Gene-environment interactions between adult lead exposure and Apolipoprotein E4 on adult hippocampal neurogenesis and cognitive behavior in mice

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Abstract

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Alzheimer's disease (AD) is characterized by progressive cognitive decline and memory loss. It has been hypothesized that environmental factors and gene-environment interactions (GXE) may increase AD risk and accelerate cognitive decline. However, there is currently little direct evidence supporting this hypothesis. Interestingly, the E4 allele of the Apolipoprotein E gene (ApoE4) is the strongest known genetic risk factor for late-onset, sporadic AD, and it is also associated with accelerated cognitive decline compared to ApoE4 non-carriers. Furthermore, the heavy metal lead is a neurotoxicant of major public health importance and is associated with persistent cognitive and behavior deficits in humans. Using transgenic knock-in (KI) mice that express the human ApoE4 allele (ApoE4-KI), I found that adult-only lead exposure is sufficient to impair cognitive behavior and that lead-exposed ApoE4-KI mice develop more severe or

exhibit earlier deficits in learning and memory compared to ApoE3-KI mice. Furthermore, these impairments in cognitive behavior are persistent and can occur long after the cessation of the lead exposure. I also found that females may be more sensitive to the effects of lead than males.

Through a process called adult hippocampal neurogenesis, adult neural precursor cells in the dentate gyrus of the hippocampus continuously generate neurons throughout adulthood. These adult-born neurons contribute to hippocampus-dependent learning and memory. Importantly, the hippocampus is one of the earliest affected brain regions in AD patients, and the perturbation of adult hippocampal neurogenesis may cause deficits in hippocampus-dependent learning and memory, accelerate cognitive decline, and contribute to AD pathogenesis. While various factors have been shown to modulate adult neurogenesis, little is known about the effects of neurotoxicants or GXE on adult neurogenesis. Using an *in vitro* model of adult neurogenesis, I found that lead significantly increases apoptosis, inhibits proliferation, and impairs the spontaneous neuronal differentiation and maturation of adult neural precursor cells. Furthermore, I found that activation of the JNK and MAPK signaling pathways are important for lead cytotoxicity. I also utilized a transgenic knock-in mouse model of human ApoE4 carriers in order to assess for a GXE between lead and ApoE4 on adult hippocampal neurogenesis and found that adult lead exposure is sufficient to impair adult-born cell proliferation *in vivo.* I also observed more significant effects on adult-born neuron maturation in lead-treated ApoE4-KI females, suggesting that a GXE between lead and ApoE4 may significantly

reduce the total number of adult-born neurons as well as perturb the maturation and dendritic complexity of adult-born neurons in the dentate gyrus of the hippocampus.

Together, these data suggest that lead can directly act on adult neural stem cells to impair critical processes in adult hippocampal neurogenesis, which may contribute to its neurotoxicity and adverse effects on cognition in adults. My characterization of cognitive behavior and neurogenesis in lead-exposed ApoE4-KI mice also provides strong evidence of a GXE between ApoE4 and lead on cognitive impairment and adult hippocampal neurogenesis and may help elucidate the role of GXE and sex differences on AD risk.

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Dedication

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Chapter 1: Introduction

1.1 Alzheimer's disease and cognitive decline

Alzheimer's disease (AD) is the most common neurodegenerative disease and is characterized by progressive memory loss and cognitive decline (Mu and Gage, 2011). Currently, AD affects approximately 13% of individuals over the age of 65 and 45% of individuals over age 85, and AD is expected to affect over 100 million people by 2050 (Qiu et al., 2009). In addition to significant cognitive decline (dementia), including impaired problem-solving, language, and attention, AD patients also develop mood and personality changes. In the central nervous system, AD is characterized by significant and irreversible neuronal loss. The pathological hallmarks of AD include intracellular neurofibrillary tangles of hyperphosphorylated tau protein and extracellular senile plaques consisting of aggregated amyloid-β protein in specific brain regions, including the hippocampus (limbic system) and cortex (Liu et al., 2013). The degree and severity of these AD pathological hallmarks correlate with the severity and duration of clinical changes and are believed to play a role in AD pathogenesis (Serrano-Pozo et al., 2011). However, it is still unclear whether some or all of these pathological changes are the cause or, rather, the product of AD pathogenesis (Swerdlow, 2007). In addition to the 'amyloid cascade' hypothesis, other mechanisms have been proposed for AD pathogenesis, including systemic mitochondrial dysfunction and impaired adult hippocampal neurogenesis (Swerdlow and Khan, 2004; Lazarov and Marr, 2010). Importantly, existing therapies for AD patients only slow the progression of the disease and ameliorate

some of the symptoms, but they do not reverse the course of AD (Schneider, 2013). Thus, additional research into the underlying pathogenesis of AD is paramount for the timely and successful development of new therapies for AD patients.

Aging is the primary risk factor for AD. However, AD is a multifactorial disease and both genetic and environmental risk factors are associated with an increased risk of AD. Dominant mutations in the genes for amyloid precursor protein, presinilin-1, and presinilin-2 account for about 5% of all AD cases, and individuals who inherit these mutations usually develop early-onset AD (≤ 65 years old) (Qiu et al., 2009). Thus, the majority of AD cases are considered sporadic and are likely due to a combination of both environmental and genetic factors. To estimate the contribution of genetics and environmental factors to AD, epidemiologists measure the concordance rates of AD in monozygotic (identical) and dizygotic (fraternal) twins (Raiha et al., 1997; Gatz et al., 2005; Ketelaar et al., 2012). High discordance rates for AD in identical twins and large differences in the age at onset for AD even in concordant identical twins (both twins have AD) support the significant role of the environment as well as gene-environment interactions (GXE) in AD (Gatz et al., 2005; Ketelaar et al., 2012). GXE mean individuals have different disease risks associated with an environmental exposure based on differences in genetic susceptibility (Ottman, 1996; Cummings and Kavlock, 2004). For example, factors such as diet and nutrients, occupational exposure to toxicants, educational attainment, and history of

traumatic brain injury are associated with increased AD risk (Qiu et al., 2009). Interestingly, both environmental and occupational exposure to heavy metals is associated with an increased risk of AD and accelerated cognitive decline (Stewart et al., 2002; Weisskopf et al., 2004; Stewart et al., 2006; Bakulski et al., 2012).

1.2 Lead as a model of heavy metal exposure

Lead is a ubiquitous environmental contaminant. While lead is a naturally-occurring heavy metal, most environmental lead contamination is from anthropogenic sources, such as leaded paint and gasoline. For example, the combustion of leaded gas in the U.S. in the 20th century released approximately 4 million metric tons of lead into the environment, and until the 1970s, lead comprised as much as 50% of some leaded paints (Toscano and Guilarte, 2005). Lead is found in the soil near older homes, roadways, industrial sites, landfills, smelters, and hazardous waste sites, as well as in ceramic glazes, lead fishing weights, lead batteries, and ammunition (ATSDR, 2007). Ambient lead levels and median blood lead levels have significantly declined in the U.S. since the phasing out of leaded paint and gasoline (Toscano and Guilarte, 2005). However, over 500,000 U.S. children are still considered at-risk for lead-induced neurological effects by the CDC, and high levels of lead in soil are still a major concern in urbanized and industrial areas and will persist in the soil for decades (ATSDR, 2007). For example, nearly 35% of American homes still have lead-based paint (Cox, 2011), and leaded gasoline and paint are still widely used in developing countries – over

75% of paints sampled from SE Asia exceeded the U.S. EPA limit of 90 ppm lead; 37% had over 10,000 ppm lead (Brosche, 2014).

People are exposed to lead through the ingestion of food or water, breathing air, or swallowing dust that contains lead (Toscano and Guilarte, 2005). The inorganic form of lead is the focus of most human health-related concerns because organic lead is converted to inorganic lead in the human body, combustion of organic lead results in the release and deposition of inorganic lead in the environment, and the lead found in hazardous waste sites is primarily of the inorganic form (ATSDR, 2007). Lead exposure can cause toxicity in multiple tissues and perturb various physiological processes in humans. These toxic effects depend on the level and duration of lead exposure, and include hematological and cardiovascular effects, gastrointestinal distress, and impaired renal function (ATSDR, 2007). However, the nervous system is the primary target for the deleterious effects of lead. Lead is a well-known childhood neurotoxicant, causing persistent cognitive and behavioral deficits (Needleman et al., 1979; ATSDR, 2007). In addition, chronic lead exposure can also lead to impaired neurological function in adults; both animal and epidemiological studies have found an association between low blood lead levels (< 10 μg/dL) and accelerated cognitive decline in older adults (Stewart et al., 2002; Weisskopf et al., 2004; Basha et al., 2005; Stewart et al., 2006; Wu et al., 2008a; Bihaqi et al., 2013). Importantly, the aging population in the U.S. experienced high lead exposure in early life. For example, the cross-sectional U.S. National Health and Nutrition

Examination Survey found that the geometric mean of blood lead levels (BLLs) for all populations declined significantly between 1976-1999 (12.8 to 2.8 μg/dL) and it declined even further (2.3 to 1.6 μg/dL) between the 1992-1994 and the 1999-2002 survey periods (ATSDR, 2007). Among adults, the geometric mean of BLLs was 1.5 and 2.2 μg/dL for adults age 20-59 and > 60, respectively, during the 1999-2002 survey period. While 77.8% of adults had elevated BLLs (≥ 10 μg/dL) in 1976, less than 1% of adults had elevated BLLs in the 1999-2002 survey period (Centers for Disease Control and Prevention, 2005).

Importantly, lead is deposited in and slowly released from the bone, providing a potential mechanism for continued, low-level lead exposure long after the initial exposure. While the half-life of lead in the bone is approximately 36 days (and 40 days in soft tissues), the half-life for lead in bone is 27 years (ATSDR, 2007). Physiological and pathological processes such as pregnancy, menopause, or osteoporosis accelerate lead release from the bone and exacerbate chronic lead exposure. For example, a longitudinal study of a cohort of non-occupationally exposed elderly men found an association between low blood (mean 5.5 μg/dL) and patella lead levels and increased cognitive decline (Payton et al., 1998; Weisskopf et al., 2004). Furthermore, several studies have assessed the effect of early life lead exposure on mRNA and gene expression later in life and found that lead may also exert effects long after the initial lead exposure through epigenetic changes, including altered DNA methyltransferase expression and histone acetylation and methylation (Basha et al., 2005). The

societal, financial, and health care burdens associated with cognitive decline and neurodegenerative diseases are immense. Thus, it is important to study how lead may impair hippocampus-dependent learning and memory, accelerate cognitive decline, and potentially contribute to an increased risk of AD. Interestingly, lead may interact with specific, genetic risk factors, and through these GXE, lead to more severe impairments in learning and memory and/or an earlier onset of cognitive behavior deficits (Stewart et al., 2002).

1.3 Apolipoprotein E4 as a genetic risk factor for Alzheimer's disease and cognitive decline

Apolipoprotein E (ApoE) mediates lipid homeostasis and has three isoforms (E2, E3, and E4), which differ in the cysteine or arginine residues at position 112 and 158 of the amino acid sequence (Mahley and Rall, 2000). In the central nervous system, ApoE is primarily expressed by astrocytes, but it is also expressed under basal conditions by adult neural precursor cells in the subgranular zone of the dentate gyrus of the hippocampus and by neurons under stress conditions (Liu et al., 2013). Importantly, the E4 allele of the ApoE gene (ApoE4) is the strongest genetic risk factor for late-onset AD (Liu et al., 2013). The frequency of AD among ApoE4 homozygotes is 91% with a mean age at onset of 68 years of age, while the frequency of AD among E4 non-carriers is only 20% with a mean age at onset of 84 years of age (Liu et al., 2013). In addition to its effect on AD risk, ApoE4 is also associated with a worse prognosis following traumatic brain injury (Verghese et al., 2011), a higher prevalence of

mild cognitive impairment (Cosentino et al., 2008; Ramakers et al., 2008; Whitehair et al., 2010), and accelerated cognitive decline (Mortensen and Hogh, 2001; Blair et al., 2005; Beydoun et al., 2012).

ApoE does have isoform specific effects on amyloid-β metabolism and may contribute to AD in humans through both amyloid-β dependent and independent mechanisms (Liu et al., 2013). For example, ApoE4 is associated with decreased endosomal recycling and impaired AB clearance across the blood-brain barrier. Furthermore, ApoE4 is more susceptible to proteolysis and the generation of toxic ApoE fragments, and these ApoE4 fragments associate with hallmarks of AD pathology, including neurofibrillary tangles and amyloid-β plaques (Huang, 2011). In humans, ApoE4 co-localizes with senile plaques and ApoE4 carriers experience increased amyloid-β deposition and have more senile plaques (Schmechel et al., 1993; Polvikoski et al., 1995; Kok et al., 2009). Furthermore, primates exposed to lead during development exhibited increased expression of amyloid precursor protein in old age, suggesting that lead and ApoE4 may interact with amyloid and tau pathology (Bihaqi et al., 2014a). Thus, due to the general, negative effects of ApoE4 on cognition, ApoE4 carriers may be more susceptible (*e.g.*, accelerated progression or earlier age at onset) to insults from neurotoxicants such as lead (Cannon and Greenamyre, 2011). However, whether lead and ApoE4 may interact to cause more severe impairments in cognitive behavior has not been established in an experimental model.

1.4 Adult hippocampal neurogenesis

Adult neurogenesis is a process that leads to the generation of functional neurons in the adult brain. Adult neurogenesis in the rodent hippocampus was initially described by Altman and Das (1965) and later in the HVC nucleus of songbirds by Goldman and Nottebohm (1983). However, adult neurogenesis in the mammalian brain was not widely accepted until much later (1990s) with the characterization of multipotent cells in the rodent hippocampus by Fred Gage and Theo Palmer (Gage et al., 1995; Palmer et al., 1995; Palmer et al., 1997; Gage et al., 1998; Palmer et al., 1999; Palmer et al., 2000) and the first report of adult hippocampal neurogenesis in humans published by Peter Eriksson (Eriksson et al., 1998).

There are two well-characterized neurogenic regions in the adult mammalian brain. One region is the subventricular zone (SVZ) of the lateral ventricles and the other neurogenic region is in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Adult neurogenesis in the hippocampus proceeds through a series of four primary stages, starting with the proliferation of adult neural stem and progenitor cells in the SGZ, followed by the neuronal fate determination of daughter cells, these daughter cells then exit the cell cycle and migrate as neuroblasts into the granule cell layer, and the final step is the maturation and functional integration of adult-born granule cells into existing neuronal circuits (Kempermann, 2011).

Importantly, newborn neurons contribute to synaptic plasticity within the DG. Adult-born granule cells exhibit increased excitability and plasticity, and reduced inhibition from GABAergic interneurons, compared to the mature, existing neurons in the DG (Snyder et al., 2001; Esposito et al., 2005; Marin-Burgin and Schinder, 2012). Importantly, the increased responsiveness and excitability of newborn granule cells may give these adult-born cells a specific and significant role in learning and memory (Deng et al., 2010). In rats, a greater proportion of young, adult-born neurons are activated during spatial exploration compared to existing granule cells (Ramirez-Amaya et al., 2006; Sandoval et al., 2011). Similarly, the selective loss of immature neurons impaired long-term spatial memory retention and contextual fear extinction (Deng et al., 2009).

The survival and integration of adult-born neurons is activity-dependent and occurs during a critical window, at approximately three weeks after neuronal birth (Tashiro et al., 2006; Marin-Burgin and Schinder, 2012). Several different cell types and inputs facilitate the survival and maturation of adult-born cells during this window. One such input is the GABAergic interneurons, which facilitate the survival and maturation of adult-born cells during this window. For newborn granule cells, GABAergic synapses develop earlier than glutamaterigc synapses, and the tonic activation by GABA may exert trophic effects on adult-born immature neuron survival and maturation (Tozuka et al., 2005; Ge et al., 2006; Kim et al., 2012). Another input is glutamate signaling via the *N*-methyl-D-

aspartate-type (NMDA) receptor. The absence of functional NMDA receptors at 3-7 weeks after the birth of adult-born neurons is associated with reduced adult-born neuron survival, thus, the survival and synaptogenesis of adult-born neurons during this window may be dependent on synaptic NMDA receptor activity (Tashiro et al., 2006).

Notably, humanized transgenic knock-in mice of the three ApoE alleles have been developed and express the human E2, E3, or E4 allele at physiological levels under control of the endogenous mouse ApoE promoter (Xu et al., 1996; Sullivan et al., 1997; Sullivan et al., 1998; Bour et al., 2008). Studies using the ApoE3-KI and ApoE4-KI mouse models have found that ApoE4 impairs the survival and maturation of adult-born granule cells and is associated with impaired dendritic development and differentiation of adult-born neurons compared to ApoE3-KI mice (Li et al., 2009). Li and colleagues hypothesize that these effects of ApoE4 on adult hippocampal neurogenesis are non-cell autonomous and are due to the increased generation of toxic ApoE4 fragments which subsequently impair the survival of GABAergic interneurons and delay the maturation of adult-born granule cells (Li et al., 2009) Furthermore, ApoE4-KI mice exhibit age- and sex-dependent deficits in hippocampus-dependent learning and memory (Andrews-Zwilling et al., 2010; Leung et al., 2012). In addition, studies on lead neurotoxicity in rats have reported that developmental lead exposure is associated with impaired adult-born neuron proliferation, survival, and maturation (Gilbert et al., 2005; Jaako-Movits et al., 2005; Verina et al.,

2007). Thus, one plausible mechanism through which a GXE between lead and ApoE4 may contribute to deficits in cognitive behavior and accelerate cognitive decline is through perturbation of adult hippocampal neurogenesis.

1.5 Sex differences in Alzheimer's disease and adult hippocampal neurogenesis

Epidemiological reports suggest that women have an increased risk of AD, experience more rapid decline in cognition following an AD diagnosis, and that women with mild cognitive impairment (an intermediate stage between normal, aging-related cognitive decline and AD) experience increased cognitive decline relative to males (Bour et al., 2008]{Andrews-Zwilling, 2010 #5889]{Leung, 2012 #5890). Importantly, while the prevalence of AD is higher among females, there is no difference in the incidence of AD between males and females (Mielke et al., 2014). This may be due to the fact that women, on average, live longer than men (Mielke et al., 2014). However, there is no systematic study of preclinical cognitive decline in the literature, so it is still unclear whether there are sex differences in either normal (aging-related) or pathological cognitive decline (Bretsky et al., 1999; Fleisher et al., 2005). Interestingly, there are also known sex differences in adult hippocampal neurogenesis (Barha et al., 2011; Roughton et al., 2012; Hillerer et al., 2013) and hippocampus-dependent spatial learning and memory (Maren et al., 1994; Parsons et al., 2004; Postma et al., 2004; Woolley et al., 2010). For example, females are thought to have slightly higher

rates of adult-born cell proliferation (depending on the estrous cycle) (Tanapat et al., 1999; Duarte-Guterman et al., 2015) while males are thought to have increased number of immature neurons (Hillerer et al., 2013). Thus, given the potential role of sex differences on AD risk, learning and memory, and adult hippocampal neurogenesis, we thought it was important to include both males and females in our *in vivo* studies in order to determine whether there are sex differences in susceptibility to a GXE between lead and ApoE4.

1.6 Knowledge gaps and goals of dissertation research

Importantly, everyone experiences some degree of cognitive changes as they age. However, some people have a steeper decline in cognitive function, and still others may develop mild cognitive impairment or dementia and AD. Thus, there is a large range of functional cognitive ability between physiological vs pathological cognitive decline. It has been hypothesized that genetic risk factors like ApoE4 may interact with different non-genetic risk factors to increase AD risk and contribute to cognitive decline. However, this is still a hypothesis and there is no direct evidence for gene-environment interactions on AD risk. Thus, one of the overarching goals of my project was to use an experimental model to determine whether and how environmental exposures and/or gene-environment interactions may facilitate or accelerate cognitive decline and potentially increase AD risk. Thus, the specific aims for this dissertation were to: **(1) Determine whether lead impairs adult neurogenesis *in vitro,* (2) determine if sub-chronic, adult-only lead exposure impairs adult hippocampal neurogenesis**

in vivo **and induces cognitive behavior deficits, and (3) determine if there is a GXE between ApoE4 and lead exposure on adult hippocampal neurogenesis and cognitive behavior.**

Chapter 2: Lead impairs adult neurogenesis in vitro

2.1 Abstract

Adult hippocampal neurogenesis is the process whereby adult neural precursor cells (aNPCs) in the subgranular zone (SGZ) of the dentate gyrus (DG) generate adult-born, functional neurons in the hippocampus. This process is modulated by various extracellular and intracellular stimuli, and the adult-born neurons have been implicated in hippocampus-dependent learning and memory. However, studies on how neurotoxic agents affect this process and the underlying mechanisms are limited. The goal of this study was to determine whether lead, a heavy metal, directly impairs critical processes in adult neurogenesis and to characterize the underlying signaling pathways using primary cultured SGZ-aNPCs isolated from adult mice. We report here that lead significantly increases apoptosis and inhibits proliferation in SGZ-aNPCs. In addition, lead significantly impairs spontaneous neuronal differentiation and maturation. Furthermore, we found that activation of the c-Jun NH_2-terminal kinase (JNK) and p38 mitogen activated protein (MAP) kinase signaling pathways are important for lead cytotoxicity. Our data suggest that lead can directly act on adult neural stem cells and impair critical processes in adult hippocampal neurogenesis, which may contribute to its neurotoxicity and adverse effects on cognition in adults.

2.2 Introduction

Adult hippocampal neurogenesis is the process whereby adult neural precursor cells (aNPCs) in the subgranular zone (SGZ) of the dentate gyrus (DG) lead to the generation and functional integration of adult-born neurons in the hippocampus (Ming and Song, 2011b). These adult-born neurons can influence certain forms of hippocampus-dependent learning and memory formation (Clelland et al., 2009; Deng et al., 2009; Garthe et al., 2009; Pan et al., 2012d; Pan et al., 2012b; Wang et al., 2014). Importantly, the various stages and cell types involved in adult hippocampal neurogenesis can be modulated by various physiological and pathological factors, including other cell types in the neurogenic niche, growth factors, cytokines, neurotrophins, and processes such as mating, aging, stress, and exercise (Ming and Song, 2011; Pan *et al.,* 2012a,b; Pan *et al*., 2013b; Wang *et al*., 2014). However, the effects of neurotoxicant exposure on adult hippocampal neurogenesis have not been studied extensively.

The heavy metal lead is a ubiquitous environmental contaminant and a major public health concern. The combustion of leaded gas in the U.S. in the 20th century released approximately 4 million metric tons of lead into the environment (Toscano and Guilarte, 2005). The phasing out of leaded paint and gasoline has contributed to a significant decline in ambient lead levels as well as mean blood lead levels (12.8 to 1.6 μg/dL from 1976 to 2002) in the U.S. population (Toscano and Guilarte, 2005; ATSDR, 2007). However, lead can persist in the soil for decades and no level of lead is considered safe (White et al., 2007). In addition to its well-characterized developmental neurotoxicity, cumulative lead exposure

can also cause neurological impairment in adults (van Wijngaarden et al., 2009). Monkeys and rats exposed to low concentrations of lead have increased cognitive decline and AD-associated neuropathology later in life (Basha et al., 2005; Wu et al., 2008a; Bihaqi et al., 2013; Grossman, 2014). Furthermore, longitudinal studies from a cohort of non-occupationally exposed elderly men found an association between relatively low blood (mean 5.5 μg/dL) and/or patella lead levels and increased cognitive decline (Payton et al., 1998; Weisskopf et al., 2004). These blood lead levels are comparable to background blood lead levels in the adult U.S. population (1.5 and 2.2 μg/dL among 20-59 and ≥ 60 year-olds, respectively) (ATSDR, 2007). Thus, lead may contribute to increased or accelerated cognitive decline at environmentally relevant exposure levels.

Lead may facilitate and accelerate cognitive decline through impaired adult hippocampal neurogenesis. Several studies have examined the effect of early life lead exposure on adult hippocampal neurogenesis and found that developmental lead exposure is associated with altered proliferation, survival, and dendritic morphology of adult-born neurons in the hippocampus and altered hippocampus learning and memory in rats (Verina et al., 2007) (Jaako-Movits et al., 2005). However, results have not been entirely consistent among various studies. Gilbert *et al*. (2005) found that lead-treated rats have reduced adult-born cell ($BrdU^+$) survival but no change in cell proliferation, while a similar study reported that lead decreases adult-born cell survival as well as proliferation (Verina et al., 2007). Furthermore, Jaako-Movits *et al*. (2005) found that lead

impaired adult-born cell proliferation and neuronal maturation, while Verina *et al.* found no effect of lead on neuronal differentiation (Verina et al., 2007). Although these studies are very interesting, the inconsistent results warrant further investigation. Moreover, the early life exposure paradigms used in these *in vivo* studies may have introduced potential confounding factors due to the adverse effects of lead during development (Gilbert et al., 2005; Jaako-Movits et al., 2005; Verina et al., 2007). Only one study to date has assessed the effect of postnatal lead exposure alone on adult neurogenesis (Schneider et al., 2005). Schneider *et al*. (2005) exposed male rats to 1500 ppm lead acetate for 30-35 days starting at postnatal day 25 and found that lead impaired adult-born cell proliferation in the SGZ. However, they did not assess the effect of lead on other stages of adult neurogenesis or cognitive behavior. Thus, additional research is needed to determine whether adult-only lead exposure is sufficient to impair adult hippocampal neurogenesis and to characterize the signaling mechanisms underlying lead-induced impairment in adult neurogenesis. In this study, we used primary cultured aNPCs isolated from the hippocampus (SGZ-aNPCs) of adult mice as an *in vitro* model system to test the hypothesis that lead exposure impairs adult hippocampal neurogenesis and to elucidate the underlying signaling mechanisms.

2.3 Materials and Methods

Reagents. The preparation, use, and disposal of hazardous agents were carried out according to the Environmental Health and Safety Office at the University of

Washington. Lead (II) acetate trihydrate (Cat. 316512, Sigma-Aldrich, St. Louis, MO) was dissolved in deionized distilled water (H_2O) to make a 5 mM stock solution and stored at -20°C. Z-VAD-FMK (Cat. FMK001, R&D Systems, Minneapolis, MN) was dissolved in dimethyl sulfoxide (DMSO) to make a 20 mM stock and used according the manufacturer's specifications. The p38 (Cat. SB2021990, EMD Millipore Calbiochem, Billerica, MA) and JNK (Cat. SP600125, EMD Millipore Calbiochem) inhibitors were dissolved in DMSO to yield 3 mM stock solutions and stored at -20°C. 5-bromo-2'-deoxyuridine (BrdU) was from Sigma (Cat. B9285) and stored as a 65 mM stock solution. The primary antibodies and dilutions used in immunocytochemistry were rat anti-BrdU (1:500, Bio-Rad Laboratories AbD Serotec, Raleigh, NC), mouse anti-βIII-tubulin (1:500, Promega, Madison, WI), and mouse anti-SOX2 (1:500, R&D Systems). Goat anti-rat and goat anti-mouse Alexa Fluor-conjugated secondary antibodies as well as Hoechst 33342 were from Invitrogen (Carlsbad, CA). For Western Blot analysis, the following rabbit primary antibodies from Cell Signaling (Beverly, MA) were used at a 1:1000 dilution unless otherwise specified: monoclonal anti-phospho-Akt (Cat. 4060, 1:2000), polyclonal anti-phospho-p38 (Cat. 9211), monoclonal anti-phospho JNK (Cat. 4668), monoclonal anti-JNK (Cat. 9258), polyclonal anti-phospho-c-Jun (Cat. 9164), and monoclonal anti-GAPDH (Cat. 2118). Horseradish peroxidase-conjugated secondary antibodies were from EMD Millipore (Billerica, MA). All of the primary and secondary antibodies were diluted into the appropriate blocking buffer.

Cell culture. The University of Washington Institutional Animal Care and Use Committee approved all experimental procedures. The primary aNPCs were prepared as previously described (Guo et al., 2012; Pan et al., 2013) from the SGZ of the DG from 6-7 week-old male C57BL/6J mice (Taconic, Hudson, NY). The solutions and media used during the aNPC isolation were filter sterilized. Briefly, the whole brain from four adult male mice was harvested and placed in HBSS (Invitrogen). Each brain was then sliced into 1 mm sections using an adult mouse brain matrix (Kent Scientific, Torrington, CT), and then the SGZ was isolated from these sections via microdissection under a dissection microscope. The SGZ tissue was placed in Solution A (30 mM Glucose, 26 mM $NaCO_3$, 2mM HEPES pH 7.4 (Invitrogen) in HBSS (Invitrogen)) and spun down for 10 min at 1,000 rpm. The pelleted tissue was then resuspended and a combination of mechanical and enzymatic digestion (MACS Neural Tissue Dissociation Kit, Miltenyi Biotec, San Diego, CA) was used to dissociate the tissue. To stop the digestion, DMEM/F-12 medium (Invitrogen) with 10% Fetal Bovine Serum (FBS, Invitrogen) was added and the SGZ tissue was then filtered through a cell strainer (70 μm cell strainer, Fisher Scientific, Waltham, MA) and spun down for 3 min at 1,000 rpm. The pellet was washed once with DMEM/F-12 medium with 10% FBS and once with DMEM/F-12 medium with 10% FBS plus Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) solution (1:10 Percoll in PBS) followed by spins at 1,000 rpm for 3 and 15 min, respectively. The pellet was washed once with Solution A and once with initial proliferation medium (Neurobasal medium (Invitrogen); 1X B27 supplement without Vitamin A (Invitrogen); 2mM L-

Glutamine (Invitrogen); 100 U/ml penicillin/streptomycin (Invitrogen), 20 ng/ml of epidermal growth factor (EGF; EMD Chemicals) and 10 ng/ml basic fibroblast growth factor (bFGF; EMD Chemicals) followed by 5 min spins at 1,500 rpm. The cells were then plated in a petri dish with initial proliferation medium and cultured at 37°C and 6.5% CO2. Growth factors (EGF and bFGF) were refreshed every 3-4 d unless noted otherwise. Primary neurospheres formed after 7-14 d, at which time, the neurospheres were collected, enzymatically and mechanically dissociated, and then resuspended in growth media (Advanced DMEM/F-12, 1X N2 Supplement (Invitrogen), 1X B27 Supplement, 100 U/ml Penicillin/streptomycin, 2 mM L-Glutamine, 2 µg/ml Heparin sodium salt (Sigma), 20 ng/ml EGF, and 10 ng/ml bFGF). The neurospheres were maintained in petri dishes in the growth media and passaged ≤ 10 times.

Drug treatment. For experiments, the neurospheres were dissociated (0.125% trypsin-EDTA (Invitrogen) for 5 min; 0.014% soybean trypsin inhibitor (Sigma) for 5 min) and seeded as a monolayer culture on poly-L-ornithine- (15 µg/ml) and fibronectin (1 mg/ml) (BD Biosciences, San Jose, CA)-coated ACLAR coverslips or culture plates. To assess cell number, proliferation, and apoptosis, cells were seeded at a cell density of 5 X 10^3 cells per well (48-well plate) and allowed to attach overnight. The next day, the media was changed (Advanced DMEM/F-12, 1X N2 Supplement, Penicillin/streptomycin, 2 mM L-Glutamine, 2 µg/ml Heparin sodium salt, 20 ng/ml EGF, and 10 ng/ml bFGF) and the cells were treated with agents for 0-48 h as described in the figures and figure legends. For a given

experiment, each agent was administered once (not replenished). Lead was dissolved in H_2O while the p38 and JNK inhibitors were dissolved in DMSO, so either an equal volume of H_2O or an equal concentration of DMSO was used as a vehicle control. To assess cell proliferation, BrdU was added to each well (final concentration of 10 μM) for the last 2 h of the experiment.

To assess spontaneous differentiation, the cells were seeded at a cell density of 5 X 10^3 cells per well (48-well plate) overnight. The next day, the media was replaced with EGF/bFGF-free growth media supplemented with 1 mg/ml bovine serum albumin (BSA) (Equitech Bio, Kerrville, TX), and then the cells were treated with lead or vehicle control and cultured for 5 d (each agent was administered once).

Immunocytochemistry. For immunocytochemistry, the cells were fixed by removing half of the media from each well and replacing it with an equal volume of 8% paraformaldehyde (PFA)/8% sucrose in PBS for 30 min at room temperature (RT). The fixed cells were washed for 3X5 min in PBS, permeabilized by 1X5 min in 1% SDS in PBS, and washed 3X5 min in PBS. The cells were blocked with 5% BSA in PBST (0.1% Triton-X 100 in PBS) for 30 min at RT, and then incubated with primary antibodies at 4°C overnight. For BrdU staining, the cells underwent additional processing prior to blocking: 5 min in H_2O at RT, 10 min in 1 N HCl at 4°C, 30 min in 2 N HCl at 37°C, 2X15 min in 0.1 M borate buffer (pH 8.5). Following incubation with primary antibodies, the cells were washed 3X10 min with PBST and then incubated with secondary antibodies

for 2 h at RT. The cells were washed 3X10 min with PBST, incubated with 2.5 µg/ml Hoechst 33342 for 30 min, washed 1X with PBST, and then mounted onto slides using anti-fade Aqua Poly/Mount (Polysciences) solution.

Imaging and quantification of immunostained cells. All images were captured using a fluorescence microscope (Zeiss) equipped with a camera with a 10x or 20x objective (Zeiss). Images were uniformly adjusted for color, brightness, and contrast with Adobe Photoshop CS4 (Adobe Systems Inc.). A cell with nuclear condensation or fragmentation was scored as apoptotic. A cell was scored as marker$^+$ if the cell had a uniformly stained Hoechst$^+$ nucleus as well as marker expression in the nucleus (BrdU) or cell body and neurites (βIII-tubulin$^+$ neurites ≥ length of soma). All quantification was conducted by an experimenter blinded to treatment. At least 250 cells per coverslip per treatment were quantified from at least 10 randomly selected fields on each coverslip.

Western Blot analysis. The cells were seeded at 2 X 10^5 cells per well in poly-L-ornithine and fibronectin-coated 12-well plates for 24 h. Cells were then treated as described in the figure legends and then washed with ice-cold PBS followed by Triton-X cell lysis buffer with protease inhibitors. The cell lysates were clarified by centrifugation and stored at -80°C. The protein concentration was measured using the BCA protein assay (Thermo Scientific, Waltham, MA). Samples containing 5 µg protein were separated by gel electrophoresis on a 12.5% SDS-PAGE gel and transferred to a PVDF membrane (EMD Millipore). Following

antibody incubation, the protein of interest was detected with ECL Prime (GE Healthcare Life Sciences) using a ChemiDoc XRS Imaging System (BioRad). ImageJ (NIH) was used for the densitometry analysis and determination of fold induction normalized to a loading control (total protein or GAPDH).

Statistical Analysis. Statistical analyses were conducted using GraphPad Prism software (version 5.0c for Mac, GraphPad Software Inc., San Diego, CA, USA). All of the immunocytochemical data are from at least two independent experiments with triplicates or quadruplicates for each data point ($n \geq 2$ independent experiments for each data point). The Western blot data are representative data from two independent experiments with duplicates. For dose-response experiments, a Student's *t* test with two-tailed analysis ($\alpha = 0.05$) was used for pair-wise comparison of the means. One-way ANOVA with a Bonferroni *post-hoc* analysis ($\alpha = 0.05$) was used to analyze all of the drug treatment data (Z-VAD-FMK, MAPK inhibitors). Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.4 Results

SGZ-aNPCs are maintained as neurospheres in vitro and retain their stem cell characteristics

After isolation from the dentate gyrus of the hippocampus of adult C57Bl/6J male mice, the SGZ-aNPCs are maintained as neurospheres in culture (Fig. 2.1A). Upon dissociation and seeding as a monolayer in growth media, at

least 98.5% of cells continue to express the stem cell marker, SOX2 (Fig. 2.1B), after more than six passages. Thus, these SGZ-aNPCs are an appropriate *in vitro* model for studying the effects of lead on the stem/progenitor pool in the hippocampus because these cells proliferate and retain their stem cell characteristics after multiple passages.

Lead significantly decreases the total cell number and increases apoptosis in SGZ–aNPCs To determine whether lead is cytotoxic and induces apoptosis in aNPCs, cells were treated with 0 to 2 μM lead for 48 h. Lead significantly decreased the total cell number in a dose-dependent manner (Fig. 2.2A-D) starting at 0.1 μM. Furthermore, lead significantly increased the percent of apoptotic cells, quantified by nuclear condensation and/or fragmentation (Fig. 2.2E), beginning at 0.1 μM, the lowest concentration tested. A 2 h pretreatment with 5 μM Z-VAD-FMK, a pan-caspase inhibitor, almost completely blocked lead (0.5 μM)-induced cell loss (Fig. 2.2F) and apoptosis (Fig. 2.2G). These data suggest that lead is toxic to SGZ-aNPCs and induces caspase-dependent apoptosis in these cells.

Lead decreases proliferation in SGZ–aNPCs

To determine whether lead decreases aNPC cell proliferation, we pulsed the cells with BrdU during the final 2 h of a 48 h lead treatment. BrdU is a thymidine analog and it is incorporated into the DNA of actively replicating cells during S phase of the cell cycle. Starting at 0.5 μM lead, we observed a

significant decrease in the number of $BrdU^+$ cells after a 48 h-treatment (Fig. 2.3). Combined with data shown in Fig. 2, these results suggest that lead inhibits cell proliferation of aNPCs at higher concentrations. However, at lower concentrations (< 0.5 μM), lead causes cell loss primarily through apoptosis.

Lead decreases spontaneous neuronal differentiation and maturation of SGZ-aNPCs

We also examined whether lead disrupts neuronal differentiation and maturation, another critical step in adult neurogenesis. SGZ-aNPCs were seeded overnight, and then the cells were cultured in EGF/bFGF-free growth media containing vehicle or lead for 5 d to allow spontaneous neuronal differentiation in the absence of mitogens. We assessed neuronal differentiation by immunostaining for βIII-tubulin (also known as Tuj-1), a marker of immature neurons. Treatment with low concentrations (≥ 0.1 μM) of lead significantly decreased the percent βIII-tubulin$^+$ cells (βIII-tubulin$^+$ soma and neurites ≥ length of soma) (Fig. 2.4A-G).

To determine whether lead may also impair neuronal maturation, we assessed the effect of lead on neurite morphology and complexity. Using the ImageJ Simple Neurite Tracer plug-in, we traced all of the neurites from 12-16 randomly chosen fields (20X magnification) per treatment and calculated the average neurite length and average number of branching points per βIII-tubulin$^+$ cell. Lead significantly decreased the mean neurite length (Fig. 2.5A-E) and the mean number of branching points (Fig. 2.5A-D, F) per βIII-tubulin$^+$ cell. These

data suggest that lead impairs neuronal differentiation and maturation of SGZ-aNPCs.

Lead inhibits activation of the Akt signaling pathway

To begin to elucidate the molecular mechanisms underlying lead toxicity, we determined if lead inhibits antiapoptotic signaling pathways, such as the Akt pathway. Akt plays a critical role in regulating cell survival and growth, and Akt activation can prevent stress-induced apoptosis (Song et al., 2005). Lead significantly decreased Akt phosphorylation starting at 4 h (Fig. 2.6), indicative of reduced Akt activation. Thus, lead-induced cell death may involve inhibition of the prosurvival Akt pathway.

Activation of the JNK signaling pathway contributes to lead-induced cytotoxicity

The c-*Jun* NH_2-terminal kinase (JNK) is a member of the mitogen activated protein kinase (MAPK) family, and it couples various external stimuli, such as stress and growth factors, to a variety of biological responses, including apoptosis and cell proliferation, respectively (Davis, 2000; Dhanasekaran and Reddy, 2008). Thus, to begin elucidating signaling mechanisms underlying lead-induced apoptosis, we first determined the effect of lead on JNK and c-Jun phosphorylation. SGZ–aNPCs were treated with 2 μM lead for 0-12 h, and the cell lysates were subjected to Western blot analysis. Treatment with lead for 8 h or longer significantly increased JNK phosphorylation, indicative of JNK

activation (Fig. 2.7A, B). Furthermore, lead treatment for 12 h caused a significant increase in the phosphorylation of c-Jun, the prototypical JNK substrate (Fig. 2.7C, D). To determine whether inhibition of JNK is sufficient to attenuate the effect of lead on cell number and apoptosis, SGZ–aNPCs were pretreated with 0.5 μM of the ATP-competitive, pan-JNK inhibitor SP600125 for 1 h and then treated with 0.5 μM lead for an additional 48 h. Pretreatment with the JNK inhibitor without lead co-treatment had no effect on total cell number (Fig. 2.7E) or the number of $BrdU^+$ cells (data not shown), suggesting that the endogenous basal JNK activity does not play a major role in cell proliferation under normal proliferation conditions in the presence of growth factors. However, pretreatment with the JNK inhibitor almost completely reversed lead-induced cell loss and apoptosis (Fig. 2.7E, F). These data suggest that activation of the JNK signaling pathway contributes to lead-induced apoptosis.

Activation of the p38 MAP kinase signaling pathway is also important for lead cytotoxicity

p38 is another member of the stress activated MAP kinases (Kyriakis and Avruch, 2012). Lead significantly increased p38 phosphorylation [6.25 ± 0.14 (SEM)-fold increase] after treatment for 8 h (Fig. 2.8A, B), indicative of p38 activation. Pretreatment with 0.5 μM SB2021990, a p38 inhibitor, had no effect by itself on total cell number (Fig. 2.8C), apoptosis (Fig. 2.8D), or the number of $BrdU^+$ cells (data not shown), but completely blocked the adverse effect of lead

on cell number and apoptosis. Together, the data in Fig. 7 and Fig. 8 suggest that activation of both JNK and p38 are critical for lead-induced apoptosis.

2.5 Discussion

Various extracellular and intracellular stimuli have been shown to modulate the survival, proliferation, and differentiation of adult-born cells in the hippocampus (Clelland et al., 2009; Deng et al., 2009; Garthe et al., 2009; Ming and Song, 2011b; Pan et al., 2013; Wang et al., 2013b). However, the effects of toxicants on adult neurogenesis are not well understood. A recent study from our lab found that a hydroxylated metabolite of PBDE-47, a brominated flame retardant, interferes with the differentiation, proliferation, and survival of primary cultured aNPCs isolated from the subventricular zone of the lateral ventricle (Li et al., 2013). In addition, several studies have examined the effect of developmental lead exposure on adult hippocampal neurogenesis, although the results have been inconsistent (Gilbert *et al.,* 2005; Jaako-Movits *et al*., 2005; Verina *et al*., 2007). In addition, their use of early life exposure paradigms makes it difficult to conclude that these observations are not due to the cumulative effects of lead on both developmental and adult neurogenesis (Gilbert *et al.,* 2005; Jaako-Movits *et al*., 2005; Verina *et al*., 2007).Thus, additional studies are needed to determine whether adult-only lead exposure is sufficient to impair adult hippocampal neurogenesis and learning and memory. Most importantly, the signaling mechanisms underlying lead-induced impairment in adult neurogenesis have not been investigated. Thus, the goal of this study was to determine

whether lead directly impairs critical processes in adult neurogenesis using primary cultured SGZ-aNPCs as a model system and to characterize the underlying signaling pathways.

Our data demonstrate that lead impairs several key processes in adult neurogenesis. Under proliferation conditions, lead significantly reduced aNPC total cell number and increased apoptosis at concentrations as low as 0.1 μM, the lowest tested concentration. It also significantly decreased BrdU incorporation, a measure of cell proliferation, starting at 0.5 μM. Under differentiation conditions when cells were cultured in media lacking the mitogenic growth factors EGF/bFGF, lead significantly impaired spontaneous neuronal differentiation and neuronal maturation, starting at 0.1 μM. These data are consistent with the notion that adult lead exposure, alone, may be sufficient to directly impair multiple aspects of adult neurogenesis (Schneider et al., 2005), with apoptosis and neuronal differentiation being most sensitive.

The underlying mechanisms for the effects of lead on adult neurogenesis have not been well characterized. JNK and p38 are stress-activated MAP kinases (Barger et al., 1993; Davis, 2000; Dhanasekaran and Reddy, 2008), and their activation has been implicated in toxicant-induced neuronal apoptosis (Xia et al., 1995; Newhouse et al., 2004; Giordano et al., 2007; Klintworth et al., 2007; Choi et al., 2010). JNK and p38 are also activated by growth factors and mediate cell proliferation (Davis, 2000; Kyriakis and Avruch, 2012). Here we show that pharmacological inhibition of JNK or p38 had no effect on total cell number or BrdU incorporation, suggesting that JNK or p38 do not play a major role in the

cell proliferation of cultured SGZ-aNPCs. However, lead increases JNK and p38 phosphorylation as well as phosphorylation of the transcription factor c-Jun, a downstream target of JNK. These results are consistent with the findings from another study in which acute treatment with 1-10 μM lead acetate increased p38 phosphorylation in a human derived cell line (SH-SY5Y cells), although cell survival and apoptosis were not assessed (Leal et al., 2002). Furthermore, in our study, we found that pharmacological inhibition of either JNK or p38 was sufficient to prevent lead-induced cell loss and apoptosis. Thus, lead-induced cell loss and apoptosis may require activation of both JNK and p38 MAP kinase signal transduction pathways.

Akt is important for the regulation of cell growth and survival, and Akt activation can prevent stress-induced apoptosis (Kim et al., 2001; Tobiume et al., 2001; Yoon et al., 2002; Song et al., 2005). We found that lead treatment significantly decreased Akt phosphorylation starting at 4 h. Interestingly, Akt phosphorylates and negatively regulates the MAPKKK apoptosis signal-regulating kinase 1 (Ask1) (Tobiume et al., 2001). Inhibition of Akt results in the dissociation of Ask1 and the subsequent activation of downstream targets, including p38 and JNK (Yoon et al., 2002). Thus, lead-induced cell death may also be mediated through the inhibition of the pro-survival Akt pathway. Endoplasmic reticulum (ER) stress, oxidative stress, and disrupted calcium homeostasis have also been implicated in lead-induced neurotoxicity and other target organ toxicities (Toscano and Guilarte, 2005; White et al., 2007; Baranowska-Bosiacka et al., 2013; Liu et al., 2013; Akande et al., 2014). For

example, lead may bind to sulfhydryl groups, leading to a reduction in cellular antioxidant capacity through the depletion of the cellular thiol status and inhibition of antioxidant enzymes, inducing oxidative stress (Ercal et al., 2001). Increased oxidative stress is associated with reduced cell viability and cell proliferation in embryonic and adult neural progenitor cells (Sava et al., 2007; Choi et al., 2014). In our study, we found that lead increased JNK and p38 activation in SGZ-aNPCs. Importantly, both of these MAP kinases can be activated by oxidative stress (Davis, 2000), and JNK may be activated via the IRE1/JNK pathway in response to lead-induced ER stress (Qian et al., 2001; Qian and Tiffany-Castiglioni, 2003). Thus, additional research is needed to determine whether oxidative and ER stress may underlie lead toxicity in SGZ-aNPCs.

Both animal and epidemiological studies have found an association between low blood lead levels (~ 10 μg/dL) and increased cognitive decline in older adults (Stewart et al., 2002; Weisskopf et al., 2004; Stewart et al., 2006; Wu et al., 2008a). Although we cannot directly compare the lead concentrations we used to human blood lead levels, we observed lead toxicity in SGZ-aNPCs at 0.1 μM lead, which is equivalent to 2.05 μg lead/dL media. Thus, lead may exert toxic effects on adult neurogenesis under environmentally relevant exposure conditions.

In summary, using an *in vitro* model system, we provide evidence that lead significantly impairs aNPC survival, proliferation, and differentiation in a dose-dependent manner. In addition, we show that the activation of proapoptotic JNK and p38 MAP kinase and inhibition of the prosurvival Akt signaling may

mediate lead toxicity. Because adult hippocampal neurogenesis plays an important role for hippocampus-dependent learning and memory, impairment in adult neurogenesis may underlie cognitive decline in adults exposed to lead.

2.6 Figures

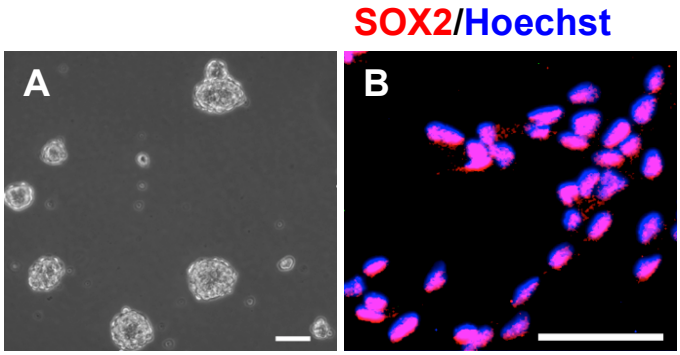

Figure 2.1. The SGZ-aNPCs are maintained as neurospheres and retain their stem cell characteristics, *in vitro*. *A,* SGZ-aNPCs continue to proliferate as neurospheres after six passages. ***B,*** SGZ-aNPCs continue to express SOX2 (red), a stem cell marker, when dissociated and seeded as a monolayer in proliferative conditions. Scale bars, 50 μm.

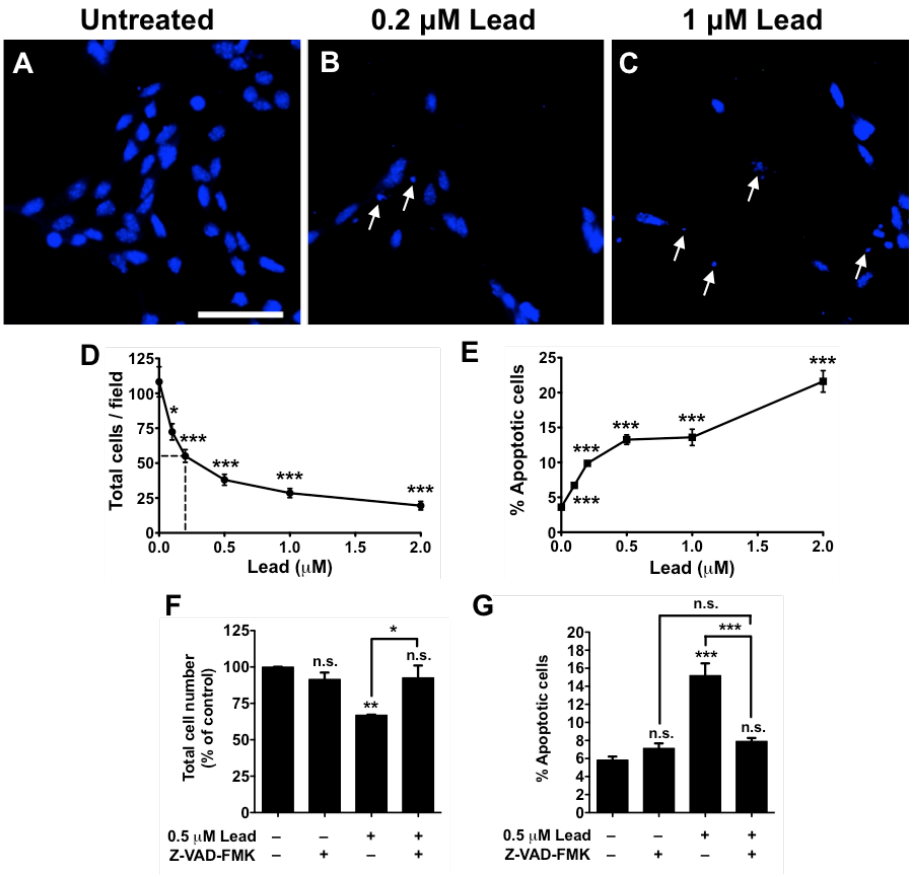

Figure 2.2. Lead significantly decreases the total cell number and increases apoptosis in SGZ–aNPCs. *A-E*, SGZ-aNPCs were treated with 0, 0.1, 0.2, 0.5, 1, or 2 µM lead for 48 h. Representative Hoechst nuclei staining from (*A*) untreated, (*B*) 0.2 µM, and (*C*) 1 µM lead-treated SGZ-aNPCs. Quantification of (*D*) the total cell number and (*E*) the percent apoptotic cells. ***F-G*,** SGZ-aNPCs were pretreated with 5 µM Z-VAD-FMK, a pan-caspase inhibitor, for 2 h followed by 0.5 µM lead for 48 h and (*F*) the total cell number and (*G*) the percent apoptotic cells were quantified. Arrows: nuclear condensation and/or fragmentation. Hoechst: nuclei staining. Scale bar: 50 µm. *n* = 2-3 independent experiments for a total of 4-8 coverslips per data point. Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

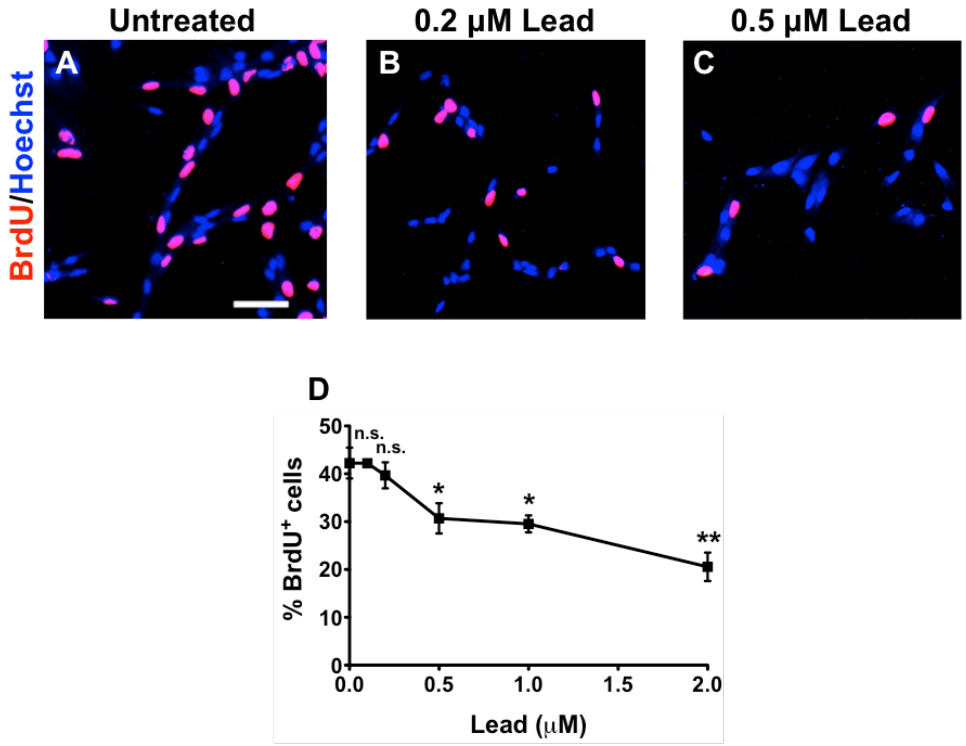

Figure 2.3. Lead decreases proliferation in SGZ–aNPCs. *A-D*, SGZ-aNPCs were treated with 0, 0.1, 0.2, 0.5, 1, or 2 µM lead for 48 h. Representative BrdU and Hoechst co-staining from (*A*) untreated, (*B*) 0.2 µM, and (*C*) 0.5 µM lead-treated SGZ-aNPCs, and quantification of (*D*) the percent $BrdU^+$ cells. BrdU: a marker for cells in S phase of the cell cycle. Scale bars: 50 µm. $n = 2$ independent experiments for a total of 8 coverslips per data point. Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$.

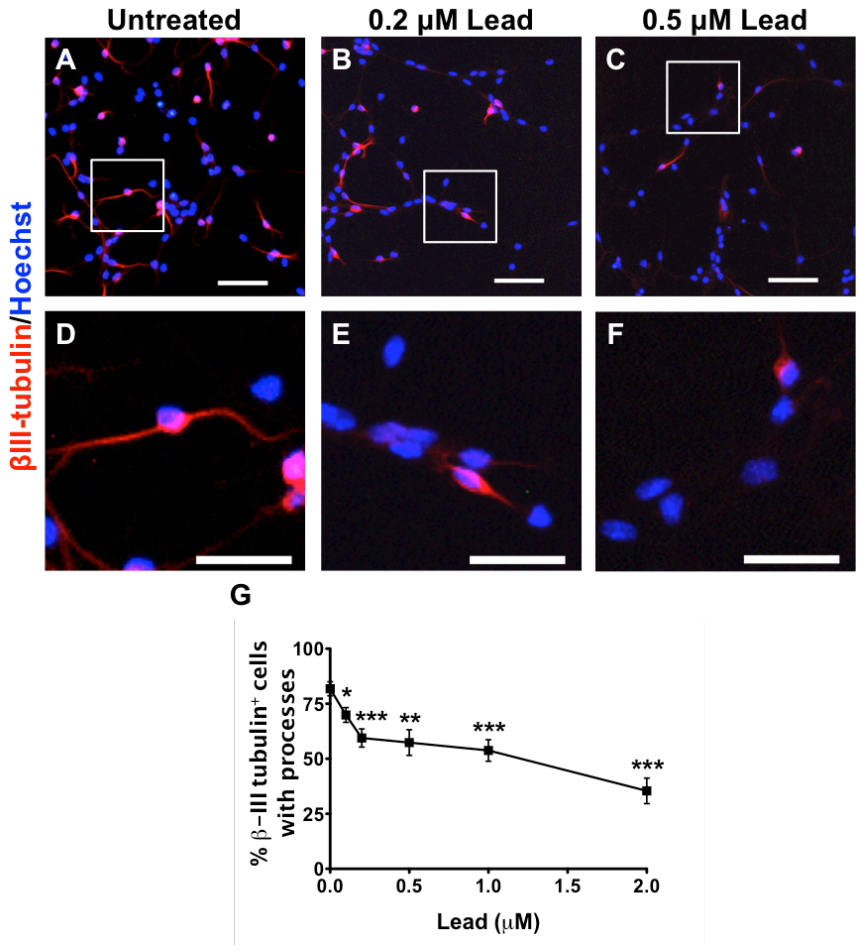

Figure 2.4. Lead decreases spontaneous neuronal differentiation of SGZ-aNPCs. *A-G*, SGZ-aNPCs were treated with 0, 0.1, 0.2, 0.5, 1, and 2 µM lead for 5 d in EGF/bFGF-free growth media. Representative βIII-tubulin and Hoechst co-staining from (*A,D*) untreated, (*B,E*) 0.2 µM, and (*C,F*) 0.5 µM lead-treated SGZ-aNPCs. Images *D-F* correspond to the boxed regions in images *A-C*, respectively. Quantification of (*G*) the percent βIII-tubulin$^+$ cells with processes. βIII-tubulin$^+$: immature neuron marker. Scale bars for A-C: 50 µm; D-F: 25 µm. *n* = 2 independent experiments for a total of 8 coverslips per data point. Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.00$

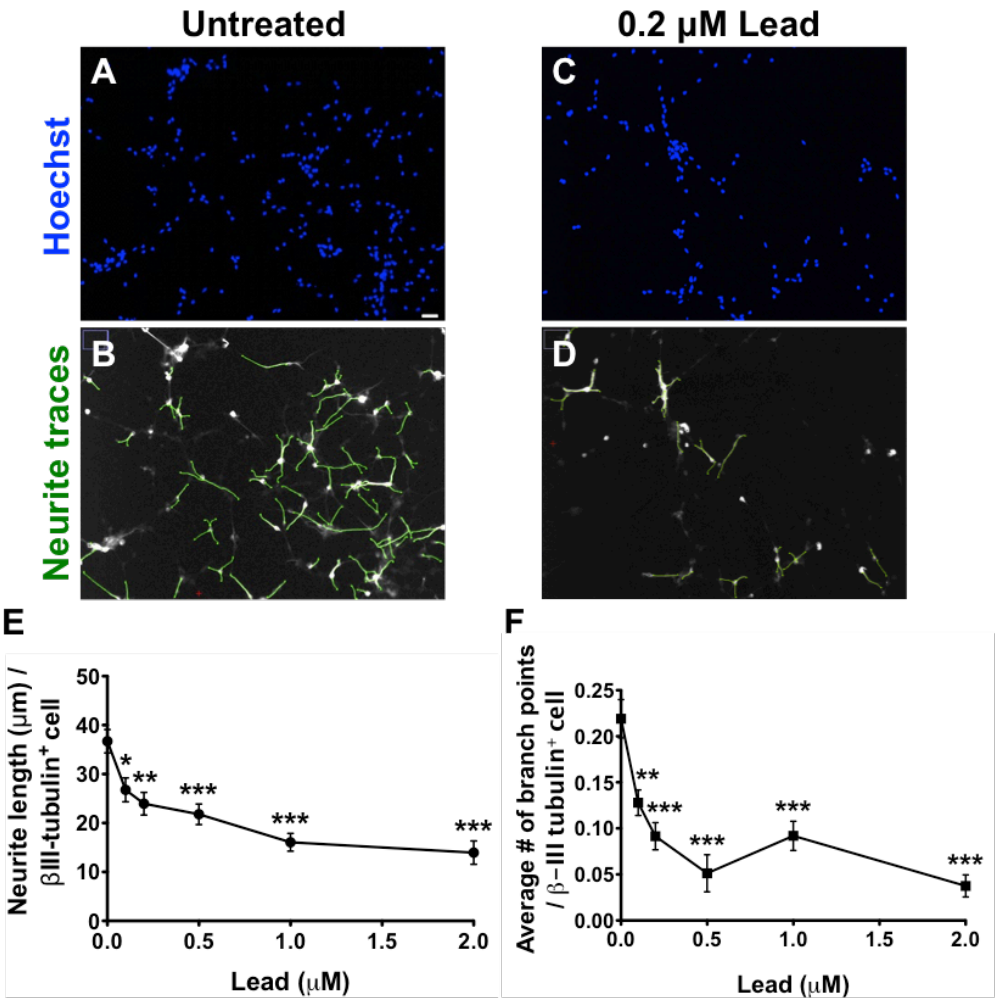

Figure 2.5. Lead decreases the neuronal maturation of SGZ-aNPCs. *A-F*, SGZ-aNPCs were treated with 0, 0.1, 0.2, 0.5, 1, and 2 µM lead for 5 d in EGF/bFGF-free growth media. Representative (*A, C)* Hoechst staining and (*B, D)* neurite traces from untreated and 0.2 µM lead-treated SGZ-aNPCs, respectively. Quantification of (*E*) the mean neurite length per βIII-tubulin$^+$ cell and (*F*) the average number of branch points per βIII-tubulin$^+$ cell. Scale bar: 25 µm. $n = 2$ independent experiments for a total of 8 coverslips per data point. Data represent mean ± SEM.; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

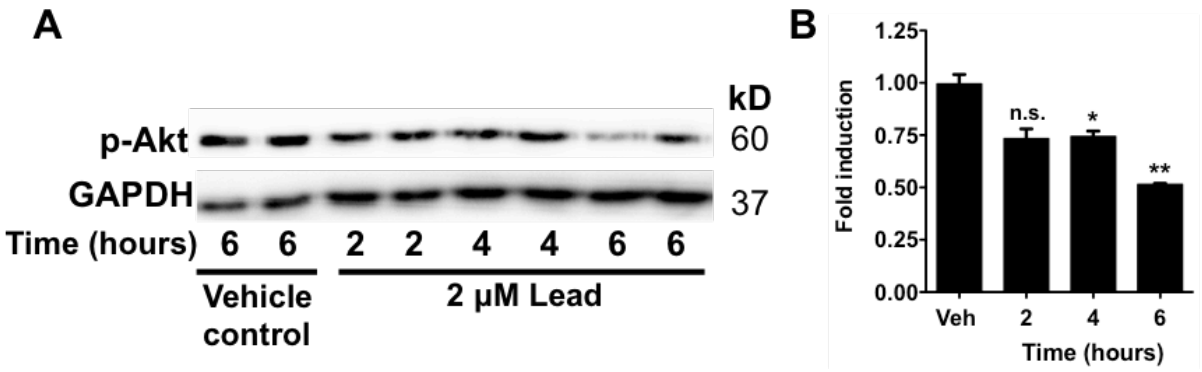

Figure 2.6. Lead inhibits activation of the Akt signaling pathway. ***A-B*****,** SGZ-aNPCs were treated with 2 μM lead for the indicated amount of time, and the cell lysates were subjected to Western blot analysis for **(***A***)** phosphorylated-Akt. **(***B***)** The fold induction of Akt normalized to GAPDH compared to DMSO controls. *n* = 2 independent experiments with duplicates. Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$.

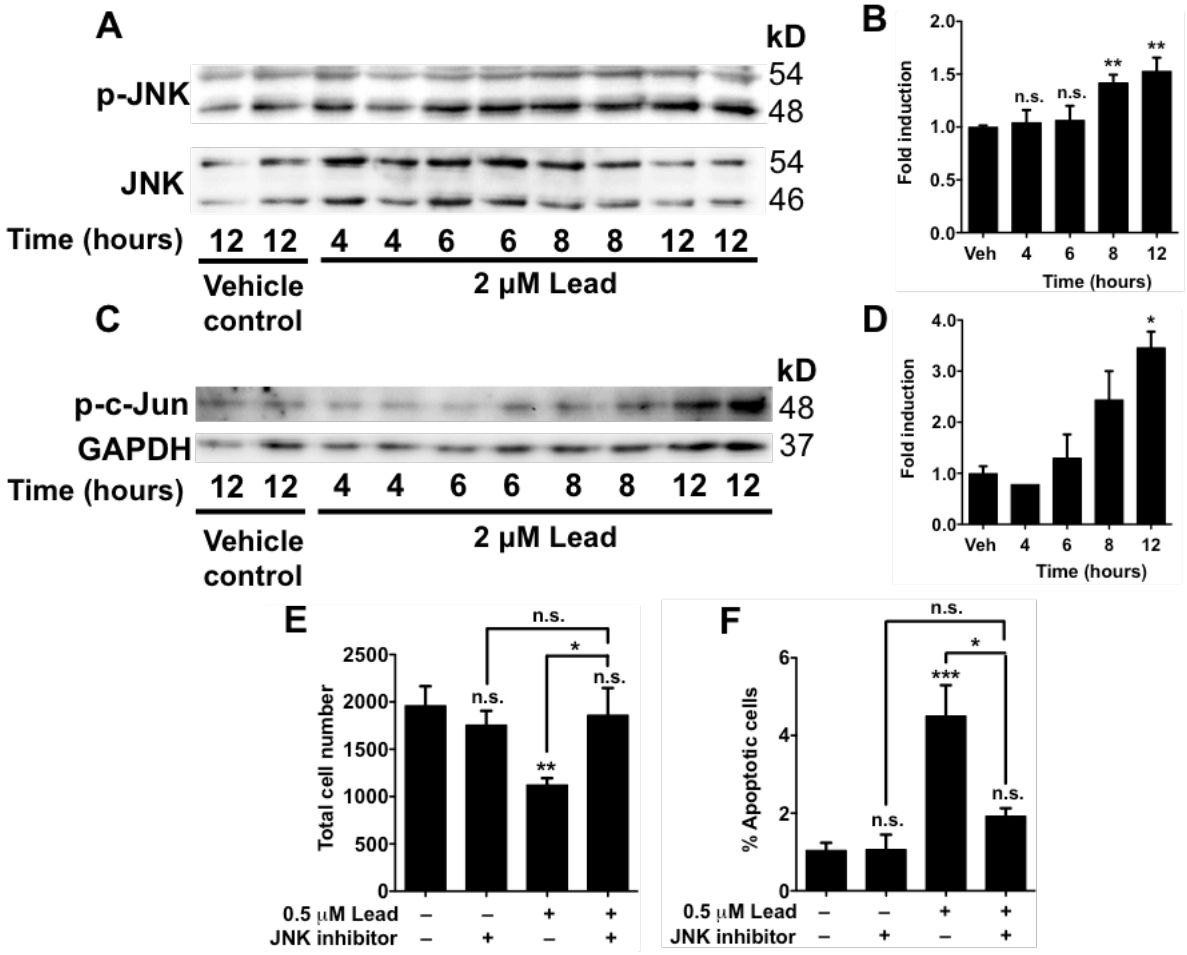

Figure 2.7. Activation of the JNK signaling pathway contributes to lead-induced cytotoxicity. ***A-D*,** SGZ–aNPCs were treated with 2 μM lead for the indicated amount of time. The cell lysates were subjected to Western blot analysis for (*A*) phosphorylated-JNK and (*C*) phosphorylated c-Jun, and the fold induction of (*B*) p-JNK (normalized to total JNK) and (*D*) p-c-Jun (normalized to GAPDH) in lead-treated cells compared to DMSO controls was quantified. ***E-F*,** SGZ–aNPCs were pretreated with 0.5 μM of the ATP-competitive, pan-JNK inhibitor SP600125 for 1 h and then treated with 0.5 μM lead for an additional 48 h. Quantification of (*E*) the total cell number and (*F*) the percent apoptotic cells. *A-D: n* = 2 independent experiments with duplicates. *E-F: n* = 3 independent experiments for a total of 9 coverslips per data point. Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

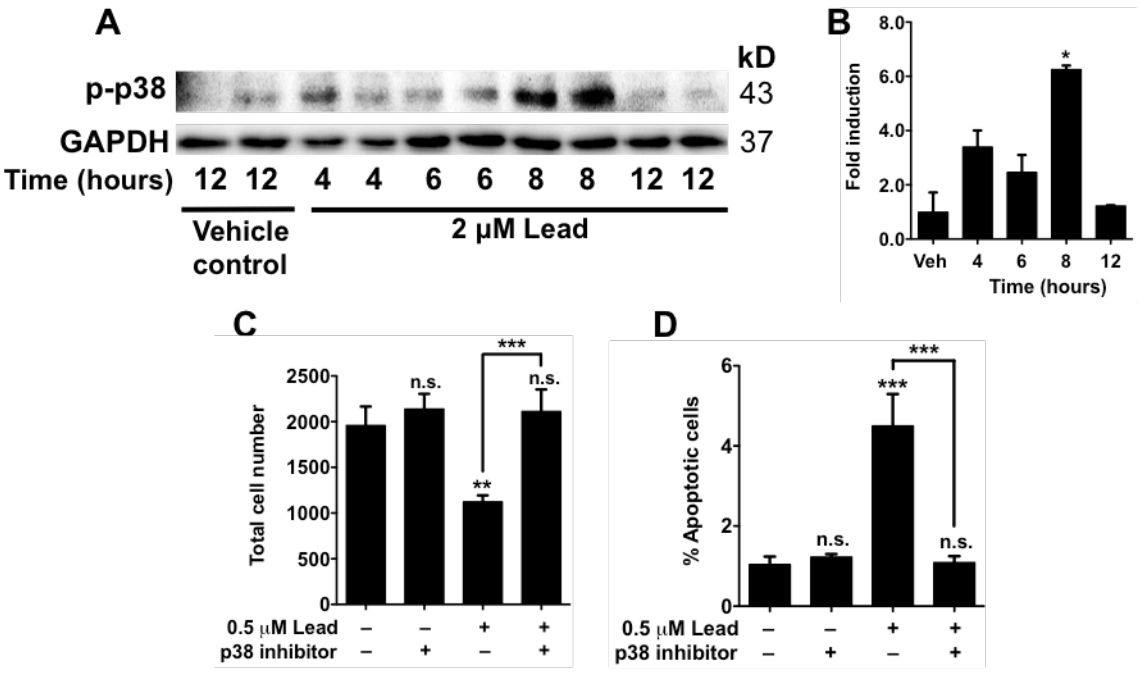

Figure 2.8. Activation of the p38 MAP kinase signaling pathway is also important for lead cytotoxicity. ***A-B*,** SGZ-aNPCs were treated with 2 μM lead for the indicated amount of time, and the cell lysates were subjected to Western blot analysis for **(***A***)** phosphorylated-p38. **(***B***)** The fold induction of p-38 normalized to GAPDH compared to DMSO controls. ***C-D*,** SGZ–aNPCs were pretreated with 0.5 μM of a p38 inhibitor for 1 h and then treated with 0.5 μM lead for 48 h. Quantification of **(***C***)** the total cell number and **(***D***)** apoptosis. *A-B: n* = 2 independent experiments with duplicates. *C-D: n* = 3 independent experiments for a total of 9 coverslips per data point. Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Chapter 3: Gene-environment interaction between lead and ApoE4 on cognitive behavior

3.1 Abstract

Alzheimer's disease is characterized by progressive cognitive decline and memory loss. It has been hypothesized that environmental factors and gene-environment interactions (GXE) may increase Alzheimer's disease risk and accelerate cognitive decline. However, there is currently little direct evidence supporting this hypothesis. The E4 allele of the Apolipoprotein E gene (ApoE4) is a significant risk factor for late-onset Alzheimer's disease. Lead is a neurotoxic heavy metal of great public health concern and can cause cognitive impairment in humans. Here, we assessed for a GXE between lead and ApoE4 on cognitive behavior using transgenic knock-in (KI) mice that express the human ApoE4 allele (ApoE4-KI). We exposed 2-month-old male and female ApoE3-KI and ApoE4-KI mice to 0.2% lead acetate via drinking water for 3 months. We report that, although all lead-treated animals had reduced contextual fear memory, this decrease was greatest and statistically significant only in lead-treated ApoE4-KI females. Similarly, only lead-treated ApoE4-KI females exhibited a significant decrease in spontaneous alternation in the T-maze. Furthermore, all lead-treated animals eventually developed persistent deficits in spatial working memory in the novel object location test. Interestingly, this deficit manifested earlier in ApoE4-KI mice than ApoE3-KI mice and earlier in females compared to males within the same genotype; female ApoE4-KI mice exhibited the earliest deficit onset.

Together, our data show that 1) adult-only lead exposure is sufficient to impair cognitive behavior; 2) lead-exposed ApoE4-KI mice develop more severe or exhibit earlier deficits in learning and memory compared to ApoE3-KI mice; 3) these impairments are persistent and can occur long after the lead exposure; and 4) females may be more sensitive to the effects of lead than males. These results provide strong evidence of a GXE between ApoE4 and lead exposure on cognitive impairment and may help elucidate the role of GXE and sex differences on Alzheimer's disease risk.

3.2 Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder, characterized by progressive and significant cognitive decline and memory loss (Mu and Gage, 2011). The hippocampus, which contributes to hippocampus-dependent learning and memory, is one of the earliest affected brain regions in AD (Mattson, 2004). While autosomal dominant mutations are associated with familial, early-onset AD, these mutations account for less than 5% of all AD cases (Wu et al., 2008b). Thus, most AD cases are likely due to a combination of environmental and genetic risk factors (Raiha et al., 1997; Gatz et al., 2005; Ketelaar et al., 2012). It has been hypothesized that a GXE between toxicants and genetic risk factors may perturb hippocampus-dependent learning and memory, accelerate cognitive decline, and contribute to neurodegenerative disease, including AD. However, definitive evidence supporting this hypothesis is lacking.

The strongest known genetic risk factor for late-onset AD is the Apolipoprotein E4 allele (ApoE4) (Liu et al., 2013). While ApoE3 is the most common of the three human ApoE allele variants (E2, E3, and E4), ApoE4 increases the frequency and decreases the age at onset of AD in a gene dose-dependent manner (Liu et al., 2013). In addition to its effect on AD risk, ApoE4 carriers also have a worse prognosis following traumatic brain injury, a higher prevalence of mild cognitive impairment, and accelerated cognitive decline (Mortensen and Hogh, 2001; Blair et al., 2005; Cosentino et al., 2008; Whitehair et al., 2010; Verghese et al., 2011; Beydoun et al., 2012). Importantly, ApoE is expressed in the brain, including in adult neural precursor cells in the dentate gyrus (DG) of the hippocampus (Ming and Song, 2011b; Yang et al., 2011; Liu et al., 2013).

Studies using humanized, transgenic ApoE3 knock-in (KI) and ApoE4-KI mice found that female ApoE4-KI mice exhibited learning and memory deficits starting at 16 months of age, suggesting that ApoE4 expression may impair hippocampus-dependent learning and memory in an age- and sex-dependent manner (Bour et al., 2008; Andrews-Zwilling et al., 2010; Leung et al., 2012). In humans, the prevalence of AD is higher in females, but it is still unclear whether there are sex differences in cognitive decline among non-Alzheimer's cases because there is no systematic study of sex differences in preclinical cognitive decline in the literature (Mielke et al., 2014). Interestingly, female ApoE4 carriers do have an increased risk of AD, and female ApoE4 carriers with mild cognitive impairment may experience a greater loss of hippocampal volume and reduced

memory performance compared to male carriers (Bretsky et al., 1999; Fleisher et al., 2005). Thus, ApoE4 carriers may be more susceptible than ApoE4 non-carriers, and female ApoE4 carriers may be more sensitive than male ApoE4 carriers, to cognitive impairment induced by environmental exposures.

Lead is a ubiquitous environmental contaminant. Both environmental and occupational lead exposures continue to be a significant public health concern in the U.S. and globally (Cox, 2011; Brosche, 2014; Garrison, 2014; Willmsen, 2014). This is exemplified by the recent water crisis in Flint, Michigan; the drinking water supply in Flint was contaminated with high levels of lead from April to October 2015 due to lead leaching from the old water pipes. The fact that many U.S. cities have lead service pipes for water distribution underscores the continuing risk of lead exposure in the U.S. (Rabin, 2008). Lead can cross the blood-brain-barrier, accumulate, and persist in human tissues, and lead can cause persistent neuropsychological, cognitive, and other behavior deficits in humans (Bellinger et al., 1992; Tong et al., 1998; Rice and Barone Jr., 2000; Yokel, 2006). In addition, both animal and epidemiological studies have reported an association between lead exposure and accelerated cognitive decline and/or AD-associated neuropathology in adults (Stewart et al., 2002; Weisskopf et al., 2004; Basha et al., 2005; Stewart et al., 2006; Wu et al., 2008a; Bihaqi and Zawia, 2013). Interestingly, an epidemiological study reported that, among workers occupationally exposed to lead, workers with at least one ApoE4 allele experienced accelerated cognitive decline relative to ApoE4 non-carriers, (Stewart et al., 2002). These data are thought provoking and suggest that an

interaction between lead and ApoE4 may accelerate cognitive decline. However, epidemiological studies cannot exclude other uncontrolled confounding factors; thus, it is important to directly ascertain a GXE relationship between ApoE4 and lead exposure on cognitive impairment in experimental models. In this study, we utilized lead exposure of homozygous male and female ApoE3-KI and ApoE4-KI mice as a defined experimental model to address this issue and to examine any potential sex differences on disease susceptibility.

3.3 Materials and Methods

Animals. Humanized Apolipoprotein E3 and E4 knock-in (ApoE3-KI and ApoE4-KI) animals were generated as previously described (Xu et al., 1996) and provided by Dr. Nobuyo Maeda at the University of North Carolina, Chapel Hill. The human E3 or E4 allele in the ApoE3-KI or ApoE4-KI mice is expressed at physiological levels under control of the endogenous mouse ApoE promoter (Bour et al., 2008). ApoE3-KI and ApoE4-KI animals were maintained as homozygous lines and all animals were housed in standard conditions (12 h light/dark cycle) with food and water provided *ad libitum*. The University of Washington Institutional Animal Care and Use Committee approved all animal protocols.

Reagents. Animal drinking water with 0.2% lead (II) acetate (Cat. 316512, Sigma-Aldrich, St. Louis, MO) was prepared from a stock lead acetate solution and replaced weekly. The preparation, use, and disposal of hazardous agents

were carried out according to the Environmental Health and Safety Office at the University of Washington.

Lead exposure. Male and female ApoE3-KI and ApoE4-KI animals were weaned at 28 days and littermates of the same sex were randomly mixed into groups of 3-5 animals per cage. At 2 months of age, the animals were either switched to drinking water with 0.2% lead acetate (lead-treated) or kept on normal drinking water (control). Body weight was recorded every 1-2 weeks throughout the exposure. Water consumption was monitored for the first week of the exposure period. Lead-treated animals were exposed to leaded drinking water for 12 weeks, at which point all the animals were switched to normal drinking water for the remaining duration of the study. We included n= 8-13 animals per genotype/treatment/sex for behavior tests and n= 3-4 mice for the lead analysis studies

Blood lead analysis. We collected blood and brain tissue from the lead analysis cohorts at the end of lead exposure (when mice were 5 months old) as well as from a subset of 15-month-old mice from the behavior cohorts (n=3-4 per sex/genotype/treatment) at sacrifice for lead analysis. The Environmental Health Laboratory at the University of Washington measured lead in whole blood and brain tissue (one brain hemisphere of each mouse) using inductively coupled plasma mass spectrometry.

Open field test. The open field test was conducted before and after the lead exposure to assess for the development of any lead-induced locomotor deficits or anxiety. Briefly, each animal was placed into a TruScan Photo Beam Tracking arena (Colbourn Instruments, Whitehall, PA) with Plexiglas walls (10 X 10 X 16 in) and their movement was monitored with two sets of infrared breams. The animal was allowed to freely explore the arena for 20 min and the data was collected by TruScan 2.0 software (Colbourn Instruments).

Elevated plus maze. We conducted the elevated plus maze test in order to assess the effect of lead exposure on anxiety. The maze (26" x 26" x 15.25"; San Diego Instruments, San Diego, CA) consisted of four plastic arms with 7" walls (two enclosed and two open), and it was placed in the center of the behavior room. Each animal was placed into the center of the maze facing an open arm and allowed to freely explore the maze for 5 min. The open and closed arm ends were defined as the distal 1/3 of the arms. ANYmaze software (San Diego Instruments) was used to collect data on the animal's movement during the test.

Novel object location test. In order to assess hippocampus-dependent, short-term spatial working memory, we used the 1 h novel object location (NOL) test. This test was performed as previously described with a few modifications (Wang et al., 2014). Briefly, each animal was placed into an open field arena (Colbourn Instruments) with two identical objects in two different locations. During training, the animal was allowed to freely explore the two objects for 5 min and then

returned to its home cage. After 1 h, the animal was returned to the arena with the same two objects; one object remained in its original location and one object had been moved to a novel location. The time the animal spent actively investigating each object during the training and testing was quantified. Each training and testing session was recorded and scored offline by an experimenter blinded to the animal's genotype and treatment. We calculated the discrimination index by dividing the difference in exploration time between the novel (C) and familiar (A) locations by the total exploration time.

Cued-contextual fear-conditioning. Contextual fear conditioning is another form of hippocampus-dependent learning and memory (Shan et al., 2008; Pan et al., 2012d; Pan et al., 2012b). We tested 5-6-month-old animals and used a weak footshock conditioning paradigm (3 x 0.3 mA, 2 s shocks with 2 min inter-trial intervals) as previously described (Pan et al., 2012d). For conditioning, the mouse was placed into the footshock context (10" x 10" x 16" in arena with grid shock floor (Colbourn Instruments) and star-shaped wallpaper) and allowed to freely explore the arena for 2 min before the presentation of a 90 dB, 30 s tone (conditioned stimulus, CS). During the last 2 s of the tone presentation, a 0.3 mA footshock (unconditioned stimulus, US) was delivered via the grid shock floor. This cycle was repeated two more times for a total of three cycles before the animal was returned to their home cage. The CS and US were automated and delivered by TruScan software (Colbourn Instruments). The contextual fear memory test was conducted 24 h after conditioning. The mouse was placed back

into the foot shock context for 2 min in the absence of any foot shock. For the cued test (performed 2 h after context test), the animal was placed into a different context (new room; hexagonal Plexiglas arena; cartoon wallpaper) and allowed to freely explore for 2 min followed by the presentation of the CS for 2 min. For the novel context test (performed 2 h after the cued test), the mouse was placed into a novel context (new room; rat cage; striped wallpaper) and allowed to freely explore without any CS or US presentation. In all three tests, persistent freezing behavior (four paws on the ground, no head or body movement besides breathing) was recorded manually every 5 s during the 2 min scoring periods by an experimenter blinded to animal genotype and treatment.

Morris water maze test. We conducted the Morris water maze (MWM) test using 6-7-month-old animals to assess hippocampus-dependent spatial learning and memory. This test was performed as previously described with slight modifications (Wang et al., 2014). We used a more challenging MWM training paradigm (2 trials/day vs. 4 trials/day in the standard MWM test) because adult neurogenesis is believed to be required for more challenging forms of hippocampus-dependent learning and memory (Garthe et al., 2009; Deng et al., 2010; Garthe and Kempermann, 2013; Wang et al., 2014). The MWM tank consisted of a circular steel tank (120 cm diameter) filled with room temperature water made opaque with nontoxic white paint. The Plexiglas hidden escape platform (10 x 10 x 19.5 cm) was submerged 1-2 cm below the water surface in one of the tank quadrants. Four extra-maze cues were placed at regular intervals

around the tank and only indirect lighting was used in the room. For each trial or probe test, the mouse was placed into the tank facing the wall at a drop zone. For each trial, the mouse was given a maximum of 40 s to find the hidden escape platform. If the mouse did not reach the platform within 40 s, they were guided to the platform and assigned a latency of 40 s for that trial. Once the mouse reached or was guided to the platform, they were allowed to stay on the platform for 15 s and then dried and returned to their home cage. Each mouse was given 2 trials/day for 8-10 consecutive days. Twenty-four hours after the last training day, the hidden escape platform was removed from the tank and each animal completed a single, 60 s probe test. Reversal training began 24 h after the initial probe test in which the hidden escape platform was placed in the opposite quadrant, and a reversal probe test was conducted 24 h after the last reversal training day. A visible platform test (4 trials/animal) was conducted 2-4 h after the reversal probe test in which a black plastic pedestal was attached to the hidden escape platform and placed in a new quadrant. The mice were dropped from a different drop zone for each trial and allowed to locate the visible escape platform. There was a 20 min inter-trial interval for all trials. All the trials and tests were performed by an experimenter blinded to genotype and treatment, and all the data from the training trials and probe test were collected using ANYmaze software (San Diego Instruments).

T-maze continuous alternation task. We assessed spontaneous alternation in 12-13- month-old animals using a continuous alternation T-maze protocol with slight

modifications (Spowart-Manning and van der Staay, 2004). Briefly, the black, plastic T-maze had two goal arms and a start arm (31 x 11.5 x 21 cm), and was placed on a platform (57.5 cm) in the center of a room. The test consisted of a first-forced trial followed by 14 free-choice trials. For the first-forced trial, one of the goal arms was (randomly) blocked with a plastic guillotine door. The animal was sequestered in the distal one-third of the start arm for 5 s before the guillotine door was raised and the animal was allowed to enter the unblocked goal arm. Once the animal returned to the start arm, it was sequestered in the start arm for 5 s before the start of the 14 free-choice trials. For each free-choice trial, the mouse was allowed to enter either of the unblocked goal arms; once it entered a goal arm, the other goal arm was blocked with a guillotine door. The animal eventually returned to the start arm and was sequestered for 5 s while all of the goal arms were unblocked. This was repeated for a total of 14 free-choice trials. The alternation percentage was calculated by dividing the number of times the animal entered alternating arms by 14 (free-choice trials). We defined arm entry as the animal's tail tip entering the arm and defined repetitive arm entries as an animal re-entering the same arm three times in a row (e.g., 5 sequential entries into the same arm is 3 repetitive entries). An experimenter blinded to animal genotype and treatment scored each test.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism software (version 6.0h for Mac, GraphPad Software Inc., San Diego, CA, USA) and Stata (version 12.0 for Mac, StataCorp LP, College Station, TX, USA). Two-

way analysis of variance (ANOVA) with the Holm-Sidak *post-hoc* test (α = 0.05) was used to analyze the blood and brain lead, open field, elevated plus maze, MWM platform and visible platform, contextual fear, and T-maze data in order to account for the main effect of genotype (ApoE3-KI vs. ApoE4-KI), treatment (control vs. lead), or time. One-way ANOVA with the Holm-Sidak *post-hoc* test (α = 0.05) was used to analyze the MWM probe test data. Two-way ANOVA with repeated measures with *post-hoc* analysis (α = 0.05) was used to analyze the body weight and MWM training data. Two-tailed *t* test (α = 0.05) was used for within-genotype comparisons for the NOL data. Multilevel mixed-effects linear regression (α = 0.05) was used for longitudinal analysis of within-genotype NOL discrimination index data. All data represent mean ± SEM, n.s. not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.4 Results

Blood and brain lead levels

Eight-week-old ApoE3-KI and ApoE4-KI animals, both males and females, were exposed to 0.2% lead acetate (lead treated) or normal drinking water (control) for 12 weeks. We used 0.2% lead acetate in our study because this lead concentration has been used extensively in the literature to study lead neurotoxicity in mice (Dosunmu et al., 2012; Patkova et al., 2012; Bihaqi et al., 2014b; Liu et al., 2014; Yu et al., 2014; Masoud et al., 2016). We exposed mice to lead through drinking water for 12 weeks to model sub-chronic environmental and occupational exposures through ingestion. We measured blood and brain

lead levels in a group of animals after the cessation of the lead exposure, and blood lead levels from a subset of animals at 15 months of age (i.e. 10 months post-lead). Blood and brain lead results are summarized in Table 3.1. Lead exposure significantly raised blood lead levels in all animals relative to their controls at the end of the 12 week exposure, and the lead levels were higher in exposed females than males of the same genotype. Lead exposure also increased lead levels in the brains of all animals. However, there was no direct correlation between increases of blood vs. brain lead. For example, although the blood lead was highest in ApoE3-KI females, the brain lead was higher in ApoE4-KI males and females. At 15 months of age (10 months post-lead), blood lead levels had greatly reduced relative to the end of the 12 week exposure, although still significantly higher than controls.

ApoE3-KI and ApoE4-KI mice do not exhibit weight loss, locomotor deficits, or anxiety following lead exposure

We recorded body weight every 1-2 weeks during the lead exposure (Fig. 3.1). We did not observe any weight loss in any of the lead-exposed animals at any time during the lead exposure (Fig. 3.2). Lead-treated ApoE3-KI mice were slightly heavier than their controls at several time points, but there was no significant difference in body weight between control and lead-treated ApoE4-KI mice. Thus, adult-only lead exposure does not cause weight loss, and while the lead-treated ApoE3-KI mice were heavier than control ApoE3-KI mice at several

time-points, the ApoE3-KI lead-treated mice were not obese and this weight gain was not associated with any locomotor deficits.

We conducted the open field test to assess for locomotor activity and anxiety before and after lead exposure. The pre-exposure test was performed to exclude any intrinsic differences between the control and lead-treated cohorts that could complicate data interpretation later. Prior to the lead exposure, there were no significant differences in the open field activities between control and lead-treated ApoE3-KI or ApoE4-KI animals (data not shown). There were some small differences in locomotor activity between the two genotypes, with ApoE4-KI females and males exhibiting slightly higher baseline activity compared to the ApoE3-KI mice (data not shown).

After lead exposure, the lead-treated ApoE3-KI and/or ApoE4-KI females travelled further, spent more time moving, and moved slightly faster than controls (Fig. 3.3). There was no significant effect of lead on locomotor activity in male mice. It is important to note that there were no significant differences in locomotor activities between lead-treated ApoE3-KI and ApoE4-KI mice, either females or males.

We also used the open field test to determine if lead treatment caused anxiety, measured as more time or distance in the margin, less time or distance in the center, or reduced center entries. Lead treatment did not change these parameters for males (Fig. 3.4 A-F). Lead-treated ApoE3-KI females travelled a greater distance in the center of the open field and made more center entries compared to ApoE3-KI control females (Fig. 3.4D-F). Notably, there were no

significant differences in center distance travelled and center entry between lead-treated ApoE3-KI and ApoE4-KI females.

We also conducted the elevated plus maze test to assess anxiety after the cessation of the lead exposure (Fig. 3.4G-J). The elevated plus maze is commonly used to assess the anxiogenic and anxiolytic effects of toxicological and pharmacological agents, and it relies on a mouse's natural avoidance of heights and open spaces and preference for dark, sheltered spaces (Walf and Frye, 2007). There was no significant effect of genotype or lead exposure on the percent of total time or total distance males or females spent in the open arms by two-way ANOVA (Fig. 3.4G, H). In addition, there was no significant effect of genotype or treatment on the number of open arm entries or open arm end entries among males or females (Fig. 3.4I, J).

Thus, subchronic exposure of 8-week-old mice to 0.2% lead acetate did not decrease locomotor activity or cause anxiety in either male or female ApoE3-KI or ApoE4-KI mice. Although lead exposure may result in a slight hyperactive phenotype in ApoE3-KI and ApoE4-KI females and a slight anxiolytic phenotype in ApoE3-KI females, there were no significant differences between lead-treated ApoE3-KI and ApoE4-KI mice, either males or females.

Adult-only lead exposure causes minor deficits in ApoE4-KI mice in the Morris water maze (MWM) test

To investigate the effect of lead exposure and ApoE4 on hippocampus-dependent spatial learning and memory, we conducted the MWM test after the

lead exposure with both female (Fig. 3.5) and male (Fig. 6) ApoE3-KI and ApoE4-KI mice (age of mice: 6-7 months old). In the initial MWM test, all mice successfully learned the location of the hidden escape platform (Fig. 3.5 and 3.6, panels A, B) and spent significantly more time in Quadrant 3 (Q3, where the platform had previously been located) than any other quadrant (Fig. 3.5 and 3.6, panels C). There was no difference between the amounts of time any control and lead-treated mice spent in Q3.

Mice were then subjected to the reversal MWM test, which assesses learning flexibility and is functionally more challenging because it requires animals to actively forget the original platform location and learn the new platform location. During reversal learning, lead treatment did not affect the behavior of female or male ApoE3-KI mice. All ApoE3-KI mice learned the new location of the hidden platform equally well during the course of an 8 day reversal training (Fig. 3.5 and 3.6, panels D), and spent significantly more time in the new (Q1) over the old (Q3) platform location in the reversal probe test (Fig. 3.5 and 3.6, panels F). There was no difference in the amount of time control and lead-treated ApoE3-KI mice spent in Q1 (Fig. 3.5 and 3.6, panels F) and no significant effect of genotype or treatment on the Q1/Q3 ratio (panels G) or time in the target platform zone (Fig. 3.5 and 3.6, panels H) by two-way ANOVA.

Although lead-exposed ApoE4-KI mice also behaved largely the same as their controls in the reversal MWM test (Fig. 3.5 and 3.6, panels E-H), lead-treated ApoE4-KI female mice travelled further than their controls to locate the hidden platform on the last day of reversal training (Fig. 3.5E). In addition, lead-

treated ApoE4-KI males spent a similar amount of time in the old and new platform locations, rather than more time in the new platform location in the reversal probe test. Although lead-treated ApoE4-KI males had a decreased Q1/Q3 time ratio and spent less time in the target platform zone compared to their controls, neither reduction was statistically significant.

The visible platform test was conducted 2 h after the reversal probe test for all mice. Lead treatment did not affect the swim speed (Fig. 3.5I and 3.6I) or latency to reach the visible platform (Fig. 3.5J and 3.6J) for ApoE3-KI or ApoE4-KI mice, either sex. These data suggest that lead-treated ApoE4-KI mice may have a slight impairment in spatial learning in the reversal MWM test, and that the impairment was not due to deficits in swimming ability, vision, or motivation to escape water.

ApoE4-KI females exposed to lead exhibit impaired contextual fear memory

We also conducted a contextual fear-conditioning test (age of mice: 5-6 months old) to characterize the effect of lead and ApoE4 on hippocampus-dependent learning and memory (Fig. 3.7A). We used the 0.3 mA x 3 footshocks paradigm because we previously reported that this paradigm is sensitive to changes in contextual fear memory (Pan et al., 2012d). All mice showed minimal freezing behavior at baseline before footshock (Fig. 3.7B, C). We performed the context test 24 h after fear-conditioning in order to assess contextual fear memory. Interestingly, lead-treated ApoE4-KI females had significantly reduced contextual fear memory compared to ApoE4-KI controls (Fig. 3.7B). Male ApoE4-

KI mice and both male and female ApoE3-KI mice exposed to lead exhibited a statistically non-significant reduction in freezing behavior upon lead treatment (Fig. 3.7B, C). Furthermore, lead treatment did not cause any statistically significant changes in any animals in auditory-cued fear memory, a form of amygdala-dependent but hippocampus-independent memory, or in general freezing in a novel context. Thus, lead exposure impairs the formation and/or retrieval of contextual fear memory in ApoE4-KI female mice.

ApoE4-KI females exhibit decreased spontaneous alternation and increased repetitive arm entry in a T-maze

We also assessed spontaneous alternation using the T-maze in 12-13-month-old animals. Rodents spontaneously alternate the arms they enter in the T-maze, and this is likely due to a combination of novelty-elicited exploratory behavior as well as spatial working memory (Gerlai, 1998; Spowart-Manning and van der Staay, 2004). Interestingly, lead-treated ApoE4-KI females exhibited a significant reduction in spontaneous alternation compared to control mice (Fig. 3.8A). Furthermore, lead-treated ApoE4-KI females had a significant increase in repetitive arm entries compared to control ApoE4-KI females (Fig. 3.8B). These behavior changes were specific to females and were not observed in males. There were no significant differences between control and lead-treated animals on the ratio of left/right arm entries or mean session duration in either females or males (Fig. 3.8C, D). These data suggest that there may be a GXE interaction between lead and ApoE4 on spontaneous alternation and repetitive entry, and

that lead-treated female ApoE4-KI mice are more sensitive than males. These differences are not due to a bias in arm entries or significant differences in task completion time.

ApoE3-KI and ApoE4-KI mice exposed to lead exhibit a GXE interaction and sex differences in the onset of short-term spatial memory deficits in the NOL

We conducted a 1 h novel object location (NOL) test to assess hippocampus-dependent spatial working memory before, during, and after the lead exposure. Importantly, at each NOL time point (baseline through 10 months post-lead), none of the mice exhibited a preference for either object or location during the training session, and there was no difference in the total exploration time between lead-treated and control mice (data not shown).

At baseline before lead exposure, all of the ApoE3-KI and ApoE4-KI females and males spent significantly more time exploring the object in the novel vs. familiar (C vs. A) location during the test period, indicating that they remembered the original object locations (Fig. 3.9A). At 7 weeks into the lead exposure, all of the mice spent significantly more time exploring the novel location except for lead-treated ApoE4-KI females, which did not discriminate between the old vs. new object locations (Fig. 3.9B). After 11 weeks of lead exposure, both the lead-treated ApoE4-KI females and males no longer discriminated between the familiar and novel object locations while the lead-treated ApoE3-KI mice continued to spend significantly more time exploring the

novel object location (Fig. 3.9C). Three to four months after the cessation of the lead exposure, both the lead-treated ApoE4-KI females and males continued to exhibit a deficit in spatial working memory (Fig. 3.9D), and this deficit continued to persist through 6 months (Fig. 3.9E) and 10 months post-lead exposure (Fig. 3.9F). Lead-treated ApoE3-KI mice still spent statistically significantly more time exploring the novel object location at 6 months (Fig. 3.9E) and 10 months post-lead exposure (Fig. 3.9F). However, the differences of exploration time between the new vs. old object locations became smaller among the ApoE3-KI mice exposed to lead.

We calculated the discrimination index for each NOL test, and the discrimination indices at each time point are summarized by genotype and sex in Figure 3.10. There was a main effect of lead exposure on the discrimination index in all lead-exposed animals (Multiple mixed-effects linear regression: ApoE3-KI females, $p = 0.001$; ApoE4-KI females, $p < 0.0001$; ApoE3-KI males, $p = 0.019$; ApoE4-KI males, $p < 0.0001$). During the lead exposure, there was no difference in the discrimination index between control and lead-treated ApoE3-KI females (Fig. 3.10A). However, lead-treated ApoE3-KI females had a significantly lower discrimination index at 3 and 10 months post-lead exposure compared to controls. There was also a non-significant decrease in the discrimination index between lead-treated and control ApoE3-KI females at 6 months post-lead.

Interestingly, the lead-treated ApoE4-KI females had a significantly lower discrimination index than control ApoE4-KI females starting at 7 weeks into the lead exposure, and this decrease persisted through 10 months post-lead (Fig.

3.10B). The discrimination index in lead-treated ApoE4-KI females was lower than controls at 6 months post-lead but not statistically significant. Thus, lead-treated ApoE3-KI and ApoE4-KI females exhibit persistent deficits in short-term spatial memory, with ApoE4-KI females exhibiting a significant decrease in the discrimination index starting at 7 weeks into the lead exposure – much earlier than lead-treated ApoE3-KI females at 3 months post-lead exposure.

Lead-treated ApoE3-KI males had a significantly lower discrimination index at 6 and 10 months post-lead compared to controls (Fig. 3.10C), suggesting that spatial memory deficits can develop long after lead exposure. In contrast, lead-treated ApoE4-KI males had a significantly lower discrimination index than controls starting at 11 weeks into the lead exposure and this effect persisted through 10 months post-lead exposure (Fig. 3.10D). Thus, both ApoE3-KI and ApoE4-KI males exposed to lead exhibit persistent deficits in short-term spatial memory, but the lead-treated ApoE4-KI males exhibit these deficits much earlier (starting at 11 weeks into the lead exposure vs. 6 months post-lead).

Together, data in Fig. 3.9 and Fig. 3.10 demonstrate that ApoE4-KI mice exposed to lead develop impaired spatial working memory much earlier than lead-treated ApoE3-KI mice, in both males and females. When we compare males and females of the same genotype, lead-induced spatial memory manifested earlier in females than males for both ApoE3-KI and ApoE4-KI mice.

3.5 Discussion

AD currently affects nearly 35 million people worldwide, and this number is projected to triple to 117 million people by 2050 (Wu et al., 2008b; Mu and Gage, 2011; Liu et al., 2013). Currently, there is a paucity of research regarding the potential contribution of GXE to the risk of developing neurodegenerative disease and AD pathogenesis. Importantly, cognitive decline and AD are associated with significant societal, financial, and health care costs. Thus, additional investigation into how environmental factors and GXE may influence AD risk or accelerate cognitive decline is a timely issue of immense public health importance.

In this study, we used humanized transgenic knock-in mice as an animal model of human ApoE4 carriers in order to assess whether there is a GXE between lead and ApoE4 on cognitive behavior. Together, our data (summarized in Table 2) show that (1) adult-only lead exposure is sufficient to lead to deficits in certain forms of hippocampus-dependent learning and memory in mice, (2) these deficits persist and can occur long after the cessation of the lead exposure, (3) transgenic mice expressing the human ApoE4 allele experience more severe – or an earlier onset of – learning and memory deficits compared to ApoE3-KI animals, and (4) lead-induced learning and memory impairments are sex-dependent and manifest earlier or are more severe in females than males.

We chose to use an adult-only exposure window because no study to date has assessed the effect of adult-only lead exposure on hippocampus-dependent learning and memory and cognitive decline (Gilbert et al., 2005; Jaako-Movits et al., 2005; Verina et al., 2007). We exposed mice to lead starting at 8 weeks of age – when mice have reached sexual maturity and developmental

neurogenesis, including that in the hippocampus, is complete – in order to avoid the cumulative effects of lead on both the developing and adult brain (Bayer et al., 1993). We report mean peak blood lead levels of 44.4-63.4 μg/dL, similar to previous animal studies and comparable to occupational exposures many workers experienced in the second half of the 20th century (Grandjean et al., 1978; Arnvig et al., 1980; Mantere et al., 1982; Williamson and Teo, 1986; Pasternak et al., 1989; Lucchini et al., 2000; Dosunmu et al., 2012). Furthermore, elevated blood lead levels (≥ 10 μg/dL. for adults) can still be encountered among workers in the US, and Occupational Safety & Health Administration regulations do not require U.S. workers to be removed from their job sites until they have a blood lead levels ≥ 50 μg/dL (OSHA, March 25, 2005). Importantly, high levels of lead exposure from environmental sources and in occupational settings are still a major concern in developing countries (Brosche, 2014; Ilyas, 2014). Therefore 0.2% lead acetate serves as an experimental model for occupational exposures in the U.S. and for environmental exposure in developing countries.

Many Alzheimer's mouse models, including the humanized ApoE4-KI mice, do not fully recapitulate all of the features of late-onset, sporadic AD (Elder et al., 2010). However, the hippocampus is critical for learning and memory and is especially vulnerable to damage at early stages of AD. Importantly, visuospatial and working memory impairments often occur prior to a clinical diagnosis of AD in humans (Buccione et al., 2007; Johnson et al., 2009; Weintraub et al., 2012). Thus, functional deficits in learning and memory,

especially those that are hippocampus-dependent, are often used as a proxy to estimate the presence and degree of AD-associated neuropathology (Jones et al., 2011; Yassa et al., 2011). In this study, we report that lead exposure impairs hippocampus-dependent contextual fear memory in female ApoE4-KI animals. In addition, we observed decreased spontaneous alternation performance in the T-maze in female ApoE4-KI mice exposed to lead. While spontaneous alternation is not strictly hippocampus-dependent, alternation performance is very sensitive to hippocampal lesions (Gerlai, 1998; Dudchenko, 2004; Spowart-Manning and van der Staay, 2004). More importantly, impaired alternation and/or repetitive arm entry have been reported in several different AD-mouse models, including models with canonical mutations in presenilins and amyloid precursor protein (Holcomb et al., 1998; Holcomb et al., 1999; Arendash et al., 2001; Laursen et al., 2014).

Lead exposure also impaired hippocampus-dependent spatial memory in the NOL test in all animals. Interestingly, lead-exposed ApoE3-KI male mice developed memory loss in the NOL test at 24 weeks (i.e. 6 months) after the 12 week lead exposure ended. Moreover, the memory loss in all lead-exposed animals is persistent and was observed through the end of the experiments, i.e. 10 months post-lead exposure. These data suggest that sub-chronic adult lead exposure causes long lasting or irreversible impairment in memory formation, and that this behavior phenotype can occur long after exposure has ended. These findings are pertinent to human health. Irreversible loss of memory formation is characteristic of AD, senile dementia, and up to 50% of Parkinson's

disease cases (Arnaldi et al., 2012). Importantly, the aging population in the U.S. experienced high lead exposure in early life (ATSDR, 2007). While ambient lead levels and median blood lead levels have significantly declined in the U.S. since the phasing out of leaded paint and gasoline, lead from early life exposures can be mobilized from the bone over time, subjecting individuals to continuous, low level lead exposure (ATSDR, 2007). Thus, it is possible that lead exposure, even if it occurred a long time ago and did not cause cognitive impairment during or immediately after exposure, may lead to cognitive impairment later in life and contribute to cognitive decline associated with aging and neurodegeneration, such as late onset AD.

Other studies have utilized the ApoE3 and ApoE4-KI mouse models and reported that female ApoE4-KI mice develop spatial learning and memory deficits in the MWM starting at 16 months of age (Andrews-Zwilling et al., 2010; Leung et al., 2012) and impaired spatial working memory and Y-maze task performance at 15-18 months of age (Bour et al., 2008). We were interested in determining whether lead exposure could accelerate the onset of these learning and memory deficits, so we chose to assess cognitive behavior before 15-18 months of age (5-6 months for contextual fear; 6-7 months for MWM; 12-13 months for T maze, 2-15 months for NOL). If we define a GXE as a different effect of lead exposure on cognitive behavior in mice expressing the human ApoE4 allele from those expressing the human ApoE3 allele, then our data suggest that lead does interact with ApoE4 because we observed more severe deficits in contextual fear memory and spontaneous alternation in lead-treated ApoE4-KI animals. In

addition, we observed that lead caused an earlier onset of short-term spatial working memory deficits in the NOL test in lead-treated ApoE4-KI mice. Together, these data suggest that the effect of lead exposure on cognitive behavior is different depending on ApoE genotype. More specifically, a GXE between lead and ApoE4 may accelerate cognitive decline, may contribute to a more rapid progression from cognitive aging to mild cognitive impairment, and potentially contribute to clinical AD.

Epidemiological reports have suggested an increased risk of late-onset AD in women as well as increased cognitive decline in women with mild cognitive impairment, which is an intermediate stage between normal, aging-related cognitive decline and AD (Bretsky et al., 1999; Fleisher et al., 2005). Others have reported learning and memory deficits in ApoE4-KI females ≥ 16 months old but not in ApoE4-KI males or in ApoE3-KI mice (Bour et al., 2008; Andrews-Zwilling et al., 2010; Leung et al., 2012). We report here that, prior to 15 months of age, we observed more significant changes in cognitive behavior in females compared to males. Lead impaired contextual fear memory and working memory in the T-maze in female but not male ApoE4-KI mice. Furthermore, lead-induced spatial memory impairment in the NOL test manifested earlier in females than males for both ApoE3-KI and ApoE4-KI mice. These data provide strong evidence for sex differences in the susceptibility to lead-induced cognitive decline in an animal model, and may suggest that women are at higher risk to develop cognitive impairment, including AD, upon exposure to environmental neurotoxicants such as lead.

It would be interesting in future studies to elucidate mechanisms underlying the GXE interaction between ApoE4 and lead and the sex differences in lead susceptibility. The quantification of blood and brain lead burden suggests that differences in lead disposition, and specifically the levels of brain lead, may contribute to the GXE between ApoE4 and lead. Brain lead levels were higher in ApoE4-KI mice than ApoE3-KI mice and highest in ApoE4-KI female mice. At steady-state, brain lead levels (μg/100g) are usually 1-3 times higher than blood lead levels (μg/dL) in rat models (Bradbury and Deane, 1993). Similarly, we report that ApoE3-KI females and males exposed to lead have 2.5-3 times higher levels of lead in the brain vs. blood. However, the brain lead levels in ApoE4-KI mice were 5.6-5.79 times higher than blood lead levels, suggesting that E4 may influence blood-brain barrier integrity or the partitioning of lead between blood and soft tissues like the brain. Interestingly, ApoE4-KI mice exhibit decreased basement membrane thickness, reduced collagen and tight junction protein expression, and increased matrix metalloproteinase 9 activity in the blood-brain barrier, suggesting that ApoE4-KI mice may have reduced blood-brain barrier integrity and, thus experience increased lead accumulation in the brain (Bell et al., 2012; Alata et al., 2015; Tai et al., 2016). In addition, human E4 carriers with AD also exhibit a thinner basement membrane, increased fibrin extravasion, and pericyte loss in the blood-brain barrier compared to ApoE3-KI carriers with AD or non-AD controls (Salloway et al., 2002; Halliday, 2016). Thus, effects of E4 on the blood-brain barrier may increase the accumulation of lead in the brain in

ApoE4-KI mice and contribute to more severe or earlier onset of learning and memory impairments. Future dose response studies will help test this hypothesis.

Additional studies are needed to determine the underlying mechanism of sex differences in susceptibility to a GXE between lead and ApoE4 on cognitive behavior. Although the blood lead burden is higher in females than in males, there is no difference in brain lead levels, so a sex difference in the susceptibility to lead is unlikely due to differential lead disposition. Interestingly, there are known sex differences in adult hippocampal neurogenesis (Barha et al., 2011; Roughton et al., 2012; Hillerer et al., 2013); a process whereby new neurons are continuously born in the hippocampus throughout adult life and contribute to structural changes as well as neuroplasticity in the hippocampus (Deng et al., 2010; Guo et al., 2011; Pan et al., 2012a; Pan et al., 2012d; Pan et al., 2012b, 2013; Wang et al., 2014). Impaired adult hippocampal neurogenesis may contribute to deficits in hippocampus-dependent learning and memory, cognitive decline, and AD (Lazarov and Marr, 2010, 2013). Interestingly, ApoE is expressed in adult neural stem cells in the hippocampus (Li et al., 2009; Gilley et al., 2011; Yang et al., 2011), and ApoE4 alters the differentiation and maturation of adult-born neurons in the DG of the hippocampus (Bour et al., 2008; Li et al., 2009; Andrews-Zwilling et al., 2010; Leung et al., 2012). Furthermore, recent studies also suggest that adult lead exposure may interfere with adult hippocampal neurogenesis (Huang et al., 2012; Wang et al., 2013a; Engstrom et al., 2015). Thus, it would be interesting to examine in future studies whether

female ApoE3-KI and ApoE4-KI mice are more sensitive than males to lead-induced impairment of adult hippocampal neurogenesis.

In conclusion, we found that adult lead exposure is sufficient to impair hippocampus-dependent learning and memory and that these deficits are more severe or occur earlier in female ApoE4-KI mice. These findings are novel and provide some of the first strong evidence of a GXE interaction between lead and ApoE4 on cognitive impairment. These results also provide new insight into how environmental toxicants and genetic risk factors contribute to cognitive decline and neurodegeneration.

3.6 Figures

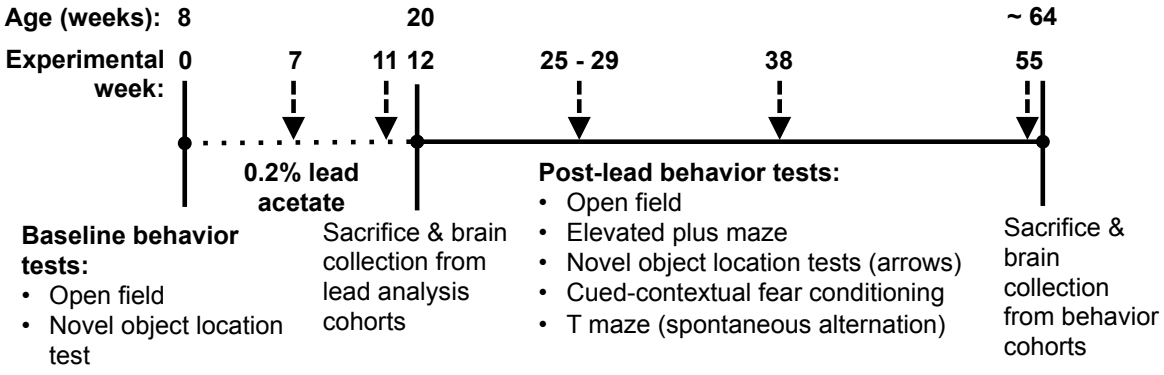

Figure 3.1. Experimental design and timeline of lead exposure, behavior tests, and tissue collection. Separate cohorts were used for behavior studies and blood lead analysis.

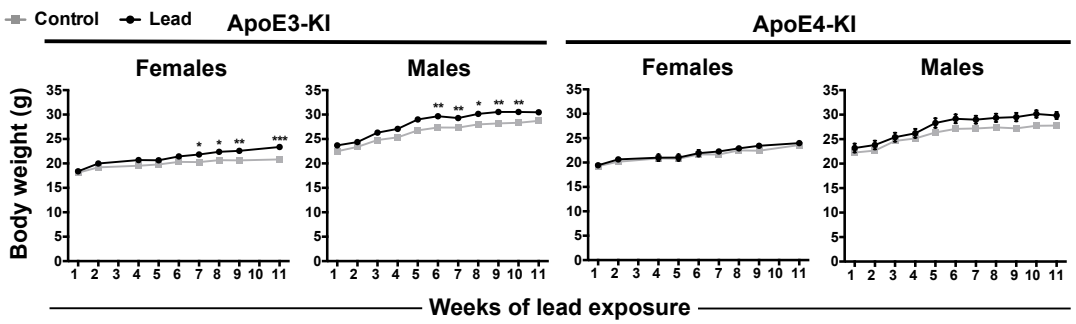

Figure 3.2. Male and female ApoE3-KI and ApoE4-KI mice do not exhibit weight loss upon exposure to 0.2% lead acetate for 12 weeks. 8-week-old male and female mice were exposed to 0.2% lead acetate via drinking water for 12 weeks and body weight was recorded every 1-2 weeks during the exposure window. (***A, B***) Lead-exposed male and female ApoE3-KI mice weighed slightly more than control ApoE3-KI mice starting at week 6 or 7 of the exposure, respectively. (***C, D***) There was no significant difference in body weight between control and lead-treated ApoE4-KI males and females during the lead exposure. Data are mean ± SEM with n= 8-13 per sex/genotype/treatment. Two-way repeated measures ANOVA with Holm-Sidak *post-hoc* tests: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

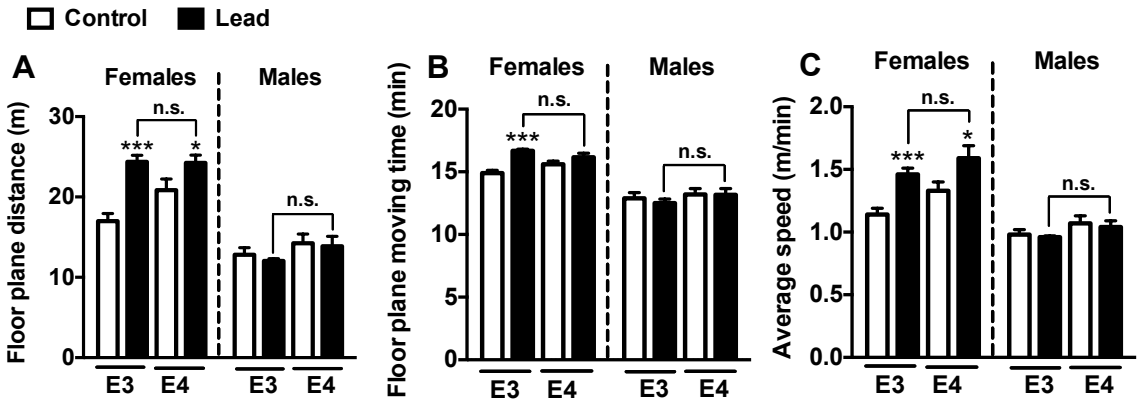

Figure 3.3. Lead-treated ApoE3-KI and ApoE4-KI male and female mice do not exhibit locomotor deficits in the open field test compared to controls. Mice were exposed to lead as previously described and the open field test was conducted after the cessation of the lead exposure. There was a significant main effect of lead treatment in female animals on floor plane distance, moving time, and average speed (Two-way ANOVA: floor plane distance, $F_{(1,31)} = 22.85$, $p < 0.0001$; moving time, $F_{(1,31)} = 20.47$, $p < 0.0001$; average speed, $F_{(1,31)} = 20.49$, $p < 0.0001$). *Post-hoc* analyses found that lead-treated ApoE3-KI and/or ApoE4-KI females travelled a (***A***) greater distance, (***B***) spent the same or more time moving, and (***C***) travelled slightly faster than controls. There were no significant differences between lead and control ApoE3-KI and ApoE4-KI males or between lead-treated ApoE3-KI and ApoE4-KI mice (males or females) in any of the locomotor endpoints. Data are mean ± SEM with n= 8-13 per genotype/treatment. Two-way ANOVA with Holm-Sidak *post-hoc* tests: n.s., not significant; * $p < 0.05$; *** $p < 0.001$.

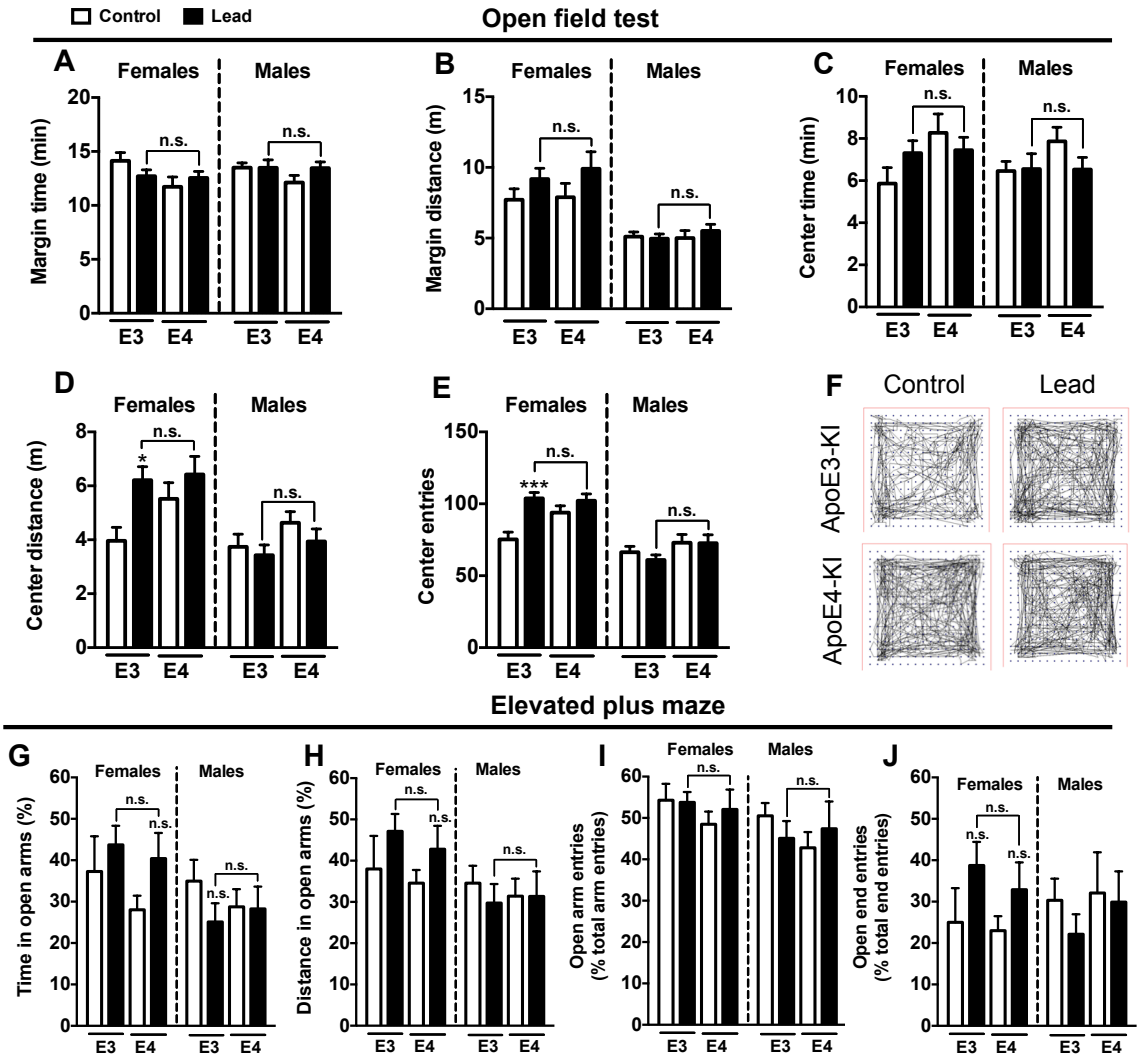

Figure 3.4. Male and female ApoE3-KI and ApoE4-KI mice exposed to lead do not exhibit overt anxiety in the open field test or elevated plus maze. The open field test was used to determine if lead treatment caused anxiety, measured as more time or distance in the margin, less time or distance in the center, or reduced center entries. There was a significant main effect of lead treatment in female mice on center distance and center entries (Two-way ANOVA: center distance, $F_{(1,31)} = 5.936$, $p = 0.0208$; center entries, $F_{(1,31)} = 14.76$, $p = 0.0006$). *Post-hoc* analyses found that there were no significant differences between lead-treated ApoE3-KI and ApoE4-KI mice (males and females) on the (***A***) time or (***B***) distance travelled in the margins, the (***C***) time or (***D***) distance travelled in the center, or the (**E**) number of center entries in the open field test. *D*, ApoE3-KI females treated with lead travelled a slightly greater distance in the center and *E*, made more center entries than controls. There were no significant differences between control and lead-treated ApoE4-KI females, ApoE3-KI males, or ApoE4-KI males in the open field test. (***F***) Representative open field track plots from female control and lead-treated ApoE3-KI and ApoE4-KI mice. In the elevated

plus maze, both male and female ApoE3-KI and ApoE4-KI mice exposed to lead spent a (***G***) similar or greater amount of time and travelled a (***H***) similar or greater distance in the open arms of the maze compared to controls. Lead-treated males and females did not exhibit reduced (***I***) open arm or (***J***) open arm end entries compared to control animals. Data are mean ± SEM with n= 8-13 per genotype/treatment. Two-way ANOVA with Holm-Sidak *post-hoc* tests: n.s., not significant; * $p < 0.05$; *** $p < 0.001$.

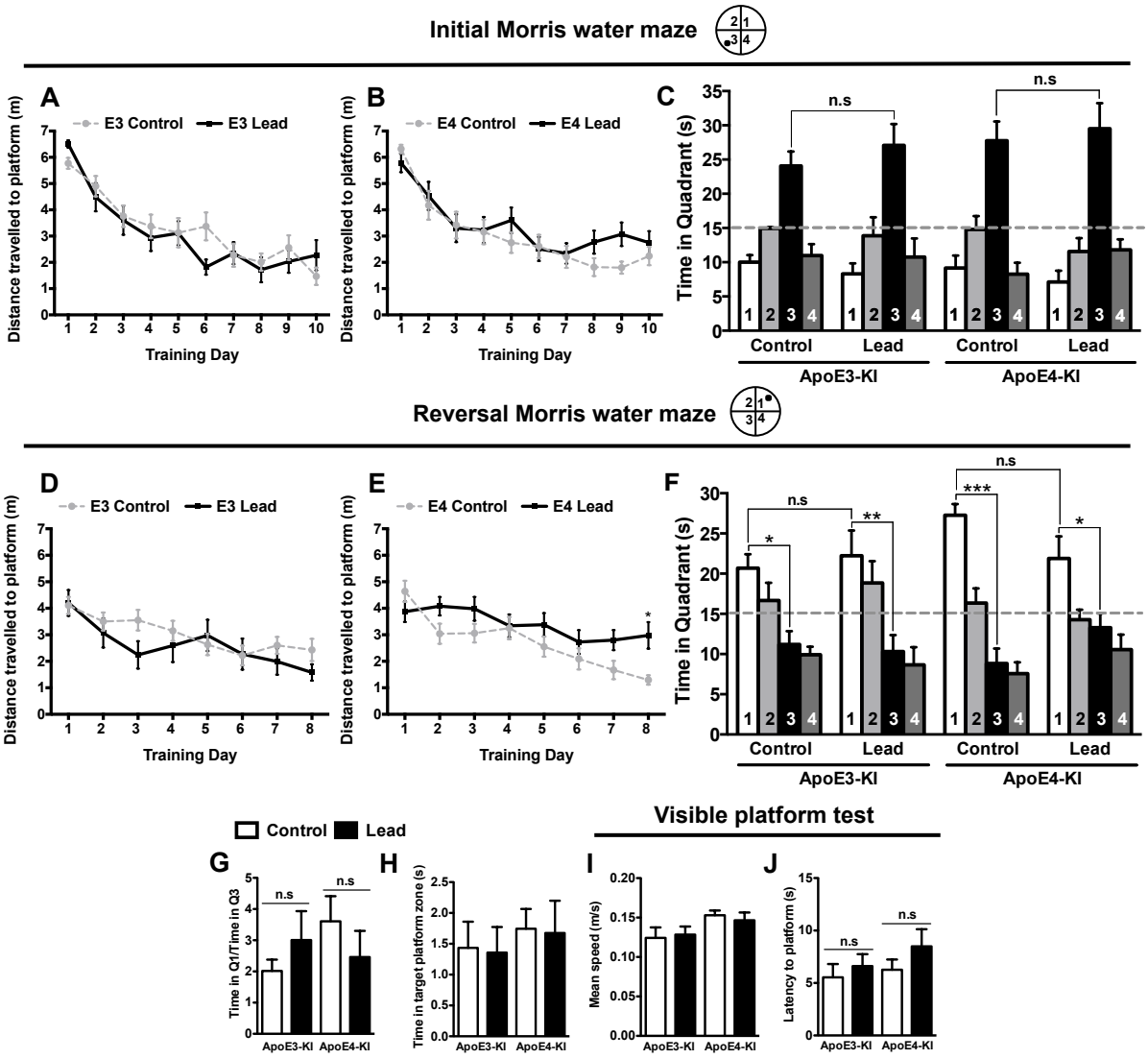

Figure 3.5. Lead-treated ApoE4-KI females exhibit minor spatial learning and memory deficit in the MWM at 6-months-old. (***A***), Lead-treated ApoE3-KI and (***B***) ApoE4-KI females did not exhibit deficits in spatial learning during training, travelling a similar distance to reach the hidden escape platform. (***C***) In the initial probe test 24 h after the last training day, all of the females had intact spatial memory, spending significantly more time in quadrant three (Q3) where the platform had previously been located. During reversal training, the lead-treated and control ApoE3-KI mice (***D***) learned the new location of the hidden escape platform. Similarly, the lead-treated and control ApoE4-KI mice had (***E***) similar learning curves but lead-treated mice travelled a greater distance to reach the platform on the last few training days compared to controls, and the difference was statistically significant on day 8 (One-way ANOVA with *post-hoc* test: ApoE3-KI females day 8, $F_{(1,144)} = 2.525$, $p = 0.0127$). All of the female mice spent (***F***) significantly more time in Q1 (new platform location) during the reversal probe test. There was no significant difference in the specificity of the spatial

memory between control and lead-treated animals because they spent a (***G***) similar amount of time in the new vs. old platform location and (***H***) in the platform zone during the reversal probe test. The visible platform test was conducted 2 h after the reversal probe test. There was no difference in (***I***) mean speed or (***J***) latency to reach the visible platform. Data are mean ± SEM with n= 8-10 per genotype/treatment. *A,B,D,E*: Two-way repeated measures ANOVA. *C,F*: One-way ANOVA. *G-J*: Two-way ANOVA. Holm-Sidak *post-hoc* tests. n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

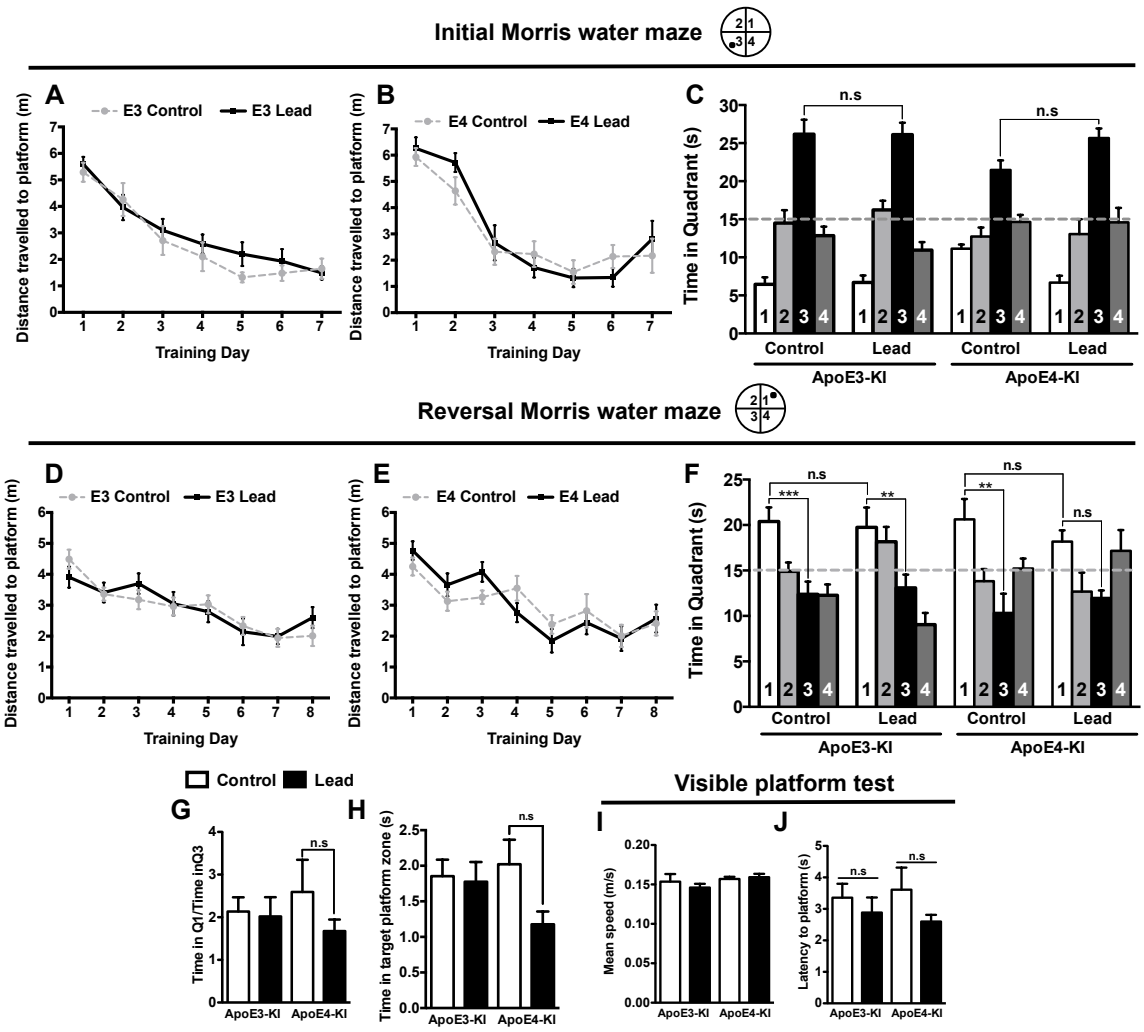

Figure 3.6. Lead-treated ApoE4-KI males also exhibit small spatial learning and memory deficits in the MWM at 6 months of age. (***A***), Lead-treated ApoE3-KI and (***B***) ApoE4-KI males performed comparably to controls during the initial learning test. (***C***) In the initial probe test 24 h after the last training day, all of the males had intact spatial memory, spending significantly more time in quadrant three (Q3), where the platform was located during training. During reversal training, (***D***) all of the ApoE3-KI and (***E***) ApoE4-KI mice learned the new location of the hidden escape platform. During the reversal probe test, all of the males – except for the ApoE4-KI male mice – spent significantly more time in Q1 (new platform location) than the other quadrants (One-way ANOVA with *post-hoc* test: ApoE4-KI males, $F_{(1,64)} = 2.393$, $p = 0.0946$) (***F***). Nevertheless, the lead-treated and control mice spent a (***G***) similar amount of time in the new vs. old platform location and (***H***) in the platform zone during the reversal probe test. The visible platform test was conducted 2 h after the reversal probe test. There was no difference in (***I***) mean speed or (***J***) latency to reach the visible platform. Data are mean ± SEM with n= 10-13 per genotype/treatment. *A,B,D,E*: Two-way

repeated measures ANOVA. *C,F:* One-way ANOVA. *G-J*: Two-way ANOVA. All with Holm-Sidak *post-hoc* tests: n.s. not significant; ** $p < 0.01$; *** $p < 0.001$.

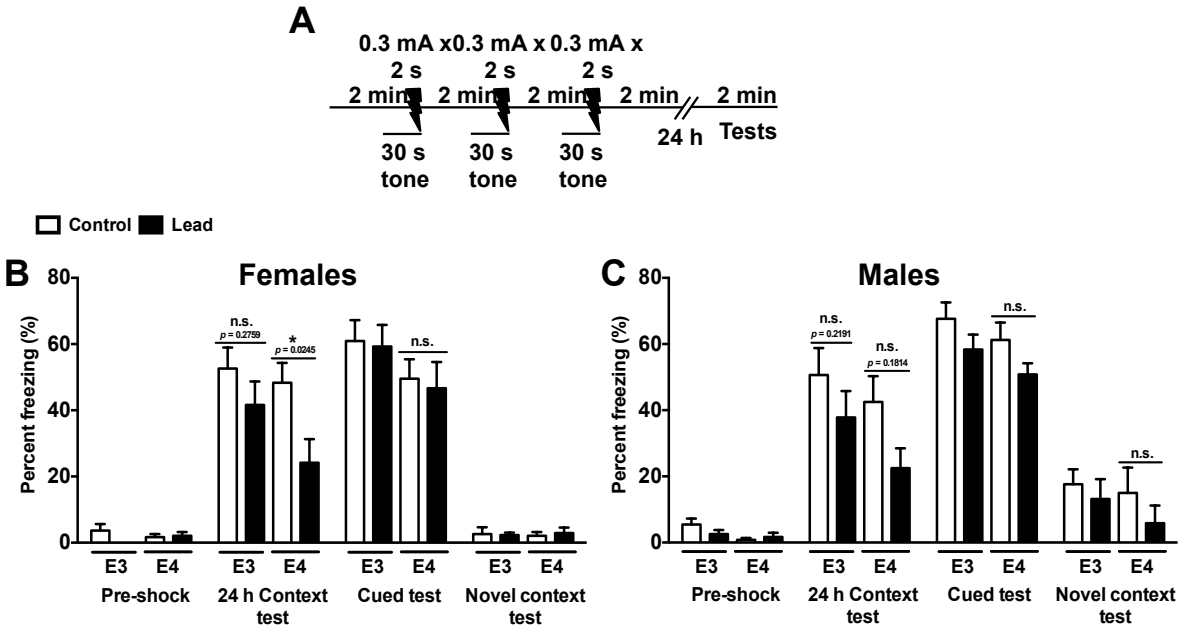

Figure 3.7. Lead impairs contextual fear memory in lead-treated ApoE4-KI females at 6 months of age. (***A***) Schematic of cued and contextual fear conditioning test performed post-lead exposure. (***B***, ***C***) Female and male mice had low baseline freezing behavior (Pre-shock). In the 24 h Context test, there was a significant main effect of lead treatment on freezing behavior in both males and females (Two-way ANOVA: females, $F_{(1,33)} = 6.803$, $p = 0.0136$; males, $F_{(1,42)} = 4.435$, $p = 0.0412$). Although lead treatment reduced contextual memory in all animals (manifested as reduced freezing 24 h after fear conditioning) *post-hoc* tests revealed that this was only statistically significant between control and lead-treated ApoE4-KI females (Holm-Sidak *post-hoc* test: $F_{(1,33)} = 2.648$, $p = 0.0245$). Auditory-cued (hippocampus-independent) fear memory was not affected (Cued test) in any lead-exposed animals. All animals did not freeze when placed into a novel, non-shock context (Novel context test). Data are mean ± SEM with n= 8-13 per sex/genotype/treatment. Two-way ANOVA with Holm-Sidak *post-hoc* test. n.s. not significant; * $p < 0.05$.

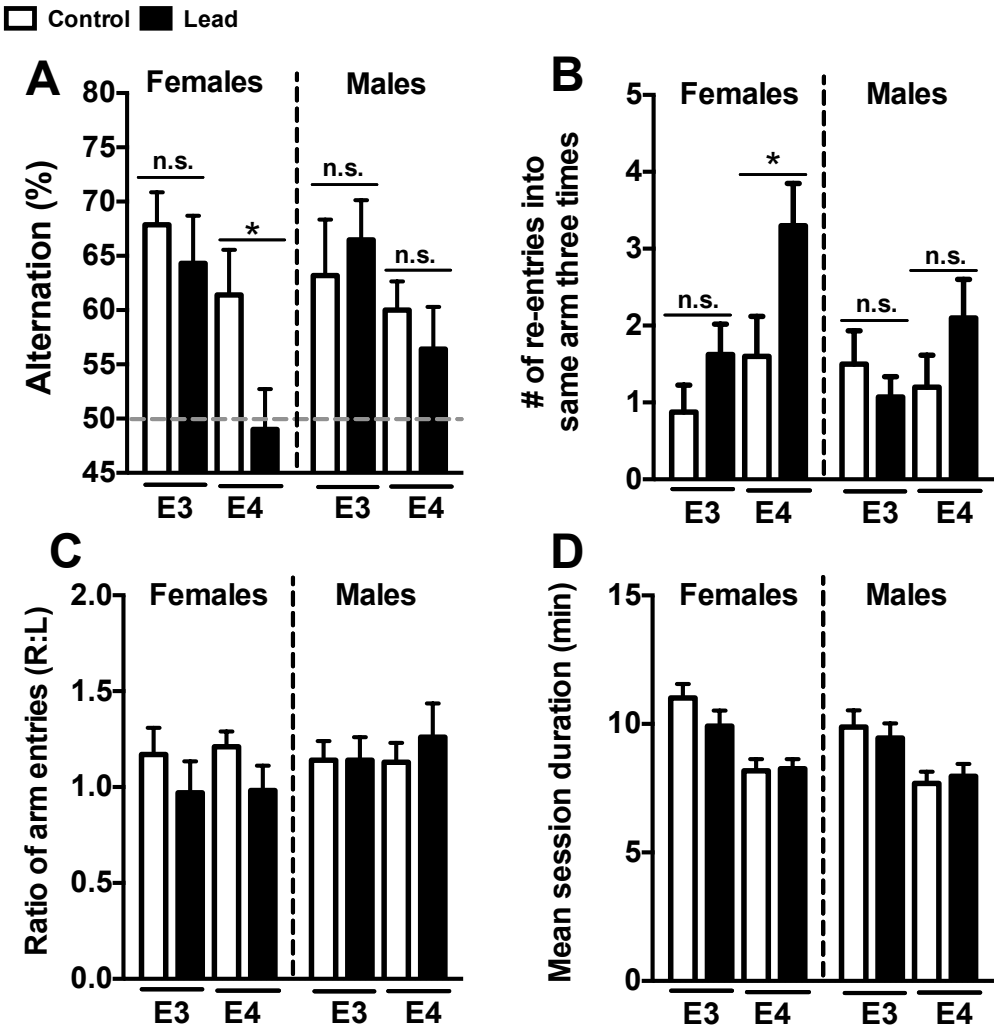

Figure 3.8. Lead impairs spontaneous alternation and increases repetitive arm entry in lead-treated ApoE4-KI females. Spontaneous alternation was assessed using the T-maze at 12-13 months of age. (***A***) There was a significant main effect of genotype on spontaneous alternation in females (Two-way ANOVA: genotype, $F_{(1,32)} = 7.666$, $p = 0.0093$). Lead-treated ApoE4-KI female mice exhibited reduced spontaneous alternation compared to female ApoE4-KI control mice (Holm-Sidak *post-test*: $F_{(1,32)} = 2.356$, $p = 0.0490$). (***B***) There was a significant main effect of both genotype and treatment on repetitive arm entries in females (Two-way ANOVA: genotype, $F_{(1,32)} = 5.915$, $p = 0.0208$; treatment, $F_{(1,32)} = 6.164$, $p = 0.0185$). ApoE4-KI females exposed to lead exhibited significantly increased repetitive arm entries compared to control ApoE4-KI female mice (Holm-Sidak *post-test:* $F_{(1,32)} = 2.584$, p 0.0289). There was no significant difference in spontaneous alternation or arm re-entries in lead-treated ApoE3-KI and ApoE4-KI male mice. (***C***) Female and male mice did not exhibit any arm preference. (***D***) There was a significant main effect of genotype on mean session duration in both the females and males, with ApoE4-KI mice completing the alternation task slightly faster than ApoE3-KI mice of the same sex (Two-way

ANOVA: females, $F_{(1,30)} = 19.50$, $p = 0.0001$; males $F_{(1,41)} = 9.803$, $p = 0.0032$). There was no significant difference in mean session duration between control and lead-treated animals of the same genotype and sex. Data are mean ± SEM with n= 8-13 per sex/genotype/treatment. n.s. not significant; * $p < 0.05$.

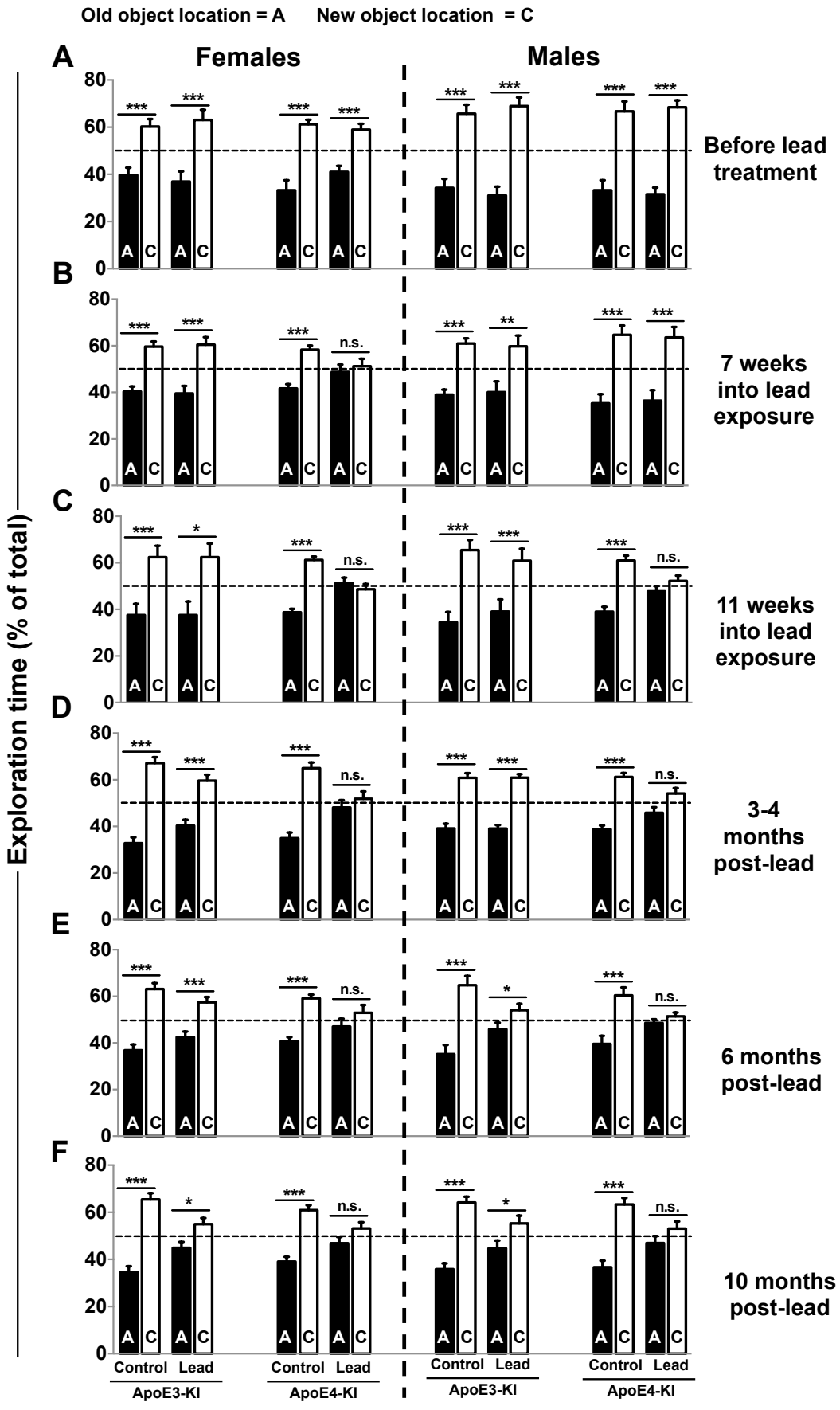
Old object location = A
New object location = C
A
Females
Males
Before lead treatment
B
7 weeks into lead exposure
C
11 weeks into lead exposure
D
3-4 months post-lead
E
6 months post-lead
F
10 months post-lead
Exploration time (% of total)
Control
Lead
ApoE3-KI
ApoE4-KI

Figure 3.9. The effect of lead on short-term spatial memory in the NOL test. The NOL test was performed **(*A*)** before**, (*B*)** 7 weeks and **(*C*)** 11 weeks into lead exposure, and **(*D*)** 3-4 months**, (*E*)** 6 months, and **(*F*)** 10 months after the lead exposure to assess for spatial working memory deficits. The time animals spent investigating the object in the old (location A) vs. new (location C) locations was quantified. More time spent exploring the object in the novel vs. old location indicates memory for the old location. *A*, All the animals had intact spatial memory prior to the lead exposure. *B,* At 7 weeks into the lead exposure, only the lead-treated ApoE4-KI females did not discriminate between the old vs. new object locations (Two-tailed *t*-test (A vs. C): $p = 1.000$). *C,* At 11 weeks, both the lead-treated ApoE4-KI females and males no longer discriminated between the object locations (females, $p = 0.4042$; males, $p = 0.1959$) while the ApoE3-KI females and males spent significantly more time exploring the novel object location (females, $p = 0.0107$; males, $p = 0.0098$). *D-F*, lead-treated ApoE4-KI females and males continued to exhibit a deficit in spatial working memory at 3-4 months (females, $p = 0.3867$; males, $p = 0.1032$), 6 months (females, $p = 0.2078$; males, $p = 0.2456$), and 10 months post-lead exposure (females, $p = 0.0961$; males, $p = 0.1855$). Lead-treated ApoE3-KI mice still spent statistically significantly more time exploring the novel object location at 6 months (males, $p = 0.0482$) and 10 months post-lead exposure (females, $p = 0.0186$; males, $p = 0.0320$). Data are mean ± SEM with n= 8-13 per genotype/treatment. Two-tailed *t*–test: n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

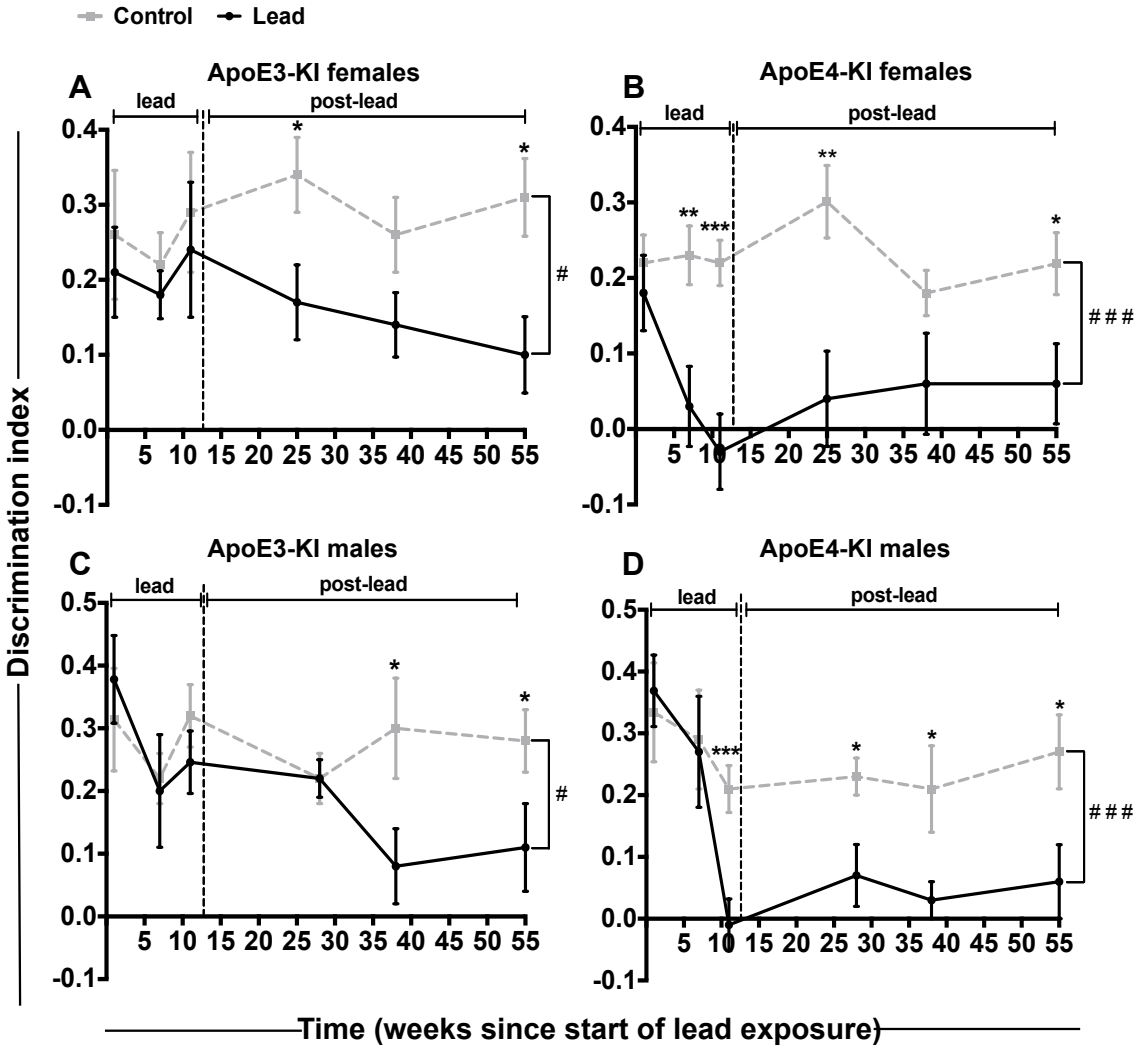

Figure 3.10. Genotype and sex differences in lead-induced reduction of discrimination index over time. The discrimination index in the NOL test was calculated by dividing the difference in exploration time between the novel (C) and familiar (A) locations by the total exploration time, data obtained in figure 9, and used to compare changes of spatial memory over time. (***A-D***) There was a main effect of lead exposure on the discrimination index in all lead-exposed animals (Multiple mixed-effects linear regression: ApoE3-KI females, $p = 0.001$; ApoE4-KI females, $p < 0.0001$; ApoE3-KI males, $p = 0.019$; ApoE4-KI males, $p < 0.0001$). *A*, During the lead exposure, there was no difference in the discrimination index between control and lead-treated ApoE3-KI females (Two-tailed *t*-test: 7 week, $p = 0.3046$; 11 week, $p = 0.9977$). Lead-treated ApoE3-KI females had a significantly lower discrimination index at 3 and 10 months post-lead exposure (3 months, $p = 0.0268$; 10 months, $p = 0.0156$) and a non-significant decrease 6 months post-lead ($p = 0.0901$) compared to controls. *B*, Lead-treated ApoE4-KI females had a significantly lower discrimination index than ApoE4-KI control mice starting at 7 weeks into the lead exposure and this persisted through 10 months post-lead (7 weeks, $p = 0.0016$; 11 weeks, $p =$

0.0002; 3 months, p = 0.0030; 10 months, p = 0.0269). The lead-treated ApoE4-KI discrimination index was lower than controls at 6 months post-lead but not statistically significant (p = 0.0713). *C,* Lead-treated ApoE3-KI males had a significantly lower discrimination index at 6 and 10 months post-lead compared to controls (6 months, p = 0.0356; 10 months, p = 0.0418). *D*, In contrast, lead-treated ApoE4-KI males had a significantly lower discrimination index than controls starting at 11 weeks into the lead exposure and this effect persisted through 10 months post-lead exposure (11 weeks, p = 0.0064; 4 months, p = 0.0433; 6 months, p = 0.0382; 10 months, p = 0.0275). Data are mean ± SEM with n= 8-13 per sex/genotype/treatment. Multi-level mixed-effects linear regression; significant effect of treatment: # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$. Two-tailed *t*-test; significant difference between control and lead: n.s., not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.6 Tables

Blood or brain lead measurement	ApoE3-KI females	ApoE4-KI females	ApoE3-KI males	ApoE4-KI males
Blood lead levels (µg/dl) immediately after lead exposure				
Control	3.817 ± 0.325	3.657 ± 0.307	3.13 ± 0.070	3.855 ± 0.868
Lead-treated	63.400 ± 2.503 ***, ^^	54.833 ± 2.492 ***, #, ^^	48.033 ± 4.725 ***	44.375 ± 2.584 ***
Brain lead levels after (µg/100g) immediately after lead exposure				
Control	0.340 ± 0.152	0.915 ± 0.683	0.147 ± 0.043	0.278 ± 0.103
Lead-treated	159.000 ± 22.898 ***	317.333 ± 15.624 ***, ###	152.067 ± 32.416 *	250.000 ± 67.625 **
Blood lead levels (µg/dl) 10-months post-lead exposure				
Control	3.587 ± 0.828	4.407 ± 2.587	1.607 ± 0.094	1.783 ± 0.015
Lead-treated	10.583 ± 0.322 **	13.067 ± 1.049 **	7.723 ± 0.887 *	10.173 ± 2.676 **

The data are mean ± SE (n= 3-4 per genotype/sex/treatment)
Significance – control vs. lead-treated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$
Significance – lead ApoE3-KI vs. lead ApoE4-KI within the same sex: # $p < 0.05$; ### $p < 0.001$
Significance – females vs. males within the same genotype: ^^ $p < 0.01$

Table 3.1: Summary of blood lead (µg/dL) and brain lead (µg/100g) levels in male and female ApoE3-KI and ApoE4-KI mice immediately or 10 months after the 12 week exposure to 0.2% lead acetate

	Lead-treated vs. Control			
Behavior test	**ApoE3-KI females**	**ApoE4-KI females**	**ApoE3-KI males**	**ApoE4-KI males**
Morris water maze test				
Reversal learning - distance to platform	**n.s.**	*, day 8	**n.s.**	**n.s.**
Reversal probe test - time in new (Q1) vs. old (Q3) quadrant	***, Q1 > Q3	**, Q1 > Q3	**, Q1 > Q3	n.s., Q1 ≈ Q3
24 h Contextual fear test				
Decreased freezing	**n.s.**	*	**n.s.**	**n.s.**
T maze				
Decreased spontaneous alternation	**n.s.**	*	**n.s.**	**n.s.**
Increased repetitive arm entries	**n.s.**	*	**n.s.**	**n.s.**
1 h Novel object location test				
Decreased discrimination index	*, 3-months post-lead	**, 7 weeks into lead exposure	*, 6 months post-lead	***, 11 weeks into lead exposure

Significance – control vs. lead-treated: n.s. not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 3.2: Summary of behavioral differences between lead-treated and control ApoE3-KI and ApoE4-KI males and females

Chapter 4: Gene-environment interaction between lead and ApoE4 on adult hippocampal neurogenesis

4.1 Introduction

Through a process called adult hippocampal neurogenesis, adult neural precursor cells in the dentate gyrus of the hippocampus continuously generate adult-born neurons which functionally integrate into existing neuronal circuits (Ming and Song, 2011a) and contribute to hippocampus-dependent learning and memory (Clelland et al., 2009; Deng et al., 2009; Garthe et al., 2009; Pan et al., 2012d; Pan et al., 2012b; Wang et al., 2014). A variety of physiological and pathological stimuli have been shown to modulate adult neurogenesis (Ming and Song, 2011b; Pan et al., 2012c; Pan et al., 2012e; Pan et al., 2013; Wang et al., 2014). However, little is known about the effects of neurotoxicants, such as heavy metals or gene-environment interactions (GXE) on adult hippocampal neurogenesis.

The heavy metal lead is a neurotoxicant of major public health importance. There is no threshold for lead toxicity; adverse health effects, including cognitive impairment, can occur at blood lead levels below U.S. regulatory standards (ATSDR, 2007). Interestingly, epidemiological and animal studies have found an association between cumulative lead exposure and an increased risk of accelerated cognitive decline and Alzheimer's disease (AD)-associated neuropathology (Stewart et al., 2002; Weisskopf et al., 2004; Basha et al., 2005; Stewart et al., 2006; Wu et al., 2008a). The hippocampus is one of the earliest

affected brain regions in AD patients, and deficits in hippocampus-dependent spatial learning and memory may develop prior to the onset of a clinical diagnosis (Buccione et al., 2007; Johnson et al., 2009; Weintraub et al., 2012). Importantly, perturbation of adult hippocampal neurogenesis may cause hippocampus-dependent learning and memory deficits, accelerate cognitive decline, and contribute to the development of neurodegenerative disease, including AD (Lazarov and Marr, 2010). Thus, lead may potentially contribute to cognitive decline and AD though impairment of adult hippocampal neurogenesis. We previously reported that low levels of lead are sufficient to impair the proliferation, survival, and differentiation of adult neural precursor cells from the DG of the hippocampus *in vitro* (Engstrom et al., 2015). In addition, other studies have reported that developmental lead exposure may impair the proliferation, survival, and differentiation of adult-born cells *in vivo* (Gilbert et al., 2005; Jaako-Movits et al., 2005; Verina et al., 2007). However, these animal studies used developmental exposure windows so the observed effects are likely due to the cumulative effect of lead on both developmental and adult neurogenesis. Currently, no study has yet assessed the effect of adult-only lead exposure or potential sex differences in susceptibility on adult hippocampal neurogenesis in mice.

Whether GXE between toxicants, such as lead, and genetic risk factors may perturb adult hippocampal neurogenesis is unclear. Interestingly, one study found that lead workers with at least one copy of the E4 allele of the Apolipoprotein E gene (ApoE4) allele gene experienced more severe cognitive

deficits than ApoE4 non-carriers after lead exposure (Stewart et al., 2002). The ApoE4 allele is the strongest known genetic risk factor for late-onset, sporadic AD, and it is also associated with accelerated cognitive decline compared to ApoE4 non-carriers (Moffat et al., 2000; Cohen et al., 2001; Mahley et al., 2006; Caselli et al., 2009; Liu et al., 2013). Moreover, ApoE is expressed in adult neural precursor cells, and *in vivo* studies using transgenic knock-in mice expressing the human ApoE4 allele (ApoE4-KI) found that ApoE4 alters adult-born neuron survival and maturation, and impairs hippocampus-dependent learning and memory in an age- and sex-dependent fashion (Bour et al., 2008; Li et al., 2009; Andrews-Zwilling et al., 2010; Leung et al., 2012).

Thus, the cognitive deficits experienced by individuals exposed to lead or the increased AD risk among ApoE4 carriers may be due, in part, to the negative effects of lead or ApoE4 on adult hippocampal neurogenesis. Furthermore, a GXE between lead exposure and ApoE4 may further impair adult hippocampal neurogenesis and subsequently contribute to impaired learning and memory, cognitive decline, or AD. Thus, in this study, we used adult lead exposure of female and male ApoE3-KI and ApoE4-KI mice as an *in vivo* animal model in order to directly assess whether there is a GXE between lead and ApoE4 on adult hippocampal neurogenesis and to characterize the potential underlying mechanisms.

4.2 Methods

Animals. Humanized apolipoprotein E3 and E4 knock-in (ApoE3-KI and ApoE4-KI) animals were the gift of Dr. Nobuyo Maeda at the University of North Carolina, Chapel Hill and were generated as previously described (Xu et al., 1996). ApoE3- and ApoE4-KI animals were maintained as homozygous lines and all animals were housed in standard conditions (12 h light/dark cycle) with *ad libitum* food and water. The University of Washington Institutional Animal Care and Use Committee approved all animal protocols.

Lead exposure. Animal drinking water with 0.2% lead (II) acetate (Cat. 316512, Sigma-Aldrich, St. Louis, MO) was replaced weekly. The preparation, use, and disposal of hazardous agents was carried out according to the Environmental Health and Safety Office at the University of Washington. The mice were kept on normal drinking water (control) or switched to 0.2% lead acetate (lead-treated) starting at 2 months of age for 12 weeks and then either sacrificed or switched to normal drinking water for 10 months and sacrificed at 15 months of age. Body weight was recorded every 1-2 weeks throughout the exposure. For each endpoint, there were n= 3-5 animals per genotype/treatment/sex.

Blood and brain lead analysis. Blood and brain tissue were at the end of lead exposure (n= 3-4 per genotype/sex/treatment) for lead analysis. The University of Washington Environmental Health Laboratory used inductively coupled plasma mass spectrometry (ICP-MS) to measure lead in whole blood and brain tissue and laser ablation ICP-MS (LA-ICP-MS) for trace metals analysis on a 30 μm

brain hemisphere section from a 5-month-old female ApoE4-KI control and lead-treated mouse. Semi-quantitative measurement of lead and zinc levels (ppm) from LA-ICP-MS was determined using spiked protein matrix standards.

BrdU administration. 5-bromo-2'-deoxyuridine (BrdU) was from Sigma (Cat. B9285) and stored as a 20 mg/ml stock in saline with 0.007% NaOH at -20C. Mice were dosed with 100 mg/kg BrdU by intraperitoneal injection 5 times in one day (every 2 h) and sacrificed either 3 weeks or 7 weeks later to assess the number of surviving BrdU-labeled adult-born cells. A different cohort of mice were dosed with BrdU 1 X 100 mg/kg 2 h prior to sacrifice to assess for proliferative BrdU-labeled cells.

Immunohistochemistry. The primary antibodies and dilutions used in immunohistochemistry were rat monoclonal anti-BrdU (1:500, Bio-Rad Laboratories AbD Serotec, Raleigh, NC), mouse monoclonal anti-NeuN (1:1000, Millipore, Billerica, MA), mouse monoclonal anti-GAD67 (1:2000, Millipore, Billerica, MA), and donkey polyclonal anti-DCX (1:200, Santa Cruz Biotechnology, Dallas, TX). Goat anti-rat, goat anti-mouse, and donkey anti-rat Alexa Fluor-conjugated secondary antibodies as well as Hoechst 33342 (2.5 µg/ml) were from Invitrogen (Carlsbad, CA). All of the primary and secondary antibodies were diluted into the appropriate blocking buffer (10% donkey or goat serum and 1% BSA).

Mice were anesthetized with ketamine/xylazine and then killed by decapitation. One brain hemisphere was post-fixed in 10% neutral-buffered formalin for 3-4 days followed by 30% (w/v) sucrose in phosphate-buffered saline (PBS, pH 7.4) at 4°C for 3-4 days until saturated. Each hemisphere was then stored in cryoprotectant media (30% glycerol; 30% ethylene glycol; 40% PBS) at -80°C until sectioning. Coronal brain sections (30 μm thick) were used for immunohistochemistry (IHC) using the free-floating antibody staining method as previously described (Pan et al., 2012e).

Thioflavin-S staining. Thioflavin-S staining for amyloid deposition was carried out using coronal 30 μm brain hemisphere sections from 15-month-old female ApoE3-KI and ApoE4-KI mice (10 months post-lead) as previously described with modifications to allow for free-floating staining (Sun et al., 2002). Every eighth serial section (30 μm) from each animal was placed in a netwell, and washed: 1x5 min in distilled water, 1x5 min with PBS (pH 7.2), 1x4 min in 0.3% potassium permanganate (w/v), 1x5 min in distilled water, 1x5 min in 1% sodium borohydride, and 3x5 min in distilled water. Sections were then mounted onto slides, dried, and rinsed with distilled water. 0.1% Thioflavin-S solution was dropped onto the slides and the slides were incubated at RT in the dark for 8 minutes. The slides were then flooded for 2x10 s with 80% ethanol and distilled water, respectively. The slides were then incubated in phosphate buffer (411 mM NaCl, 8.1 mM KCl, 30 mM Na2HPO4, 5.2 mM KH2PO4; pH 7.2) for 30 min at 4°C and then rinsed with distilled water before incubation with Hoechst 33342

(Invitrogen, Carlsbad, CA) for 30 min and mounting with Aqua Poly/Mount (Polysciences, Inc., Warrington, PA). Thioflavin-S staining was visualized and imaged using the FITC filter.

Quantification and imaging of immunostained cells. Immunostained cells were quantified as previously described (Pan et al., 2012e; Wang et al., 2014). Every eighth serial section (30 μm) was immunostained for each IHC marker (or combination of markers). An experimenter blinded to treatment and genotype quantified marker$^+$ cells in the subgranular zone and granule cell layer of the DG. This number was multiplied by 8 in order to estimate the total number of marker$^+$ cells per DG. Marker colocalization (double-positive cell) was defined as overlapping fluorescent signals in a single cell using a Z-series stack. All the marker$^+$ cells from at least 9 coronal sections per mouse (n=3-5 mice per genotype/sex/treatment) were quantified for each immuno marker or marker combination. All images were captured with an Olympus Fluoview-1000 laser scanning confocal microscope with the following lenses: numerical aperture (NA) 0.75 10X, NA 0.75 20X, NA1.3 40X (oil), or NA 1.35 60X (oil). Optical Z-sections (1 μm thick) were collected and processed using ImageJ software (NIH, Bethesda, MD). Images were uniformly adjusted for color, brightness, and contrast with Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA). Images (10X magnification) of the DG from three sections per animal were used to measure the fluoresence intensity of GFAP immunostaining and ImageJ was used to quantify fluorescence intensity.

Quantification of dendritic morphology. The dendritic morphology of DCX^+ cells in control and lead-treated ApoE3-KI and ApoE4-KI mice (at least 13 individual neurons per genotype/sex/treatment) was assessed as previously described (Wang et al., 2014).

Kidney and liver pathology scoring. Kidney and liver tissue (in 10% NBF) from 15-month-old female and male ApoE3-KI and ApoE4-KI mice was paraffin embedded and stained with hemotoxylin and eosin by the Histology and Imaging Core at the University of Washington and a veterinary pathologist blinded to genotype and treatment scored (0-3) kidney glomerular nephropathy and livery biliary hyperplasia (n= 3 mice per genotype/sex/treatment).

Statistical analysis.

Statistical analyses were conducted using GraphPad Prism software (version 7.0a for Mac, GraphPad Software Inc., San Diego, CA, USA). Two-way analysis of variance (ANOVA) with Fisher's LSD *post-test* ($\alpha = 0.05$) was used to analyze all of the data in order to account for the main effects of genotype (ApoE3-KI vs. ApoE4-KI) or treatment (control vs. lead). All data represent mean ± SE, n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.3 Results

Adult lead exposure results in lead deposition in the brain

At the end of the 12 week exposure to 0.2% lead acetate, lead-exposed animals had significantly higher blood lead levels (44.4-63.4 vs. 3.1-3.8 μg/dl) and brain lead levels (1.52-3.17 vs. 0.00147-0.00915 μg/g) than controls. The lead exposure window did not cause weight loss or reduced water consumption (data not shown). Low-resolution LA-ICP-MS (Fig. 4.1A-F) of sections from control and lead-treated female ApoE4-KI mice illustrate that adult-only lead exposure is sufficient to cause increased lead deposition throughout the brain, while the levels of the essential metal zinc were not significantly different between control and lead-treated mice. High-resolution LA-ICP-MS shows that, within the dentate gyrus of the hippocampus, lead accumulates in the hilar region, sub-granular zone (SGZ), and molecular layer (ML) (Fig. 4.1G-I).

There is no significant amyloid deposition in the hippocampus or significant pathology in other tissues in ApoE3-KI or ApoE4-KI mice at 10-months post-lead exposure

The humanized transgenic ApoE3-KI and ApoE4-KI mouse models, alone, do not develop AD-like neuropathology (Holtzman et al., 2000; Richardson and Burns, 2002; Andrews-Zwilling et al., 2010; Elder et al., 2010). However, human ApoE4 carriers do experience increased amyloid-β deposition than non-carriers (Schmechel et al., 1993; Polvikoski et al., 1995; Kok et al., 2009) and developmental lead exposure is associated with increased AD-associated neuropathology in older primates (Bihaqi et al., 2014a). Thus, we used Thioflavin-S staining in aged female ApoE3-KI and ApoE4-KI mice to determine

whether a GXE between lead and ApoE4 may cause amyloid deposition in old age. However, we did not observe any detectable amyloid deposition (Thioflavin-S staining) in the hippocampus or cortex of either control or lead-treated ApoE3-KI or ApoE4-KI female mice at 15 months of age (Fig. 4.2A) compared to a positive control: 12-month-old female mouse expressing five mutations associated with familial AD (5xFAD) (Fig. 4.2B).

We also assessed for general pathological changes in other tissues, including the liver and kidney, in 15-month old male and female ApoE3-KI and ApoE4-KI mice (10 months post-lead). In general, the female ApoE3-KI and ApoE4-KI mice exhibited increased age-related disease than males (Fig. 4.7C-D and data not shown). However, there were no significant differences in kidney glomerular nephropathy or liver biliary hyperplasia between control and lead-treated mice of either sex or genotype (Fig. 4.7C-D), thus this concentration and duration of lead exposure did not result in significant kidney or liver toxicity. Rare intranuclear lead inclusions were observed in the livers of male and female ApoE3-KI and ApoE4-KI mice exposed to lead, but there were no significant differences in the number of inclusions between lead-treated ApoE3-KI and ApoE4-KI mice (data not shown). Thus, the effects of lead we observed in the DG of the hippocampus and the differences we observed between lead-treated ApoE3-KI and ApoE4-KI mice were not due to significant systemic toxicity.

Lead decreases proliferation of adult-born cells in the hippocampus in all lead-exposed mice

To determine if adult-only lead exposure is sufficient to impair adult-born cell proliferation, we dosed female and male ApoE3-KI and ApoE4-KI mice with BrdU 2 h prior to sacrifice at the end of the 12 week lead exposure (mice were 5 months old) in order to label actively proliferating cells in S-phase of the cell cycle (Fig. 4.2). We found that lead significantly reduced the total number of $BrdU^+$ cells in all lead-treated animals compared to controls of the same genotype and sex (Fig. 4.2A-D). The percent change in $BrdU^+$ cells in lead-treated mice compared to control mice was not statistically significantly different between female or male ApoE3-KI and ApoE4-KI mice (Fig. 4.2E-F). We also did not observe a significant difference in $BrdU^+$ cells in ApoE3-KI mice compared to ApoE4-KI mice. These data suggest that adult-only lead exposure is sufficient to significantly decrease adult-born cell proliferation in the DG of the hippocampus. However, there was not a significant difference between ApoE3-KI and ApoE4-KI mice or males and females in the susceptibility to these lead-induced effects on proliferation.

Lead decreases the total number of adult-born cells and adult-born mature neurons in the DG of ApoE4-KI mice

In order to determine the effect of lead and ApoE4 on adult-born cell survival, we dosed separate cohorts of mice with BrdU 5 times in one day (every 2 h) 7 weeks prior to sacrifice (Fig. 4.4). We found that at 7 weeks post-BrdU injection, lead-treated ApoE4-KI females and males had significantly fewer total adult-born $BrdU^+$ cells compared to ApoE4-KI control mice of the same sex (Fig.

4.4A). Furthermore, lead-treated ApoE4-KI females had significantly fewer $BrdU^+$ cells than ApoE3-KI lead-treated mice. There was no significant difference in the total number of adult-born $BrdU^+$ cells between control and lead-treated ApoE3-KI mice of the same sex (Fig. 4.4A). We also quantified the percent change in total $BrdU^+$ cells between control and lead-treated mice of the same sex and genotype. Importantly, the difference in the percent change in $BrdU^+$ cells was only significantly difference between female ApoE3-KI and ApoE4-KI mice, but not male mice, suggesting that the lead-treated ApoE4-KI females are most sensitive to the effect of lead adult-born cells.

Furthermore, we found that there was a significant decrease in the total number of surviving, adult-born neurons ($BrdU^+NeuN^+$) in lead-treated ApoE4-KI males and females at 7 weeks post-BrdU (Fig. 4.4C). There was no significant difference in the total number of $BrdU^+NeuN^+$ between ApoE3-KI control and lead-treated mice (Fig. 4.4C). Thus, in contrast to the ApoE3-KI mice, the ApoE4-KI mice exposed to lead exhibited significantly fewer adult-born, mature neurons at 7 weeks post-BrdU, suggesting that ApoE4-KI mice may be more sensitive to the effects of lead on adult born neurons.

Lead decreases adult-born immature neuron differentiation in the DG of ApoE4-KI female mice

We also assessed the survival and dendritic complexity of adult-born immature neurons in animals dosed with BrdU 3 weeks prior to sacrifice. We found that ApoE4-KI females exposed to lead had significantly fewer surviving

adult-born immature ($BrdU^+DCX^+$) neurons at 3 weeks post-BrdU compared to ApoE4-KI control mice (Fig. 4.5B). While lead-treated ApoE3-KI males also had significantly fewer adult-born immature neurons compared to controls (Fig. 4.5C), this decrease (34.8%) was much lower than the reduction in ApoE4-KI females (70.5%). There was no significant difference in adult-born immature neuron survival between control and lead-treated ApoE3-KI females or ApoE4-KI males. In addition to fewer surviving adult-born immature neurons, lead-treated ApoE4-KI females also exhibited a significant reduction in the proportion of adult-born cells that differentiated into immature neurons compared to ApoE4-KI control mice (Fig. 4.5D). There was no significant difference in adult-born neuron differentiation between control and lead-treated ApoE3-KI mice or ApoE4-KI males (Fig. 4.5D-E). Thus, in addition to the effect of lead on general adult-born cell survival, lead may also impair the survival of adult-born immature neurons. Furthermore, ApoE4-KI females exposed to lead appear to be the most sensitive to the effect of lead on the survival and differentiation of adult-born immature neurons.

Lead decreases the dendritic complexity of immature neurons in the DG of ApoE4-KI female but not male mice

To further characterize the effect of lead and a potential GXE between lead and ApoE4 on adult-born cell survival and maturation, we assessed the dendritic morphology of adult-born immature neurons in control and lead-treated ApoE3-KI and ApoE4-KI female (Fig. 4.6) and male (Fig. 4.7) mice. We found

that both ApoE3-KI and ApoE4-KI lead-treated females had decreased total dendritic length compared to control mice of the same genotype, however, this decrease was greatest and significant only in lead-treated ApoE4-KI females (Fig. 4.6B). There was no significant difference in total dendritic length among male mice (Fig. 4.7B). Furthermore, using Sholl analysis, we found that lead-treated ApoE4-KI females had significantly lower numbers of dendritic crossings than control mice, but there was no difference between ApoE3-KI control and lead-treated mice (Fig. 4.6D-E) or among male mice (Fig. 4.7D-E). Thus, the immature neurons in lead-treated ApoE4-KI females exhibited more significant impairments in dendritic morphology, including total dendritic length and dendritic crossings. These data suggest that a GXE between lead and ApoE4 may potentiate the effect of lead or ApoE4 alone on dendritic morphology and impair adult-born neuron maturation, and that ApoE4-KI females may be more sensitive than males to the effects of lead on dendritic morphology.

Lead decreases the dendritic complexity of immature neurons in the DG of ApoE4-KI female mice

Previous studies report that ApoE4-KI female mice exhibit an age-dependent decrease in the number of GABAergic interneurons in the DG of the hippocampus, starting at 6 months of age (Li et al., 2009; Andrews-Zwilling et al., 2010; Leung et al., 2012). Thus, in order to determine whether the depletion in GABAergic interneurons may be an underlying mechanism for the observed impairments on adult-born immature neuron maturation, we quantified the

number of GABAergic interneurons in 5-month-old female and male ApoE3-KI and ApoE4-KI mice. At 5 months old, there was no significant difference in the total number of GABAergic ($GAD67^+$) cells between control ApoE3-KI and ApoE4-KI females (Fig. 4.8B). However, there was a statistically significant decrease in total $GAD67^+$ cells between control and lead-treated ApoE4-KI females. In contrast, there was no significant difference between control and lead-treated male mice (Fig. 4.8B). Thus, the selective, significant reduction in GABAergic interneurons in lead-treated ApoE4-KI females suggests that the loss of GABAergic interneurons may be an underlying mechanism for a GXE between lead and ApoE4 on the maturation of adult-born neurons and a potential mechanism underlying sex differences in susceptibility.

4.4 Discussion

Disruption of adult hippocampal neurogenesis may impair hippocampus-dependent learning and memory, accelerate cognitive decline, and contribute to the development of AD (Lazarov and Marr, 2010). Yet, whether neurotoxicants and GXE between environmental exposures and genetic polymorphisms may impair adult hippocampal neurogenesis and the underlying mechanisms for these effects have not been elucidated. Here, we utilized a transgenic knock-in mouse model in order to assess whether there is a GXE between the heavy metal lead and ApoE4 on adult hippocampal neurogenesis. Our data show that adult lead exposure is sufficient to impair adult-born cell proliferation. Furthermore, our data suggest that a GXE between lead and ApoE4 may significantly reduce the total

number of adult-born neurons as well as perturb the maturation and dendritic complexity of adult-born neurons in the DG of the hippocampus.

In our study, we first assessed for extracellular amyloid-β plaques, a pathological hallmark of AD, in order to determine whether an interaction between lead and ApoE4 may cause and/or exacerbate amyloid deposition in the brain (Liu et al., 2013). Unlike transgenic mouse models of familial AD that express mutations in amyloid precursor protein and presinilin 1 and 2, the ApoE3-KI and ApoE4-KI mouse models, alone, do not develop AD-like neuropathology (Holtzman et al., 2000; Richardson and Burns, 2002; Andrews-Zwilling et al., 2010; Elder et al., 2010). However, ApoE does have isoform specific effects on amyloid-β metabolism and may contribute to AD through amyloid-β-dependent mechanisms (Liu et al., 2013). In humans, ApoE4 co-localizes with senile plaques and ApoE4 carriers experience increased amyloid-β deposition and have more senile plaques (Schmechel et al., 1993; Polvikoski et al., 1995; Kok et al., 2009). Furthermore, primates exposed to lead during development exhibited increased expression of amyloid precursor protein in old age, suggesting that lead and ApoE4 may interact with amyloid and tau pathology (Bihaqi et al., 2014a). However, we did not observe significant amyloid burden in the cortex or hippocampus of lead-treated ApoE3-KI and ApoE4-KI mice at 15 months of age. Thus, it is unlikely that lead and ApoE4 interact to facilitate amyloid deposition in the brain.

Alternative mechanisms to the ‘amyloid cascade hypothesis’, including perturbation of adult hippocampal neurogenesis, have also been proposed as

possible mechanisms underlying AD pathogenesis. Notably, no study to date has assessed the effect of adult-only lead exposure on adult hippocampal neurogenesis in mice. We previously found that lead can impair the survival, proliferations, and differentiation of adult neural precursor cells *in vitro* (Engstrom et al., 2015). In addition, several other studies have assessed the effect of developmental lead exposure on adult hippocampal neurogenesis (Gilbert et al., 2005; Jaako-Movits et al., 2005; Verina et al., 2007). For example, Jaako-Movits et al. (2005) previously reported that developmental lead exposure impairs hippocampus-dependent contextual fear memory in rats, and Verina et al. (2007) found that early life lead exposure impairs adult-born cell proliferation, survival, and dendritic morphology in the hippocampus. Importantly, these studies used developmental exposure paradigms, so it is difficult to determine whether the observed changes in neurogenesis are due to the adverse effects of lead on developmental vs. adult neurogenesis. One study by Schneider *et al*. did use an adult-only lead exposure paradigm and observed a reduction in the proliferation of adult-born cells in rats, but they did not assess any other endpoints (Schneider et al., 2005).

Unlike previous reports using the ApoE3-KI and ApoE4-KI models, we did not see a significant effect of ApoE4, alone, on adult-born cell proliferation and maturation. This may be due to the fact that the effects of ApoE4 are age-dependent and we analyzed adult-born proliferation, survival, and maturation in ApoE4-KI females 1-2 months earlier (in 5-month-old vs. 6-7-month-old mice) than previous studies (Li et al., 2009; Andrews-Zwilling et al., 2010; Leung et al.,

2012). However, we did observe more significant effects, including impaired adult-born neuron maturation as well as decreased GABAergic interneurons, in lead-treated ApoE4-KI female mice compared to control ApoE4-KI mice or lead-treated ApoE3-KI mice at 5 months of age. These data suggest that lead exposure may accelerate the age-dependent effects of ApoE4 on adult hippocampal neurogenesis.

While lead decreased adult-born cell proliferation in all lead-treated animals, lead significantly impaired adult-born immature neuron maturation specifically in lead-treated ApoE4-KI female mice. The survival and integration of adult-born neurons is activity-dependent and occurs during a critical window, at approximately three weeks after neuronal birth (Tashiro et al., 2006; Marin-Burgin and Schinder, 2012). Several different cell types and inputs facilitate the survival and maturation of adult-born cells during this window. One such input is glutamate signaling via the *N*-methyl-D-aspartate-type (NMDA) receptor. The absence of functional NMDA receptors at 3-7 weeks after the birth of adult-born neurons is associated with reduced adult-born neuron survival, thus, the survival and synaptogenesis of adult-born neurons during this window may be dependent on NMDA receptor activity (Tashiro et al., 2006). GABAergic interneurons also facilitate the survival and maturation of adult-born cells during this window. For newborn granule cells, GABAergic synapses develop earlier than glutamaterigc synapses, and the tonic activation by GABA may exert trophic effects on adult-born immature neuron survival and maturation (Tozuka et al., 2005; Ge et al., 2006; Kim et al., 2012). Importantly, female ApoE4-KI mice exhibit significantly

fewer GAD67$^+$ cells than ApoE3-KI mice starting at 6-7 months of age (Li et al., 2009). In our study, we found that lead decreased the total number of GAD67$^+$ cells in 5-month-old lead-treated ApoE3-KI and ApoE4-Ki females, and that this decrease was greatest and significant only in lead-treated ApoE4-KI females. This lead-induced reduction in GAD67$^+$ cells in 5-month-old female ApoE4-KI mice correlates closely with our observation of significantly reduced adult-born neuron maturation and dendritic complexity in ApoE4-KI females, suggesting that an interaction between lead and ApoE4 may lead to earlier impairment of GABAergic input and contribute to the observed impairment in immature neuron maturation in ApoE4-KI mice.

Importantly, newborn immature neurons contribute to synaptic plasticity within the DG. Adult-born granule cells exhibit increased excitability and plasticity and reduced inhibition from GABAergic interneurons compared to the mature, existing neurons in the DG (Snyder et al., 2001; Esposito et al., 2005; Marin-Burgin and Schinder, 2012). Notably, the increased responsiveness and excitability of newborn granule cells may give these adult-born cells a specific and significant role in learning and memory (Deng et al., 2010). In rats, a greater proportion of young, adult-born neurons are activated during spatial exploration compared to existing granule cells (Ramirez-Amaya et al., 2006; Sandoval et al., 2011). Similarly, the selective loss of immature neurons impaired long-term spatial memory retention and contextual fear extinction (Deng et al., 2009). Thus, additional characterization of hippocampus-dependent learning and memory will

be needed to assess the functional consequences of the observed impairment of immature neuron survival and maturation in lead-treated ApoE4-KI female mice.

4.5 Figures

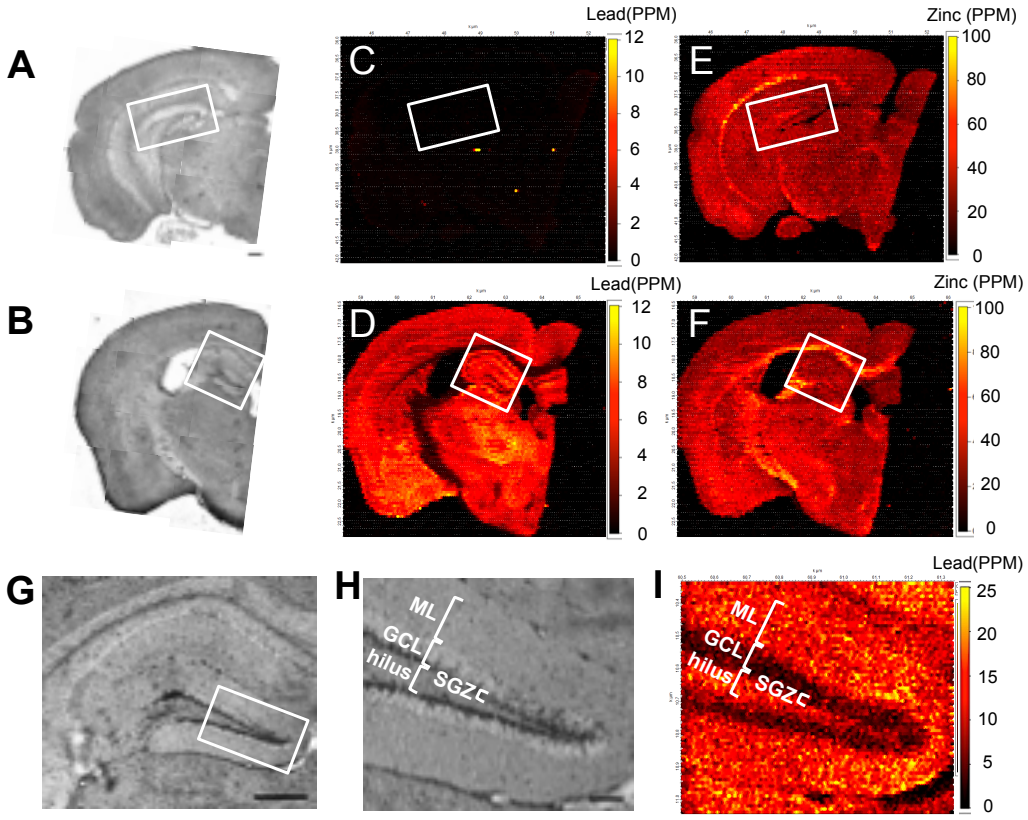

Figure 4.1. Adult-only lead exposure results in lead deposition in the brain. Brightfield images of one brain hemisphere of a 5-month-old female (***A***) control and (***B***) lead-treated ApoE4-KI mouse after the 12 week lead exposure. Semi-quantitative measurement of lead in the brain of a (***C***) control and (***D***) lead-treated ApoE4-KI mouse using LA-ICP-MS. Semi-quantitation of zinc in the same brain section from a (***E***) control and (***F***) lead-treated ApoE4-KI mouse. (***G***) Brightfeld image and (***H***) enlarged inset of the dentate gyrus of the hippocampus from a lead-treated ApoE4-KI female before high-resolution LA-ICP-MS. (***I***) Semi-quantitative measurement of lead from LA-ICP-MS of a section from a lead-treated ApoE4-KI female mouse shows that, within the dentate gyrus, lead primarily deposits in the hilus, SGZ, and ML. ML: molecular layer; GCL: granule cell layer; SGZ: sub-granular zone. Scale bar, 100 μm.

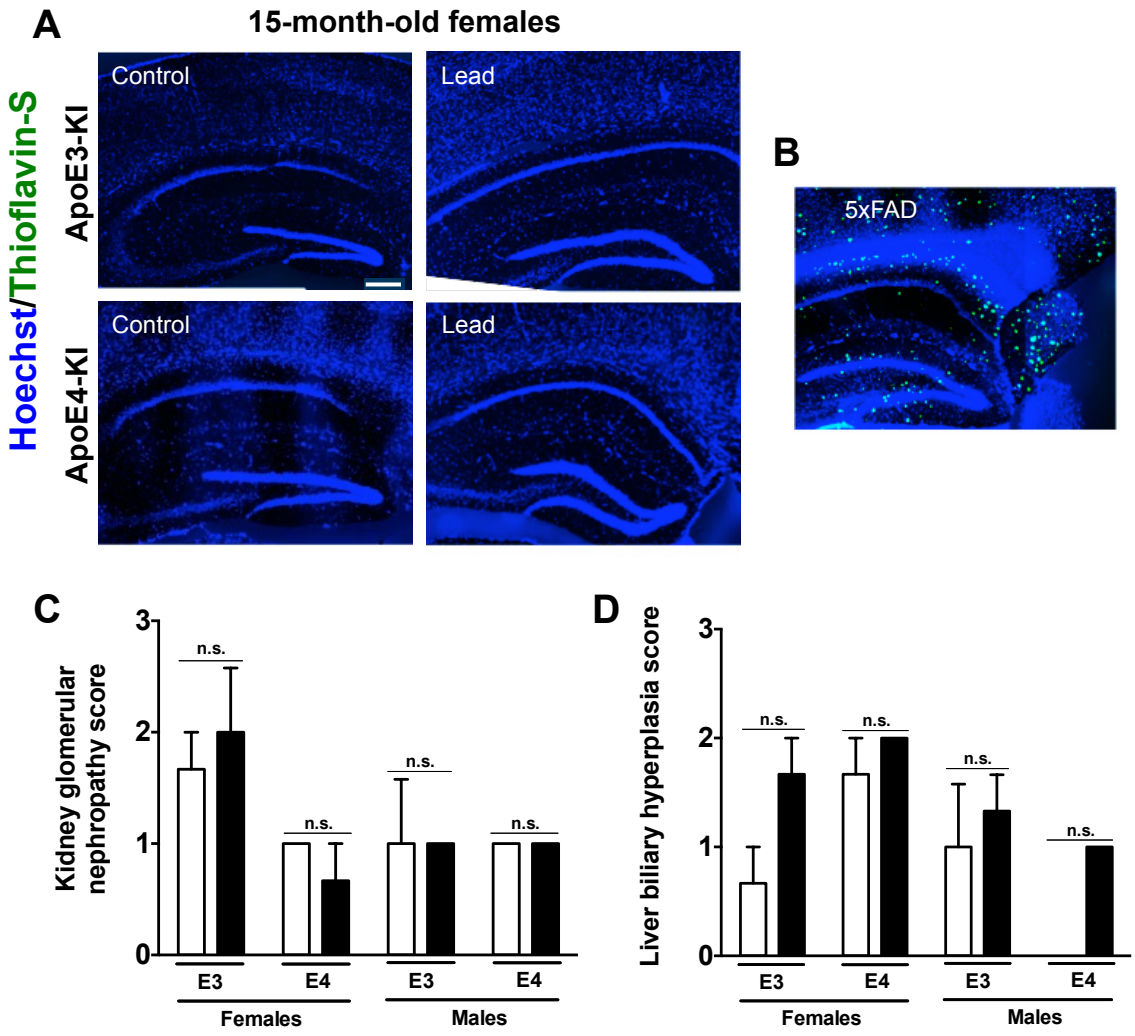

Figure 4.2. Adult lead does not cause significant liver or kidney toxicity or result in increased amyloid deposition in the brain in aged ApoE3-KI and ApoE4-KI mice. 8-week-old male and female ApoE3-KI and ApoE4-KI mice were exposed to 0.2% lead acetate for 12 weeks and then switched to normal drinking water and sacrificed 10-months post-lead (15 months old). (***A***) Representative images of Thioflavin-S (green) staining in the hippocampus of 15-month-old female ApoE3-KI and ApoE4-KI control and lead-treated mice or (***B***) a 12-month-old 5xFAD female mouse (positive control). Summary of (***C***) kidney glomerular nephropathy and (***D***) liver biliary hyperplasia scoring from hemotoxylin and eosin stained sections of 15-month-old male and female ApoE3-KI and ApoE4-Ki mice. Data are mean ± SEM. Thioflavin-S staining: n= 3-5 per genotype/treatment. Pathology scoring: n= 3 per genotype/sex/treatment. Two-way ANOVA with Fisher's LSD *post-test*: n.s., not significant. Scale bar, 100 μm.

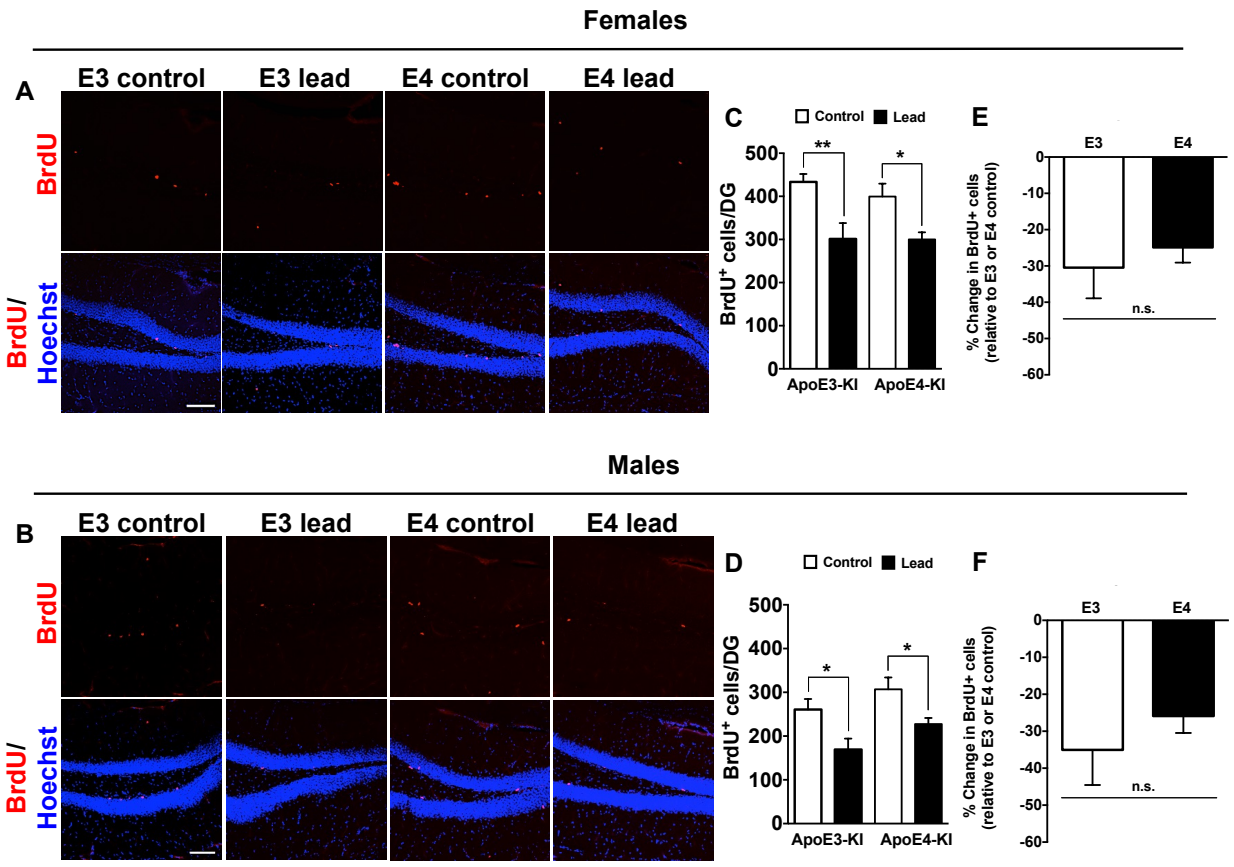

Figure 4.3. Adult lead exposure decreases adult-born cell proliferation in the DG of the hippocampus of all mice. 8-week-old male and female ApoE3- and ApoE4-KI mice were exposed to 0.2% lead acetate for 12 weeks and then sacrificed. BrdU was administered 2 h prior to sacrifice (1 x 100 mg/kg). Representative images of BrdU (red) immunostaining in the DG of 5-month-old (***A***) female and (***B***) male ApoE3-KI and ApoE4-KI control and lead-treated mice. Quantification of the total BrdU+ cells per DG in (***C***) females and (***D***) males. Quantification of the percent change in total BrdU+ cells in lead-treated mice relative to ApoE3-KI or ApoE4-KI control (***E***) females and (***F***) males. Data are mean ± SEM with n= 3-5 per genotype/sex/treatment. Two-way ANOVA with Fisher's LSD post-test: n.s., not significant; * $p < 0.05$; ** $p < 0.01$. Scale bars, 100 µm.

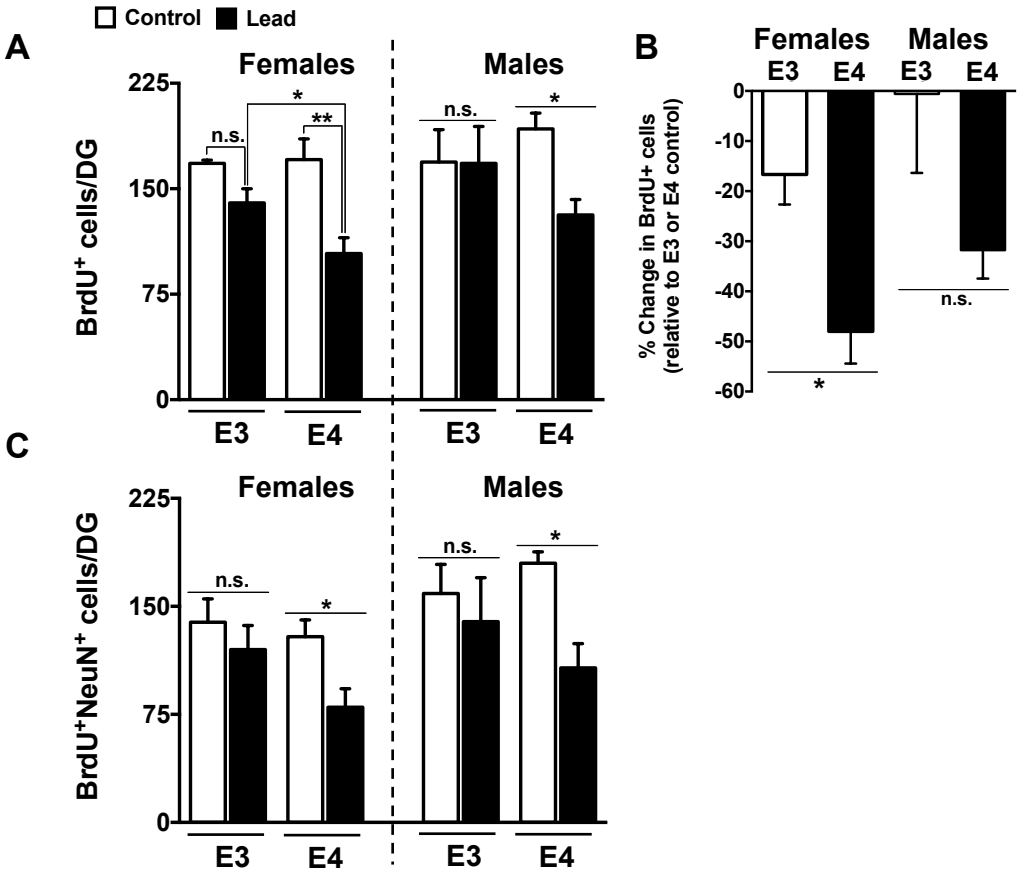

Figure 4.4. Lead decreases the total number of adult-born cells and adult-born mature neurons in the DG of ApoE4-KI males and females. 8-week-old male and female ApoE3-KI and ApoE4-KI mice were exposed to 0.2% lead acetate for 12 weeks and then sacrificed. BrdU was administered 7 weeks prior to sacrifice (5 x 100 mg/kg every 2 h in one day). Quantification of the (***A***) total number of BrdU+ cells and the (***B***) percent change in BrdU+ cells between lead-treated and control ApoE3-KI and ApoE4-KI mice 7 weeks post-BrdU. ***C***, Quantification total number of BrdU+NeuN+ cells 7 weeks post-BrdU. Data are mean ± SEM with n= 3-5 per genotype/sex/treatment. Two-way ANOVA with Fisher's LSD post-test: n.s., not significant; * $p < 0.05$; ** $p < 0.01$. Scale bar, 25 µm.

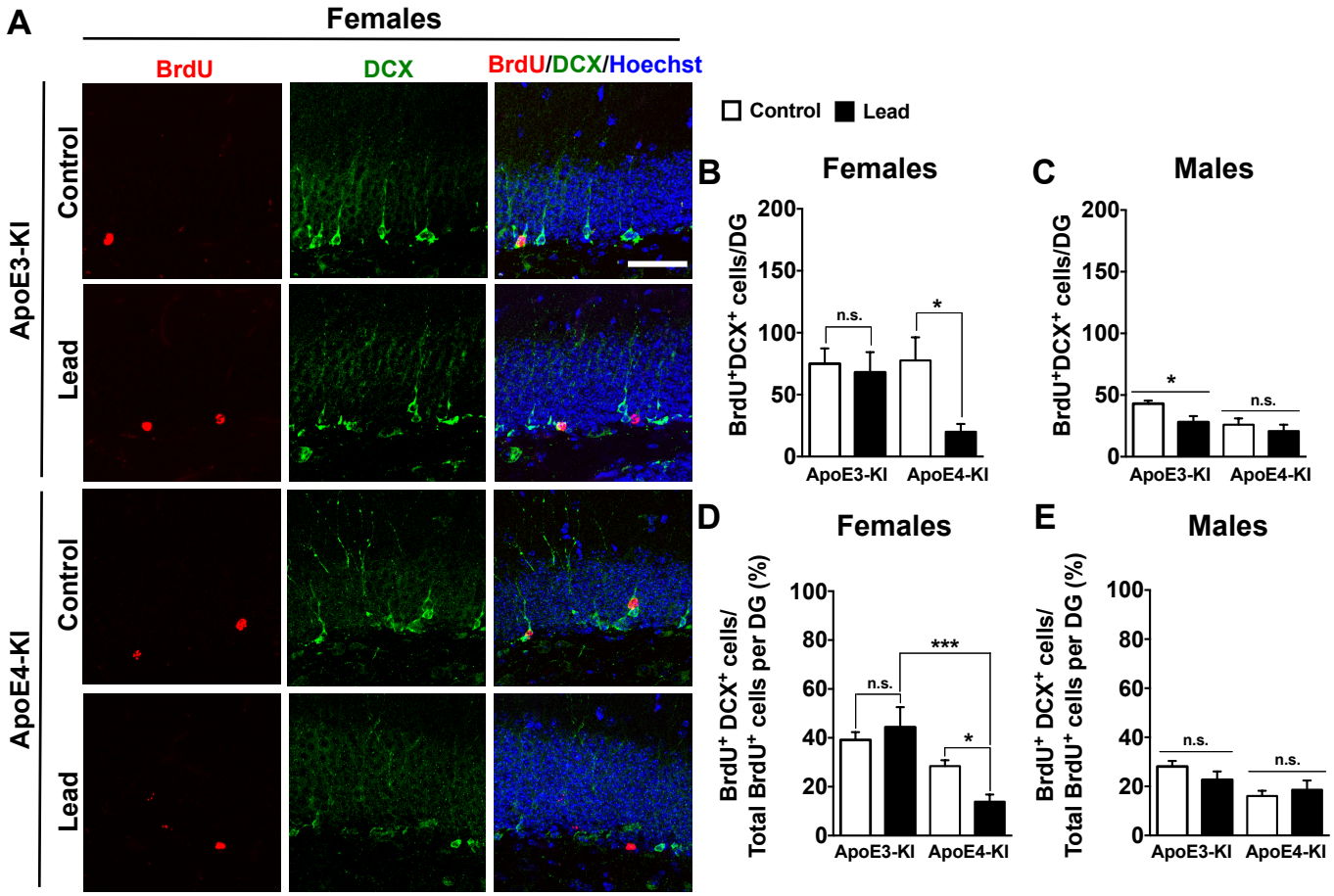

Figure 4.5. Lead decreases adult-born immature neuron differentiation in the DG of ApoE4-KI female mice. 8-week-old male and female ApoE3- and ApoE4-KI mice were exposed to 0.2% lead acetate for 12 weeks and then sacrificed. 100 mg/kg BrdU was administered 5 times in one day (every 2 h) 3 weeks prior to sacrifice (week 17). (***A***) Representative images of BrdU (red) and DCX (green) immunostaining in the DG of 5-month-oldcontrol and lead-treated female ApoE3-KI and ApoE4-KI animals. Quantification of the total number of BrdU+DCX+ cells per DG in (***B***) females and (***C***) males. Quantification of the percent of total BrdU+ cells that are BrdU+DCX+ per DG in (D) females and (E) males. Data are mean ± SEM with n= 4-5 per genotype/sex/treatment. Two-way ANOVA with Fisher's LSD post-test: n.s., not significant; * $p < 0.05$; *** $p < 0.001$. Scale bar, 50 µm.

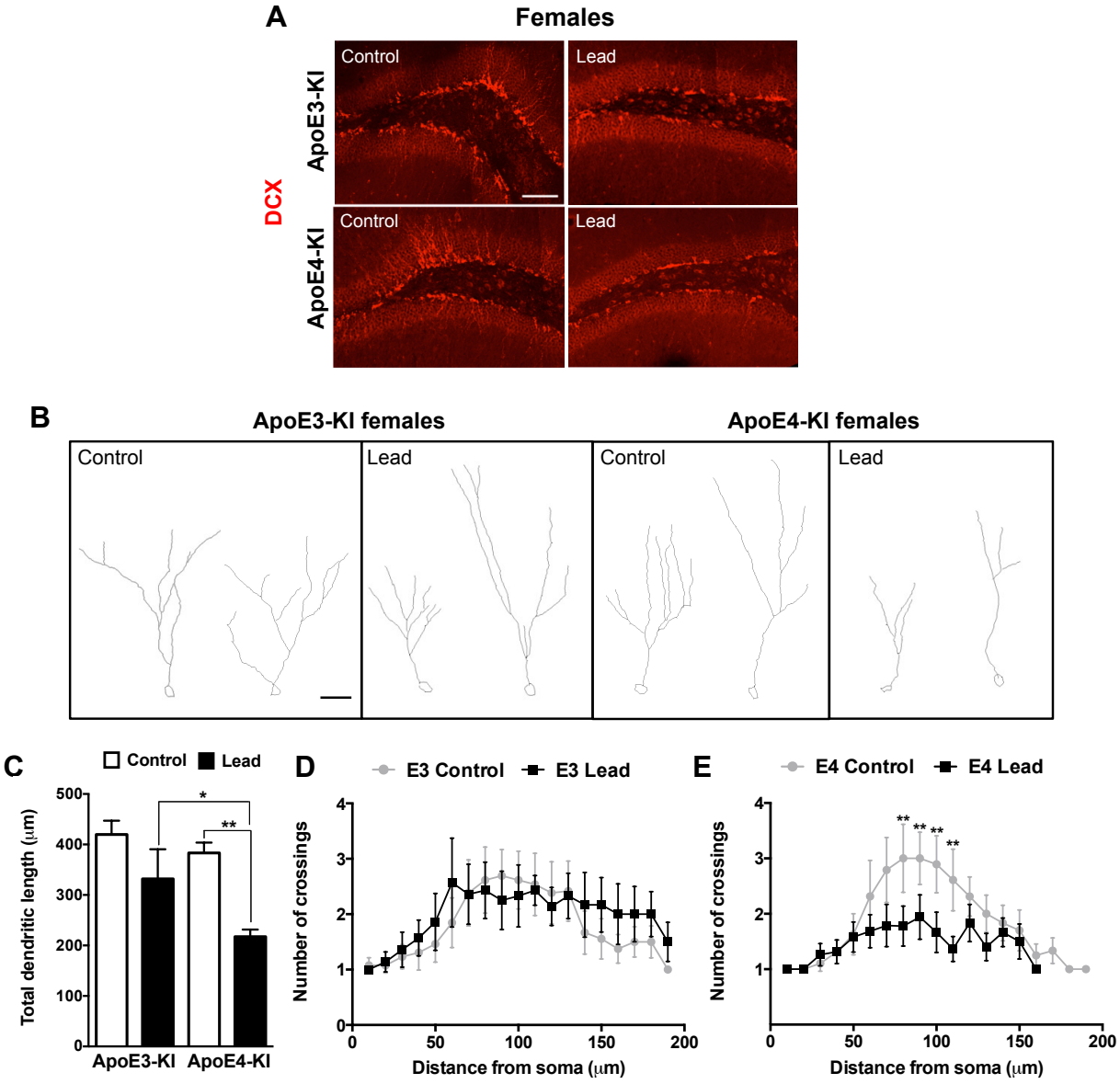

Figure 4.6. Lead decreases the dendritic complexity of immature neurons in the DG of ApoE4-KI female mice. ***A***, Representative confocal images of DCX (red) immunostaining in the DG of 5-month-old female ApoE3-KI and ApoE4-KI control and lead-treated mice (scale bar, 100 μm). ***B***, Representative examples of DCX+ neurons from female ApoE3-KI and ApoE4-KI control and lead-treated mice traced using the ImageJ Simple Neurite Tracer plug-in (scale bar, 25 μm). ***C***, Quantification of the total dendritic length of DCX+ neurons in the DG. Sholl analysis of DCX+ neurons from (***D***) ApoE3-KI and (***E***) ApoE4-KI female mice. Data are mean ± SEM with n= 4-5 per genotype/treatment. Two-way ANOVA with Fisher's LSD post-test for analysis of total dendritic length; two-tailed t-test for within genotype comparisons of the number of crossings in control vs. lead-treated mice: n.s., not significant; * $p < 0.05$; ** $p < 0.01$.

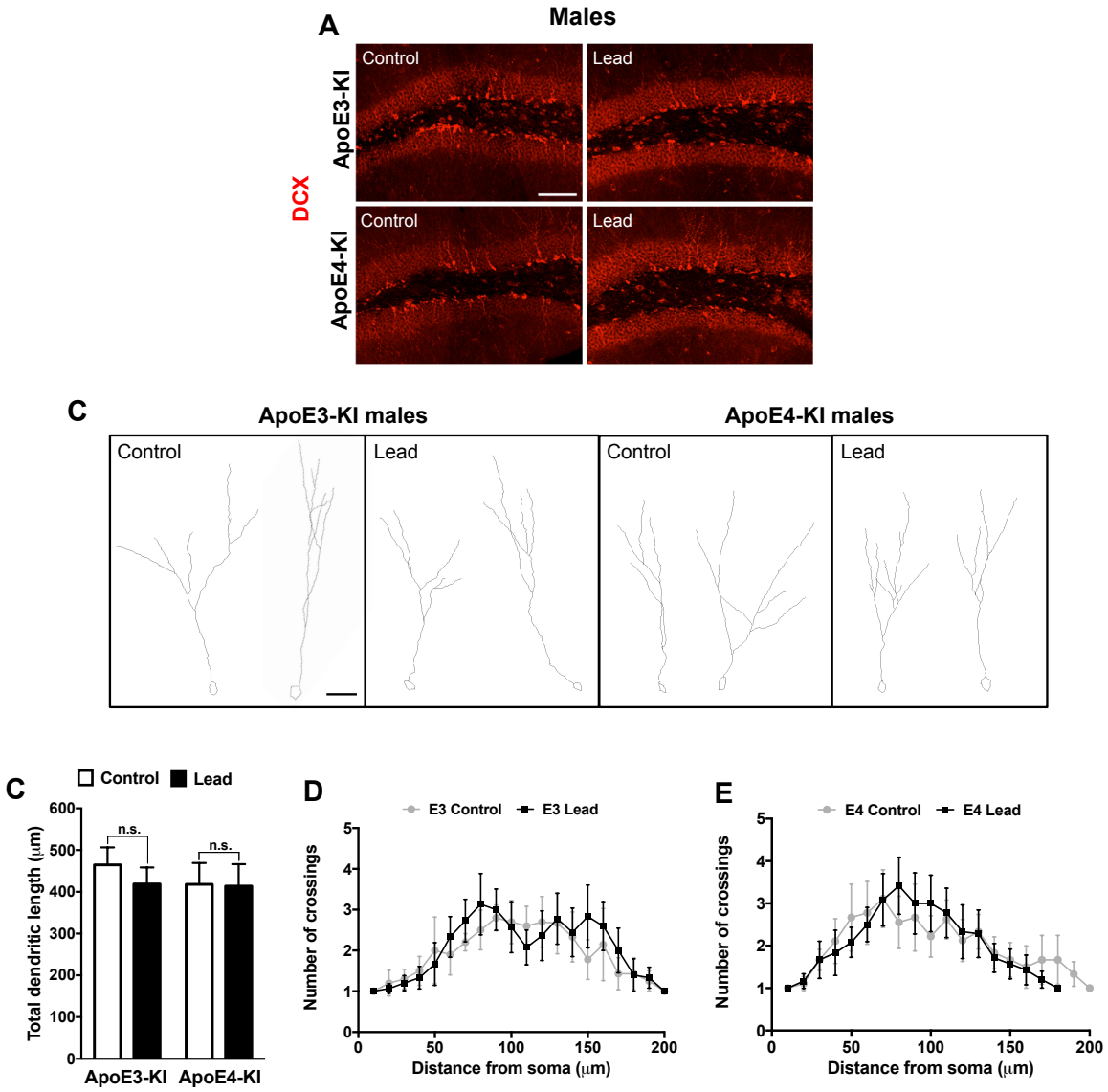

Figure 4.7. Lead does not impair the dendritic complexity of immature neurons in the DG of male mice. ***A***, Representative confocal images of DCX (red) immunostaining in the DG of 5-month-old male ApoE3-KI and ApoE4-KI control and lead-treated mice (scale bar, 100 μm). ***B***, Representative examples of DCX^+ neurons from male ApoE3-KI and ApoE4-KI control and lead-treated mice traced using the ImageJ Simple Neurite Tracer plug-in (scale bar, 25 μm). ***C***, Quantification of the total dendritic length of DCX^+ neurons in the DG. Sholl analysis of DCX^+ neurons from (***D***) ApoE3-KI and (***E***) ApoE4-KI male mice. Data are mean ± SEM with n= 4-5 per genotype/treatment. Two-way ANOVA with Fisher's LSD *post-test*: n.s., not significant; * $p < 0.05$; ** $p < 0.01$; ***, $p < 0.0001$.

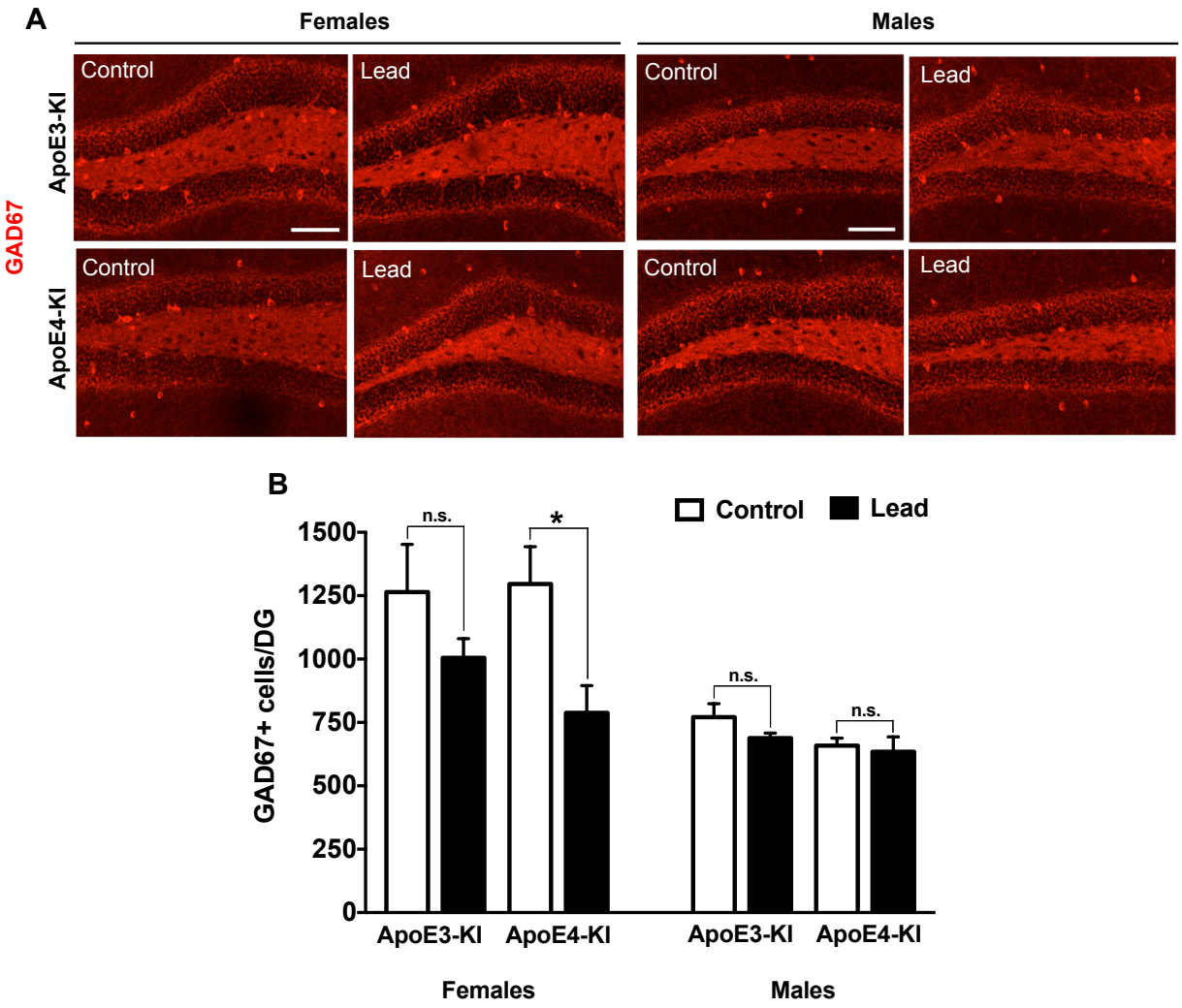

Figure 4.8. Lead decreases the total number of GAD67$^+$ GABAergic interneurons in 5-month-old female ApoE4-KI mice. 8-week-old male and female ApoE3-KI and ApoE4-KI mice were exposed to 0.2% lead acetate for 12 weeks and sacrificed. (***A***) Representative images of GAD67 (red) immunostaining in the DG of 5-month-old female and male ApoE3-KI and ApoE4-KI control and lead-treated mice. (***B***) Quantification of the total number of GAD67$^+$ cells in the DG of 5-month-old ApoE3-KI and ApoE4-KI females and males. Data are mean ± SEM with n= 3-5 per genotype/treatment. Two-way ANOVA with Fisher's LSD *post-test*: n.s., not significant; * $p < 0.05$. Scale bar, 100 µm.

Chapter 5: Conclusions and future directions

In my dissertation research, I found that environmentally relevant concentrations of lead impair the survival, proliferation, and differentiation of adult neural precursor cells *in vitro*. Furthermore, I found that these effects may be mediated by the activation of the proapoptotic JNK and p38 MAP kinases and inhibition of the prosurvival Akt signaling. In an *in vivo* pilot study assessing the effect of subchronic lead exposure in wild-type male mice (data not shown), I found that lead caused persistent deficits in spatial working memory and impairs certain stages of adult hippocampus neurogenesis. Based on these data, I wanted to determine whether a GXE between ApoE4 and lead may cause more severe or an earlier onset of learning and memory deficits and also more significant impairment of adult hippocampal neurogenesis. Through extensive cognitive behavior characterization, I observed evidence of a potential interaction between ApoE4 and lead on learning and memory, with lead-treated ApoE4-KI mice exhibiting more severe or earlier deficits in learning and memory. I also observed sex differences in these learning and memory deficits, with females exhibiting more severe or an earlier onset of deficits. Furthermore, I found that female lead-treated ApoE4-KI mice had significantly impaired adult-born immature neuron maturation and differentiation, suggesting that a GXE between lead and ApoE4 may potentiate the effects of lead or ApoE4 alone on neurogenesis. These data are exciting and novel and, together, provide important insight into how environmental toxicants and genetic risk factors may facilitate cognitive decline and neurodegeneration.

In the future, it would be interesting to assess the dose-response relationship between lead exposure and ApoE4 genotype on cognitive behavior and adult hippocampal neurogenesis. Looking at lower, environmentally relevant lead concentrations would help elucidate whether background levels of lead are sufficient to impair learning and memory and facilitate cognitive decline. In addition, extending the exposure window to include developmental lead exposure would help characterize the cumulative effects of lead on developmental and adult neurogenesis. Finally, it would be very interesting to use ApoE3/E4-KI heterozygous mice as well as the ApoE2-KI model to assess for a potential gene-dose dependent effect of E4 as well as to assess for a potential protective effect of the ApoE2 allele upon exposure to lead.

It would also be very interesting to do additional function characterization of the effects of a GXE between lead and ApoE4 on adult-born, immature neurons. Electrophysiological characterization of these adult-born cells would be very important in order to elucidate whether a GXE has functional consequences on hippocampal circuitry. In addition, additional characterization of the underlying mechanisms for the observed sex differences in susceptibility would be very interesting.

Finally, while I observed evidence of a GXE on both cognitive behavior and adult hippocampal neurogenesis, these associations are still only correlative. I cannot conclude that the observed functional deficits in lead-treated ApoE4-KI animals are directly due to GXE on adult hippocampal neurogenesis. To establish a causal link between the cellular and behavior impairments, future

studies could determine if conditional genetic stimulation of adult neurogenesis is sufficient to mitigate impairments in adult hippocampal neurogenesis and learning in lead-treated ApoE4-KI mice. Dr. Xia's lab recently generated a transgenic mouse line in which adult neurogenesis can be selectively and conditionally stimulated by activating the ERK5 MAP kinase signaling pathway upon tamoxifen induced, nestin-cre-mediated gene recombination (Wang et al., 2014). Inducible and conditional expression of one copy of constitutively active MEK5 (caMEK5), the activating kinase for ERK5, in adult neurogenic regions (under the control of the Nestin-Cre-ER^{TM} promoter) is sufficient to improve adult hippocampal neurogenesis and hippocampus-dependent learning and memory (Wang et al., 2014). Specifically, inducible and conditional activation of ERK5 promotes cell survival and neuronal differentiation, the same processes in adult neurogenesis that are impaired at 5-7 months in ApoE4-KI mice (Li et al., 2009) and that I found lead impairs in wild-type cells (Chapter 2). Our lab is currently breeding the ApoE4-KI mice with the Nestin-Cre-ER^{TM}:caMEK5-eGFP$^{lokP/loxP}$ animals, and the resulting ApoE4-KI:Nestin-Cre-ER^{TM}:caMEK5-eGFP$^{lokP/loxP}$ mice will be treated with tamoxifen to induce caMEK5 expression and to stimulate adult neurogenesis (ApoE4-KI:caMEK5). Mice treated similarly with vehicle for tamoxifen will be used as controls (ApoE4-KI:control). Thus, these future experiments will allow us to determine if genetic activation of adult neurogenesis is sufficient to rescue the observed learning and memory deficits in lead-treated ApoE4-KI animals.

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