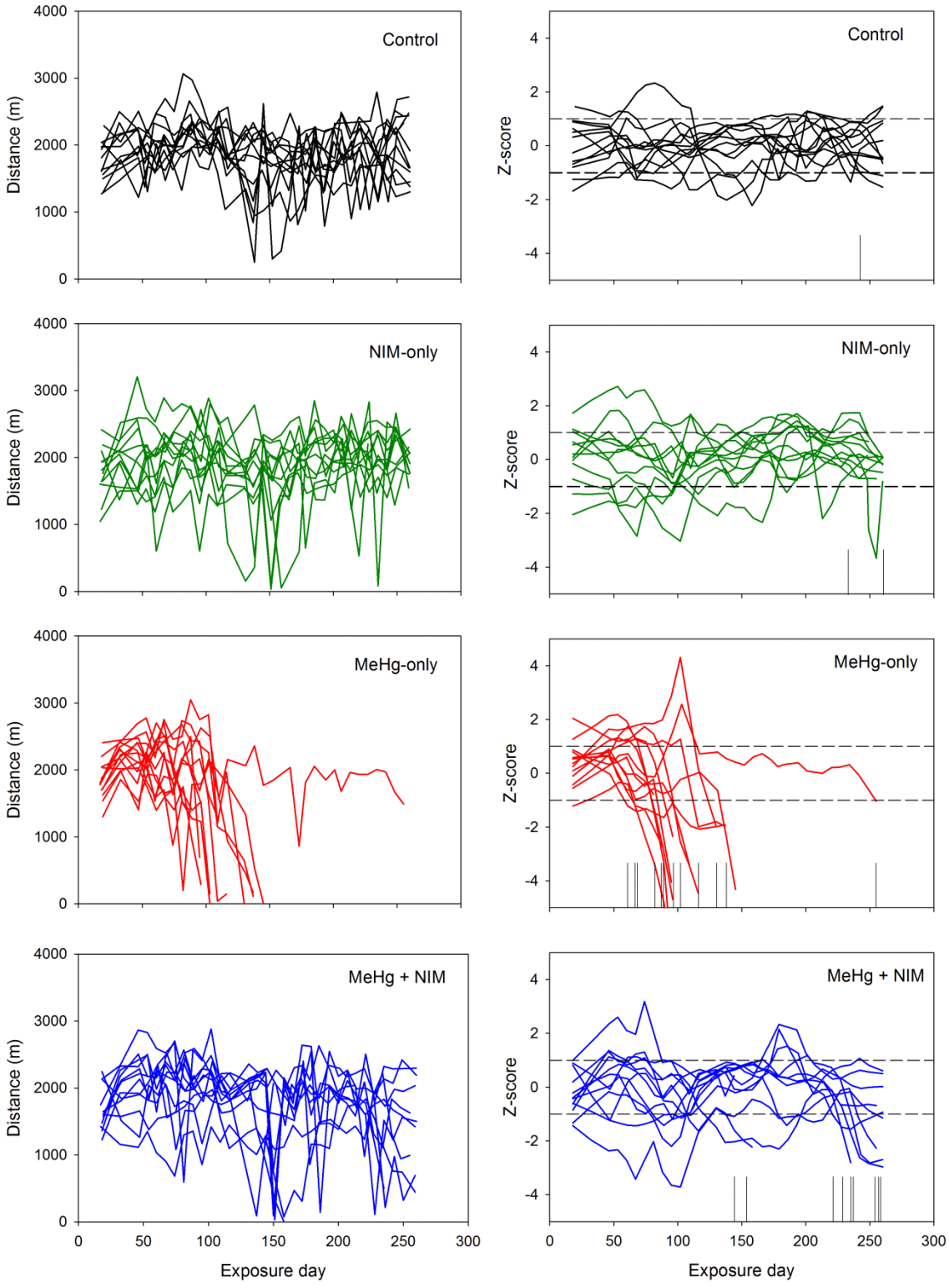

Control
NIM-only
MeHg-only
MeHg + NIM
Distance (m)
Z-score
Exposure day
0
1000
2000
3000
4000
-4
-2
0
2
4
0
50
100
150
200
250
300

Figure 3.3

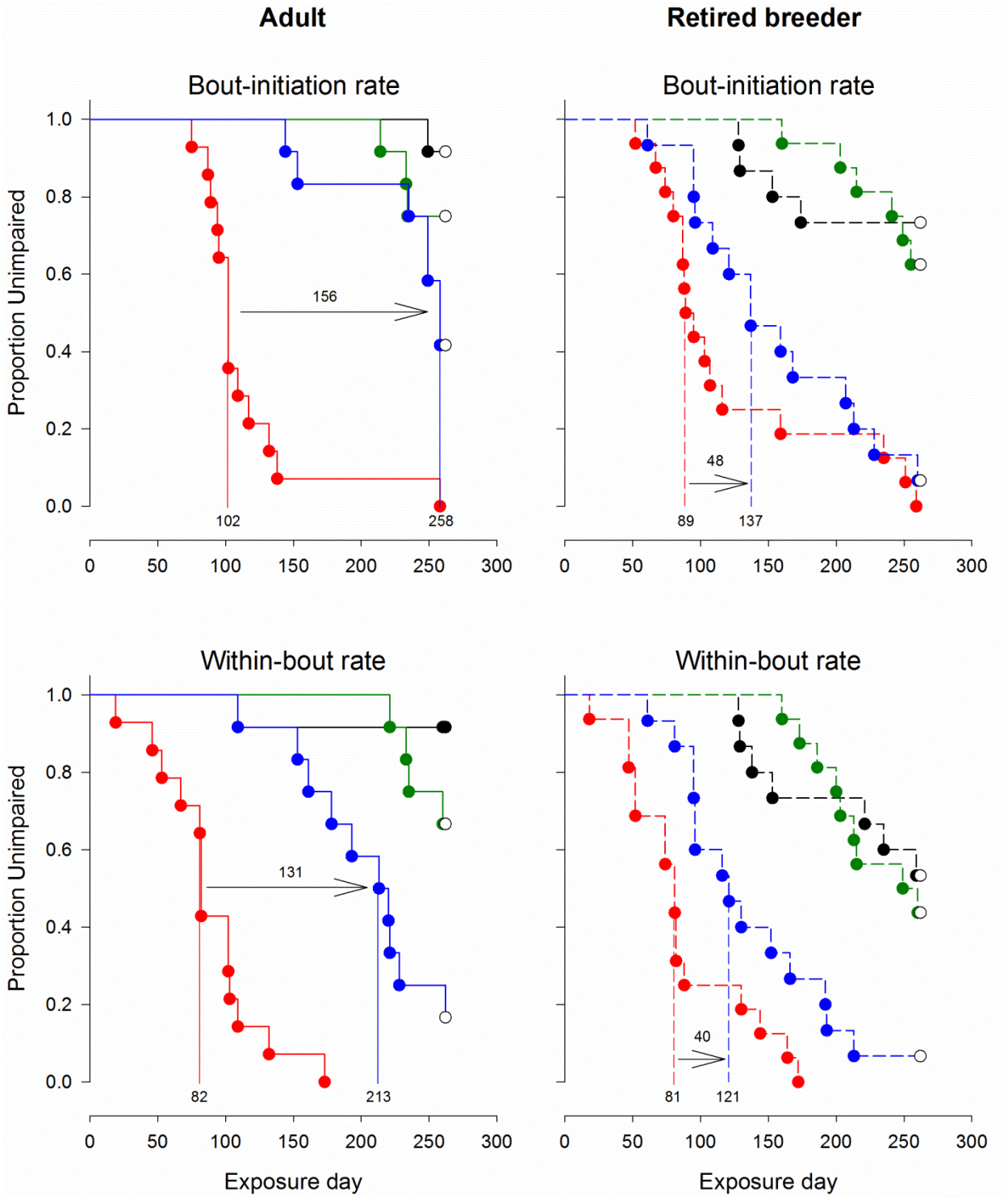

Adult
Retired breeder
Bout-initiation rate
Bout-initiation rate
Within-bout rate
Within-bout rate
Proportion Unimpaired
Proportion Unimpaired
Exposure day
Exposure day
156
102
258
48
89
137
131
82
213
40
81
121
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
200
250
300

Figure 3.4

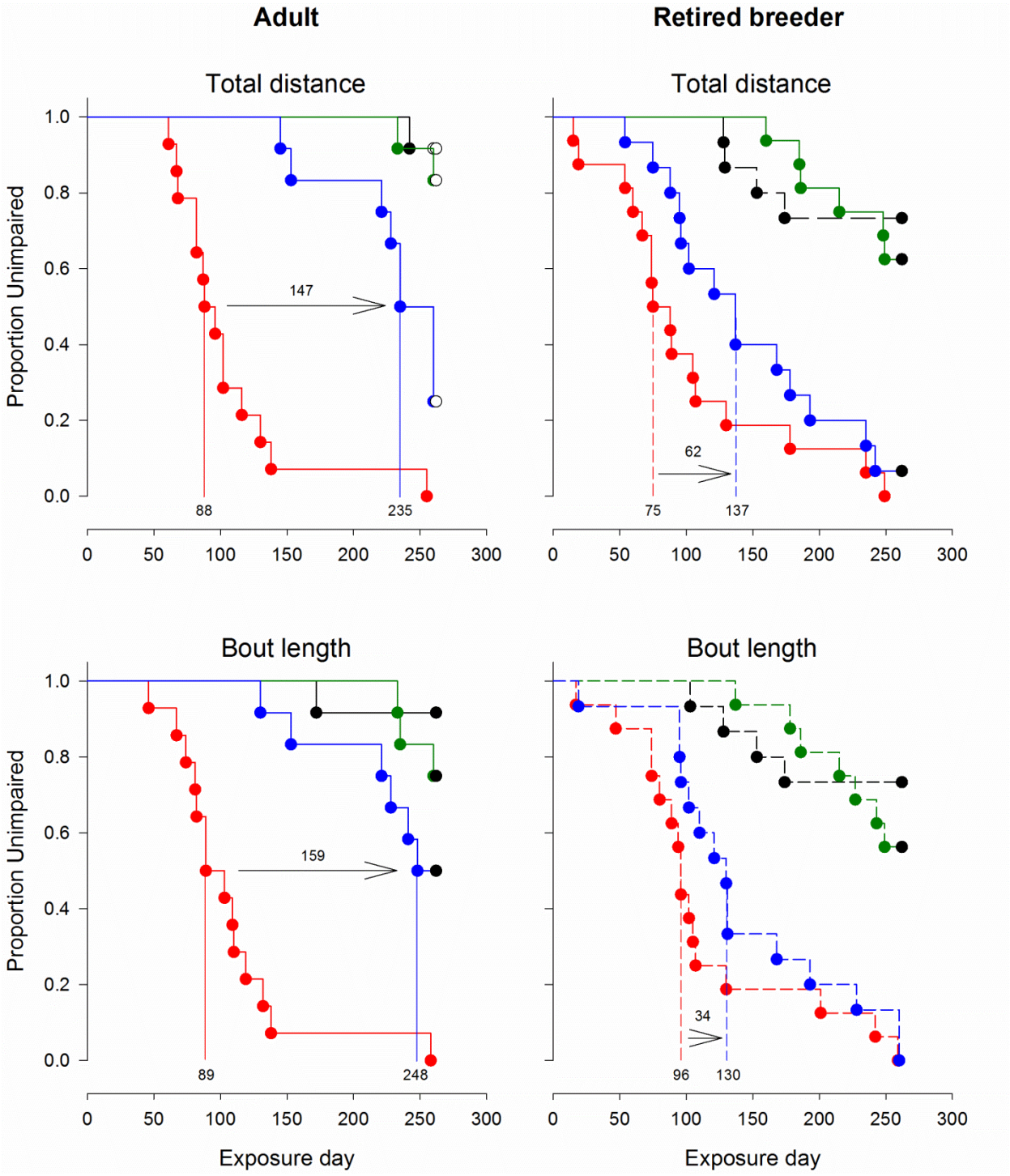

Adult
Retired breeder
Total distance
Total distance
Proportion Unimpaired
1.0
0.8
0.6
0.4
0.2
0.0
147
88
235
62
75
137
0
50
100
150
200
250
300
Bout length
Bout length
159
89
248
34
96
130
Exposure day
Exposure day

Figure 3.5

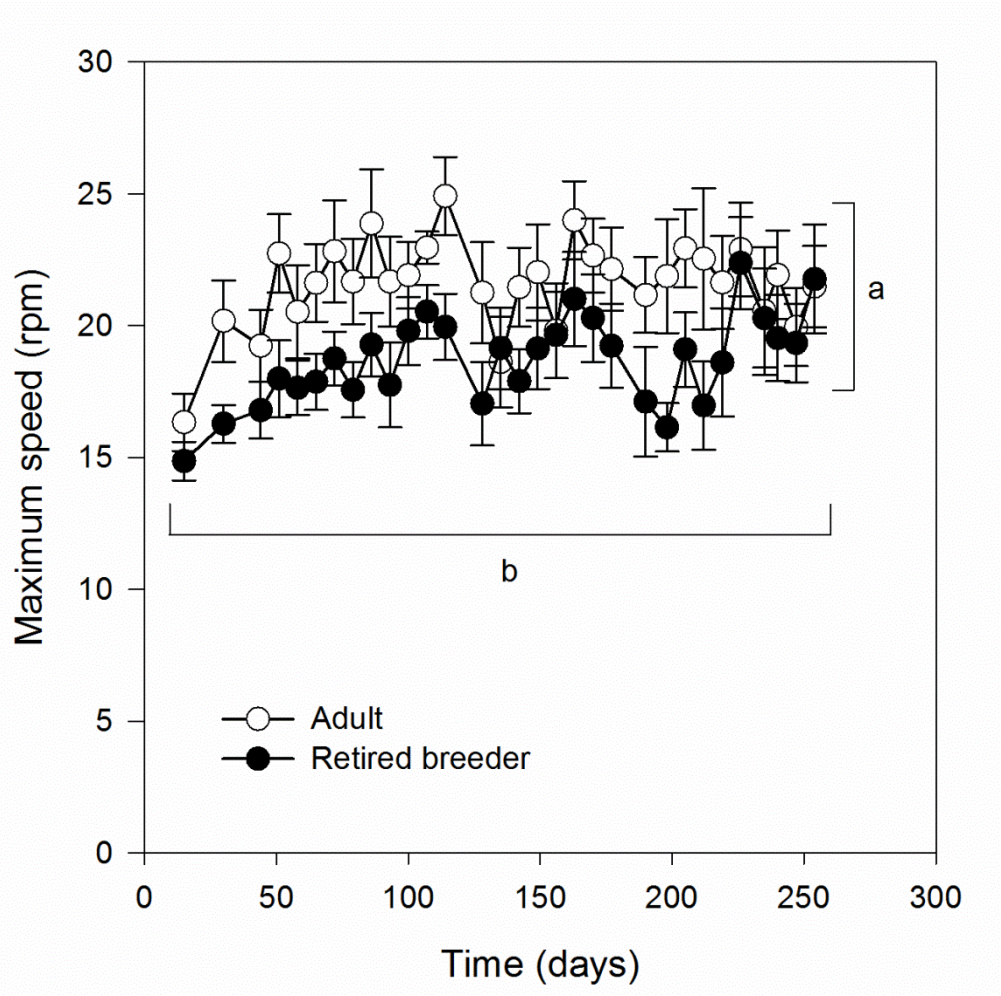

Maximum speed (rpm)
Time (days)
Adult
Retired breeder
a
b
0
5
10
15
20
25
30
0
50
100
150
200
250
300

Figure 3.6

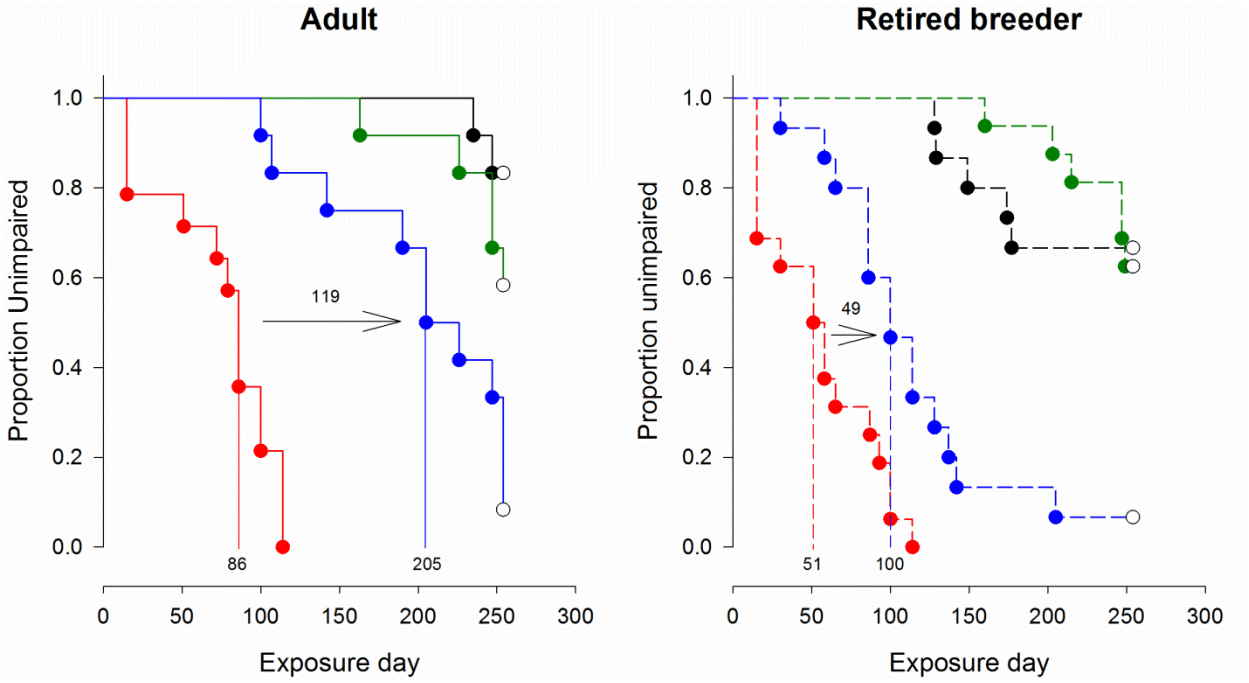
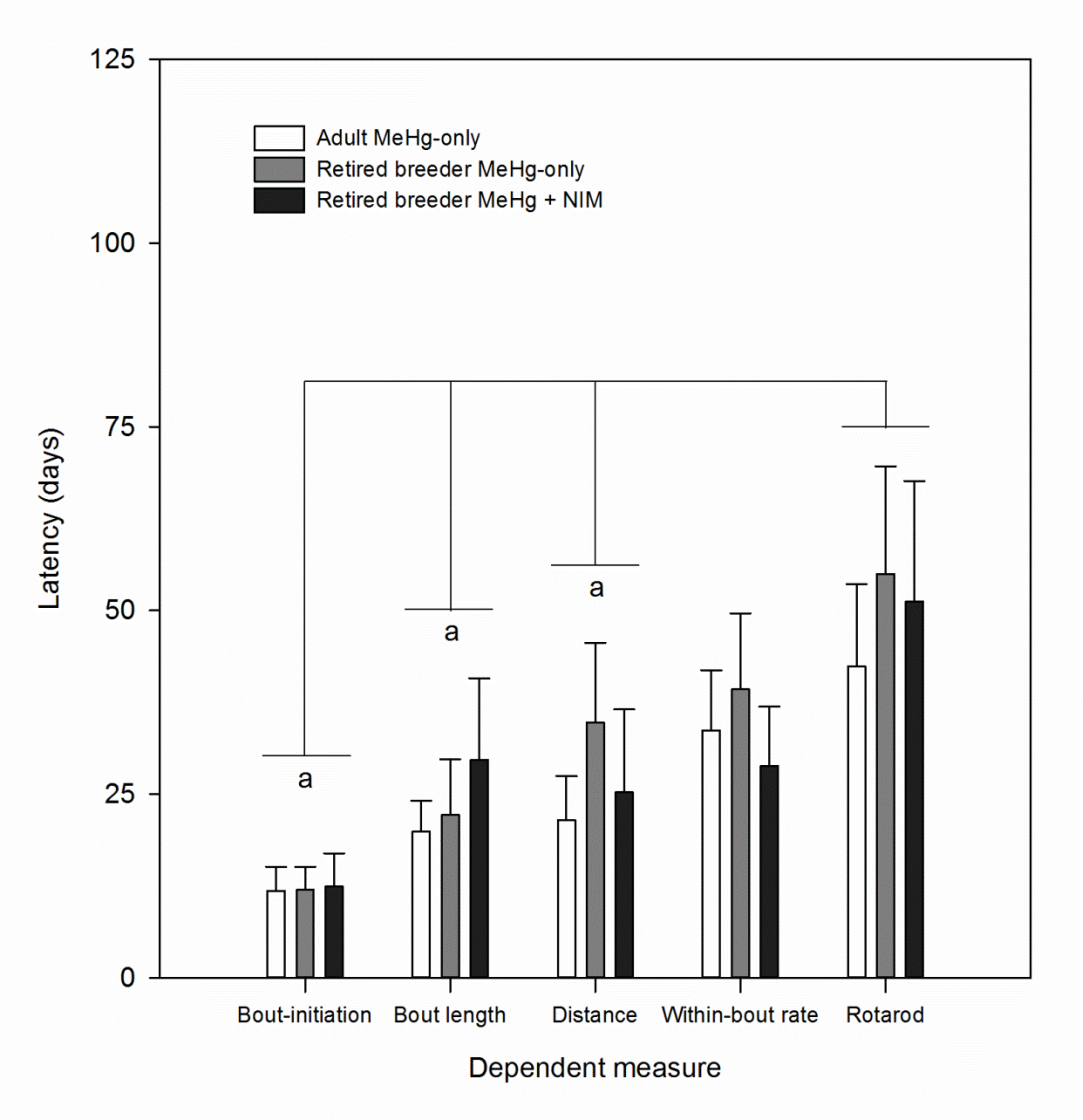

Adult
Proportion Unimpaired
1.0
0.8
0.6
0.4
0.2
0.0
119
86
205
0
50
100
150
200
250
300
Exposure day
Retired breeder
Proportion unimpaired
1.0
0.8
0.6
0.4
0.2
0.0
49
51
100
0
50
100
150
200
250
300
Exposure day

Figure 3.7


125
100
75
50
25
0
Latency (days)
Adult MeHg-only
Retired breeder MeHg-only
Retired breeder MeHg + NIM
a
a
a
Bout-initiation
Bout length
Distance
Within-bout rate
Rotarod
Dependent measure