Cadmium exposure impairs adult neurogenesis, cognition, and olfactory memory in mice

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Abstract

Cadmium exposure impairs adult neurogenesis, cognition and olfactory memory in mice

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Cadmium (Cd) is a ubiquitous toxic heavy metal of high interest to the Superfund initiative. It causes toxic effects to many organs, including kidney, liver, lung, and bone. Cd is also a potential neurotoxicant. Recently, several epidemiology studies have found an association between Cd exposure and cognitive as well as olfactory impairments in humans. However, studies in animal models are needed to establish a direct causal relationship between Cd exposure and impaired cognitive and olfactory functions. In this study, by exposing adult male mice to 3 mg/L Cd through drinking water, I found that Cd exposure induces persistent impairments of hippocampus-dependent learning and memory in the novel object location test, the T-maze test, and the contextual fear memory test, but not in the water maze test in young adult mice. In addition, I also found that Cd exposure impairs olfactory memory in mice.

Adult neurogenesis is a process that generates functional new neurons from adult neural progenitor/stem cells (aNPCs). It is spatially restricted to two specific regions in the adult brain: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) along the lateral ventricles. These adult-born neurons in the SGZ and SVZ contribute to hippocampus-dependent memory and olfaction, respectively. The process of adult neurogenesis can be modulated by various extracellular and intracellular stimuli, but the studies on how neurotoxicants affect this important process are still lacking. Using an *in vitro* model of adult neurogenesis, I found that Cd significantly increases apoptosis, inhibits proliferation, and impairs spontaneous neuronal differentiation. In addition, activation of JNK and p38 MAPK signaling pathways are involved in Cd cytotoxicity in aNPCs. Furthermore, I also observed that adult Cd exposure impairs SGZ and SVZ adult neurogenesis *in vivo.*

Additionally, by using caMEK5 mice, a gain-of-function knock-in transgenic mouse line that allows inducible and conditional activation of adult neurogenesis, I found that enhancing adult neurogenesis by genetic manipulation after Cd exposure can rescue mice from Cd-induced impairments of cognition and olfactory memory.

Together, these results suggest that Cd exposure impairs hippocampus-dependent learning and memory, as well as olfactory memory in mice. In addition, Cd can directly act on aNPCs to impair critical processes of adult neurogenesis. Furthermore, Cd-induced impairments of cognition and olfaction in mice can be rescued by inducible and conditional enhancement of adult neurogenesis. My research provides the first, clear demonstration of Cd neurotoxicity on cognitive and olfactory functions in an animal model and established a causal relationship between Cd-impaired adult

neurogenesis and cognitive as well as olfactory deficits in mice. It provides new insights regarding Cd neurotoxicity and adds useful information for Cd risk assessment.

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Chapter 1: Introduction

1.1 Cadmium as an environmental pollutant

Cadmium (Cd), one of the biologically nonessential heavy metals, is considered to be one of the top 20 hazardous substances around the world (Liao and Freedman, 1998). While Cd is a naturally occurring element of relatively poor abundance in the earth's crust (0.1-0.5 ppm), most environmental Cd contamination is from anthropogenic sources, such as the production of alkaline nickel-cadmium batteries, smelting, burning of fossil fuels, refining of metals, and the application of phosphate fertilizers (Faroon et al., 2012). The industrial Cd consumption in the world has increased steadily from 18400 tons in 2003 to 20400 tons in 2007 (Moulis and Thevenod, 2010). In the United States, the annual Cd refinery production between 2003 and 2006 declined from 1450 to 700 tons, but Cd imports of metal, alloys, and scrap increased from 112 to 288 tons from 2003 to 2005, and then declined through 2007 (Faroon et al., 2012). Cd can be released into the three major compartments of the environment – air, water, and soil, and then cause environmental pollution. So far, Cd has been identified in at least 1014 of the 1669 hazardous waste sites that have been proposed for inclusion on the EPA National priorities List (NPL) (Faroon et al., 2012). Meanwhile, Cd ranks No.7 on the Superfund hazardous waste priority list.

Due to the high rate of soil-to-plant transfer, diet is the main source of Cd exposure in non-smokers in the general population in most parts of the world (Clemens, 2006). In general, vegetables, particularly leafy vegetables, such as spinach and lettuce, contain the highest Cd concentrations (from 0.051 mg/kg-0.124 mg/kg) (Faroon et al., 2012). In addition, peanuts, soybeans, rice, shellfish, and organ meats also have high

concentrations of Cd (Clemens, 2006; Satarug et al., 2010). Based on the daily Cd intakes of males and females from 6-60 years old, age-weighted mean Cd intakes in nonsmokers are 0.35 μg/kg/day for males and 0.30 μg/kg/day for females (Choudhury et al., 2001). Since tobacco leaves naturally accumulate Cd, smoking is another important source of Cd exposure in the general population. Tobacco contains about 0.5-2.0 μg Cd per cigarette, and about 10% is inhaled when smoked (Faroon et al., 2012). Accordingly, smokers usually have higher blood Cd concentrations. In the U.S. population represented by the National Health and Nutrition Examination Survey (NHANES) 1999 - 2010, geometric mean blood Cd level was 0.37 μg/L in men, and 0.41 μg/L in women (Adams and Newcomb, 2014). For smokers, including former and current smokers, higher smoking dose was associated with elevated mean blood Cd concentrations (Adams and Newcomb, 2014). Another study found that the geometric mean blood Cd concentration of the heavy smoker in New York was 1.58 μg/L, compared to the 0.77 μg/L for all New York adults (McKelvey et al., 2007). Meanwhile, workers in certain occupations may be exposed to Cd and Cd compounds. High levels of Cd exposure are expected to occur in operations involving smelting, welding, or electroplating with Cd containing products. The primary route of occupational exposure is inhalation.

Since Cd has a long half-life (about 10-30 years) in the human body (Waisberg et al., 2003), chronic Cd exposure can induce toxic effects in various human organs. For example, kidney and bone are two major targets of Cd toxicity. Cd exposure is the major cause of Itai-itai disease. This disease affects humans by severe renal tubular and glomerular function impairments, generalized osteomalacia and osteoporosis that

result in bone fractures (Inaba et al., 2005). Lung is another target organ of Cd. Inhalation of high levels of Cd can cause respiratory stress and respiratory tract injures. Studies have found associations between Cd exposure and emphysema, as well as respiratory distress syndrome (Lampe et al., 2008; Manca et al., 1994). Cd also impairs many other organs, including liver (Goering et al., 1993; Torra et al., 1995), testis (Mao et al., 2012; Oliveira et al., 2012), and gastrointestinal tract (Tinkov et al., 2018; Waisberg et al., 2005). In addition, Cd has been classified as a Group 1 human carcinogen (particularly lung and prostate cancer) by the International Agency for Research on Cancer (IARC), based on sufficient evidence for carcinogenicity in both humans and animals (Faroon et al., 2012).

1.2 Cadmium as a potential neurotoxicant

Previous studies have suggested that Cd is a potential neurotoxicant. For example, Cd can induce cell death in different neural cells (Chen et al., 2008; Lopez et al., 2003; Wang et al., 2017; Yuan et al., 2013). Several animal studies found Cd induces impairments of the central nervous system and related behaviors in animals, such as extensive hemorrhages in the cerebral cortex and cerebellum, altered neurotransmitter function, and increased aggressive behavior and anxiety (Ali et al., 1986; Baranski and Sitarek, 1987; Gabbiani et al., 1967; Goncalves et al., 2012; Nation et al., 1984). Nevertheless, the information about Cd neurotoxicity is still very limited. Recently, several epidemiology studies have linked Cd exposure to impairments of cognition in humans (Ciesielski et al., 2013b; Ciesielski et al., 2012). A study conducted among 5662 U.S. adults found that higher cumulative Cd exposure is

associated with subtly decreased performance in tasks requiring attention and perception. Importantly, this association was observed among exposure levels (Urinary Cd concentration: 0.19-0.82 μg/L) that are considered to be without any adverse effect and common in U.S. adults (Ciesielski et al., 2013b). Researchers also suggest that children in the U.S. who have higher urinary Cd levels may have increased risk of learning disability and requiring special education (Ciesielski et al., 2012). The association was observed at Cd levels that are thought to be safe and common among children in the United States. Another study conducted in Bangladesh found that childhood Cd exposure was associated with low IQ in boys, and there were indications of altered behavior in girls (Kippler et al., 2012). Although the findings are suggestive and interesting, these studies are limited by potential confounding factors, making it difficult to conclude that Cd exposure alone, at environmentally relevant levels, can impair cognitive function.

Animals can recognize and discriminate chemical signals from the environment by the olfactory system. The olfactory system does not only provide essential information for survival, but also influences behavior and memory. For example, olfactory dysfunction is associated with the onset of certain forms of neurodegenerative diseases including Parkinson's and Alzheimer's diseases (Hawkes, 2006).

Occupational high-level Cd exposure has been associated with olfactory disorders in workers (Mascagni et al., 2003; Suruda, 2000). Animal studies also demonstrated that acute intranasal administration of Cd induces destruction of olfactory structures, decreased axonal projections, and impaired olfactory functions (Bondier et al., 2008; Czarnecki et al., 2012). Together, the human and animal studies suggest that

high dose Cd exposure may impair olfactory function. However, the information about the effects of chronic low-level Cd exposure, through ingestion, on olfactory function in mice is limited.

1.3 Adult neurogenesis

Adult neurogenesis is a process that generates functional new neurons from adult neural progenitor/stem cells (aNPCs). Neurogenesis was traditionally considered to occur only during the embryonic period. The genesis of adult-born neurons was not fully accepted despite Altman's study which provided the first evidence for the presence of newly generated cells in the hippocampus of adult rat (Altman and Das, 1965). It was many years later following the seminal work on songbird adult neurogenesis that adult neurogenesis has been accepted as a physiological phenomenon (Alvarez-Buylla et al., 1988). So far, researchers have demonstrated that neurogenesis also occurs in the brains of adult mammals (Reynolds and Weiss, 1992; Richards et al., 1992).

Under normal physiological conditions, active adult neurogenesis is restricted to two specific regions in the adult brain: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002; Alvarez-Buylla and Lois, 1995; Gage, 2000; Ming and Song, 2011). New neurons born in the SGZ extend dendrites toward the molecular layer and project axons to integrate into the hippocampal circuitry, while adult-born cells in the SVZ migrate along the rostral migratory stream (RMS) to the olfactory bulb and become interneurons there (Aimone et al., 2011; Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch et al., 1999; Gage, 2000; Gage et al., 1998).

SGZ adult neurogenesis plays an important role in hippocampus-dependent learning and memory. Dupret et al. (2008) found that reducing adult-born neurons causes a significant decline in the performance of spatial learning and memory in mice. Suppression of adult neurogenesis causes deficits in pattern separation in the radial arm maze assay, suggesting that adult neurogenesis is important for spatial pattern separation (Clelland et al., 2009; Farioli-Vecchioli et al., 2008; Pan et al., 2012a). Similarly, the selective blocking of SGZ adult neurogenesis impairs the formation and extinction of contextual fear memory (Deng et al., 2009; Imayoshi et al., 2008; Pan et al., 2012c; Saxe et al., 2006). Consistent with the idea that SGZ adult neurogenesis is important for hippocampus-dependent learning and memory, Creer et al. (2010) found that increasing adult neurogenesis by permitting mice to run on running wheels resulted in an enhanced ability of discrimination between spatial pattern differences using the visual touch-screen box (Creer et al., 2010). Wang et al. (2014) demonstrated that conditional activation of ERK5 increased SGZ adult neurogenesis and further improves the performance of spatial learning and memory and extended hippocampus-dependent long-term memory (Wang et al., 2014).

SVZ/OB adult neurogenesis is involved in olfactory functions. Breton-Provencher et al. (2009) found that a reduction of SVZ/OB adult neurogenesis reduces odor detection sensitivity and impairs short-term olfactory memory. In addition, other studies found diminishing SVZ/OB adult neurogenesis in mice impairs performance in odor discrimination task, short-term olfactory memory test, odorant detection sensitivity test, and odor-cued associative olfactory learning test (Gheusi et al., 2000; Pan et al., 2012b). Consistently, inducible activation of adult neurogenesis in SVZ/OB improves

short-term olfactory memory and odor-cued associative olfactory learning in mice (Wang et al., 2015).

The process of adult neurogenesis can be modulated by various intracellular and extracellular stimuli. Different intracellular molecules and signaling pathways have been identified to have effects on adult neurogenesis, including niche factors/receptors, transcriptional factors, cytoplasmic factors and epigenetic regulators (Ma et al., 2010; Mu et al., 2010; Ninkovic and Gotz, 2007). Meanwhile, many physiological and pathological stimuli can affect adult neurogenesis. For example, physical exercise increases aNPC proliferation, while an enriched environment increases the survival of aNPCs. In contrast, aging impairs proliferation of aNPCs both in the SVZ and SGZ (Kempermann et al., 1997; Liu et al., 2007; van Praag et al., 1999). In addition, lead (Engstrom et al., 2015) and polybrominated diphenyl ether (PBDE) (Li et al., 2013b) exposure impair adult neurogenesis, including inducing apoptosis, inhibiting proliferation, and affecting spontaneous differentiation. As discussed before, Cd is a potential neurotoxicant; it has been found that Cd can induce cell death in different neural cells and Cd exposure is associated with impairments of cognitive and olfactory function in adult humans. However, little is known about the effects of Cd on adult neurogenesis.

1.4 Extracellular Signal-Regulated Kinase 5 (ERK5) signaling pathway

ERK5 is a member of the Mitogen-Activated Protein Kinase (MAPK) family. It shares high sequence homology with ERK1/2 in its kinase domain and TEY dual phosphorylation motif. ERK5 contains a special loop-12 linker and an extended C-terminus, which makes it distinct from the other members of the MAPK family. The C-

terminus of ERK5 contains a nuclear localization signal (NLS) and a proline-rich region. ERK5 can be activated by growth factors and stress. Under normal conditions, it is localized in the cytoplasm, but upon activation by extrinsic factors, ERK5 translocates to the nucleus via the NLS sequence (English et al., 1995; Lee et al., 1995; Zhou et al., 1995).

The ERK5 signaling pathway is involved in proliferation, differentiation, and survival of various cell types, including neural cells (Cavanaugh et al., 2001; Hayashi et al., 2004; Hayashi and Lee, 2004; Liu et al., 2003). For example, Activated ERK5 promotes the survival of newborn neurons during embryonic development (Cavanaugh et al., 2001; Liu et al., 2003; Shalizi et al., 2003; Wang et al., 2006). ERK5 also specifies cortical cells toward a neuronal lineage during development (Cundiff et al., 2009; Liu et al., 2006). In addition, our lab has demonstrated that ERK5 is expressed in both the SGZ and SVZ in adult mouse brain, and inducible and conditional knock out ERK5 in the adult neurogenesis regions (SGZ and SVZ) impairs adult neurogenesis and disrupts several forms of hippocampus-dependent learning and memory, as well as olfactory functions (Li et al., 2013a; Pan et al., 2012a; Pan et al., 2012b; Pan et al., 2012c).

MEK5 is a highly specific upstream activator of ERK5. Once activated, the N-terminus of MEK5 binds to the functional domain of ERK5 and induces dual phosphorylation of the TEY motif to activate ERK5 (Drew et al., 2012). All effects of MEK5 have been attributed to the activation of ERK5. Recently, our lab has discovered that inducible and conditional activation of MEK5-ERK5 signaling pathway increases adult neurogenesis by enhancing cell survival and neuronal differentiation both in the

SGZ and SVZ/OB. Furthermore, selective and targeted activation of ERK5 also improves the performance of hippocampus-dependent spatial working memory and long-term memory, as well as short-term olfactory memory and odor-cued associative olfactory learning (Wang et al., 2015; Wang et al., 2014). These results further suggest that MEK5-ERK5 signaling pathway is important for adult neurogenesis, which plays a functional role in hippocampus-dependent memory and olfactory behaviors.

1.5 Knowledge gap and goals of dissertation study

Cd is a highly toxic heavy metal that can cause toxic effects to many organs, including kidney, liver, lung, and bone. Cd is also a potential neurotoxicant. Several epidemiology studies suggest that Cd exposure is associated with impairments of cognition and olfaction in humans. Since epidemiology studies could not rule out confounding factors, there is no direct evidence that Cd exposure alone can impair hippocampus-dependent memory and olfaction in animal models. Meanwhile, very little information is known regarding the underlying molecular and cellular mechanisms of Cd neurotoxicity.

Adult neurogenesis is a process that generates functional neurons from adult neural progenitor/stem cells (aNPCs). It is spatially restricted to two specific regions in adult brain: the subgranular zone (SGZ) in dentate gyrus, and the subventricular zone (SVZ). Adult neurogenesis in SGZ and SVZ/OB plays an important role for hippocampus-dependent memory and olfaction, respectively. The process of adult neurogenesis can be modulated by various extracellular and intracellular stimuli. But studies on how neurotoxic agents affect this process are still lacking. Based on this

background information, **the goal of my dissertation research is to test the hypothesis that Cd exposure impairs hippocampus-dependent learning and memory, and olfactory functions through affecting adult neurogenesis in mice.** The study includes 3 specific aims.

Specific aim 1: Determine if Cd exposure impairs hippocampus-dependent learning and memory, as well as olfactory functions in mice. To address this aim, I have exposed a group of adult male mice to low-level Cd through drinking water. The behavioral tests were conducted before, during, and after exposure to analyze the effects of Cd on hippocampus-dependent memory and olfactory functions.

Specific aim 2: Determine whether Cd exposure affects adult neurogenesis in SGZ and SVZ/OB. By using cultured SGZ and SVZ aNPCs, I examined the effects of Cd on adult neurogenesis *in vitro*. In addition, I investigated if Cd can impair SGZ and SVZ/OB adult neurogenesis *in vivo*.

Specific aim 3: Determine if enhancing adult neurogenesis can rescue mice from Cd-induced behavior impairments. By using caMEK5 mice, a gain-of-function knock-in mouse line that allows inducible and conditional activation of adult neurogenesis, I examined if conditionally enhancing adult neurogenesis by genetic manipulation can rescue the toxic effects of Cd on hippocampus-dependent learning and memory, as well as olfaction.

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Chapter 2: Cadmium exposure impairs cognition and olfactory memory in male C57BL/6 mice

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2.1 Introduction

Cadmium (Cd) is a ubiquitous toxic heavy metal pollutant, ranking No.7 on the US Environmental Protection Agency (EPA)'s Superfund hazardous waste priority list. Cd is released into the environment by both natural processes and human activities such as the burning of fossil fuels and wastes, refining of metals, smelting, and the use of phosphate fertilizer (United States. Agency for Toxic Substances and Disease Registry., 2008). Due to its high rate of soil-to-plant transfer compared with other metals, Cd is a common contaminant found in a variety of foods (Clemens, 2006). Currently, food and cigarette smoking are the two main sources of Cd exposure in the general population (Clemens, 2006; Satarug et al., 2010). Occupational Cd exposure is another important route of Cd exposure (Borjesson et al., 1997; Gunier et al., 2013; Lauwerys et al., 1979; Vahter et al., 1991). Since it has a long half-life in the human body (about 10-30 years) (Waisberg et al., 2003), chronic exposure to Cd can induce toxic effects in various human organs including kidney (Torra et al., 1995), liver (Goering et al., 1993; Torra, et al., 1995), bone (Chen et al., 2009; Wallin et al., 2013), lung (Manca et al., 1994; Shukla et al., 2000), and testis (Mao et al., 2012; Oliveira et al., 2012).

Other studies suggest that Cd is also a neurotoxicant. *In vitro* experimental studies have shown that Cd can induce cell death in different neural cells and primary neural stem/progenitor cells (Chen et al., 2008; Lopez et al., 2003; Wang et al., 2017;

Yuan et al., 2013). Several animal studies reported that Cd induces extensive hemorrhages in the cerebral cortex and cerebellum, affects neurotransmitter function, and impairs passive avoidance, schedule controlled responding, and conditioned suppression (Ali et al., 1986; Gabbiani et al., 1967; Goncalves et al., 2010; Goncalves et al., 2012; Maodaa et al., 2016; Nation et al., 1984; Nation et al., 1983; Wong and Klaassen, 1982). Nevertheless, the full spectrum of Cd's neurotoxic effects is still not fully established.

Several epidemiology studies have found associations between Cd exposure and impairments of cognition and olfaction in humans (Ciesielski et al., 2013b; Ciesielski et al., 2012; Mascagni et al., 2003; Rose et al., 1992). Although suggestive and very interesting, these studies are limited by potential uncontrolled confounding factors, making it difficult to conclude that Cd exposure alone, at environmentally relevant levels, causes these impairments. This study aims to establish a direct causal relationship between Cd exposure and impairments of cognition and olfaction in an animal model.

2.2 Materials and Methods

Animals and treatments

6-week old male C57BL/6J mice were purchased from Charles River Laboratories and housed (4-5 animals per cage) in standard conditions (12 h light/dark cycle) with food and water provided *ad libitum.* The mice received normal drinking water or drinking water with 3 mg/L Cd (in the form of $CdCl_2$) starting at 8 weeks of age. Animal drinking water with $CdCl_2$ (Cat. 202908, Sigma-Aldrich, St. Louis, MO) was prepared from a stock solution and replaced every week. Water consumption was

monitored every week during the whole exposure period. We used separate groups of mice in our study, one for neurobehavioral testing and one for Cd blood concentration measurement. For the neurobehavioral group (n=10 in control or treated group), the exposure time was 20 weeks, and the same group of animals were repeatedly used for all behavioral tests. For the blood Cd analysis group (n=4 in control or treated group), the exposure time was 5 and 13 weeks. The preparation, use and disposal of hazardous reagents were conducted according to the guidelines set forth by Environmental Health and Safety Office at the University of Washington. All animal care and treatments were approved by the University of Washington Institutional Animal Care and Use Committee.

Open field test

The open field test was used to assess the effects of Cd on locomotor activity and anxiety (Gould et al., 2009). Mice were placed into a 10 inches (width) × 10 inches (depth) × 16 inches (height) TruScan Photo Beam Tracking arena (Coulbourn Instruments, Holliston, MA) with clean Plexiglas sidewalls and their movement was monitored with two sets of infrared breams spaced 0.6 inch apart, providing a spatial resolution of 0.3 inch. Individual animals were allowed to freely explore the arena without prehabituation for 20 min and the data were collected by TruScan 2.0 software (Coulbourn Instruments, Holliston, MA). The total movement distance, total movement time, and average speed were used to assess the effects of Cd on locomotor activity. The time and distance spent in the margin and center, as well as center entries, were used to assess the effects of Cd on anxiety.

Elevated plus maze test

The elevated plus maze test was used to investigate the effects of Cd on anxiety (Rodgers and Johnson, 1995). The elevated plus maze apparatus (San Diego Instruments, San Diego, CA) was used for this test. The maze (26 inches × 26 inches × 15.25 inches) consists of two open arms, two closed arms, and a center area. Each closed arm has a 7-inch wall on two sides, and the center area measures 2 inches × 2 inches. During the test, the maze was placed in the center of the behavior room, and animals were placed into the center of the apparatus facing toward 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. A video camera and ANYmaze software (San Diego Instruments Co, San Diego, CA) were used to track and analyze the animal's movement during the test.

Morris water maze test

The Morris water maze (MWM) test was performed to assess hippocampus-dependent spatial learning and memory (Vorhees and Williams, 2006). This test was conducted as previously described (Wang et al., 2014). The MWM tank was a circular steel tank (47 inches diameter) filled with room temperature water made opaque with nontoxic white paint. The Plexiglas hidden platform (3.9 inches x 3.9 inches x 7.7 inches) was submerged 1-2 cm below the surface of water in one of the tank quadrants. Four extra-maze cues were placed at intervals around the tank. In this test, mice were trained 4 trials/day for 6 consecutive days. For each trial, the mouse was given a maximum of 40 s to find the platform. If the mouse did not find 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 the platform, they were allowed to stay on the platform for 15 s and then dried and returned to their home cage. Twenty-four hours after the last training day, a probe test was performed in which the hidden platform was removed and each animal completed a single, 60 s probe test. After the initial probe test, mice were subjected to reversal training for 4 trials/day and 10 consecutive days in which the hidden platform was moved from the initial quadrant (Q3) to the opposite quadrant (Q1). Twenty-four hours after the last day of reversal training, a reversal probe test was performed. Two days after the reversal probe test, a visible platform test was conducted as previously described (Wang et al., 2014). All trials and tests were performed by an experimenter blinded to treatment, and all of the data from the training trials and probe test were collected using ANYmaze software (San Diego Instruments, San Diego, CA).

Novel object location test

The novel object location (NOL) test was used to assess the effects of Cd on hippocampus-dependent spatial working memory (Mumby et al., 2002). This assay was performed as previously described with a few modifications (Wang et al., 2014). Briefly, each animal was placed into an open field arena (Coulbourn Instruments, Holliston, MA) with two identical objects placed in 2 different corners. During the training session, the mouse was allowed to freely explore the two objects for 5 min and then returned to its home cage. To exclude preference of specific location, alternating corners were used for object presentation. Testing was performed 1 h after training; the animal was then returned to the arena with the same two objects; one object remained in its original

location and the other had been moved to a new location. The time the animal spent actively investigating each object during the training and testing was recorded by camera and quantified after the test. Each training and testing session was scored and analyzed by an experimenter blinded to the animal's treatment. For each NOL test, the discrimination ratio was calculated by dividing the differences in exploration time between novel and familiar locations by the total exploration time. This test is used to compare spatial memory changes at different time points.

T-maze continuous alternation test

Spontaneous alternation is partially due to the hippocampus-dependent spatial working memory. In this study, it was assessed by using a continuous alternation T-maze protocol with minor modifications (Spowart-Manning and van der Staay, 2004). Briefly, the black, plastic T-maze apparatus had two goal arms and one start arm (12.2 inches × 4.5 inches × 8.26 inches), and was placed on a platform (22.5 inches) in the center of a behavior room. The test consists of a first, forced trial and 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. Then, the mouse was allowed to enter the unblocked goal arms. 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, no goal arm was blocked and the mouse was allowed to enter either of the goal arms; once it entered a goal arm, the other goal arm was immediately blocked with a guillotine door. When the animal eventually returned to the start arm, it was sequestered for 5 s while all of the goal arms

were unblocked. This cycle was repeated for a total of 14 times. We defined arm entry as the animal’s tail tip entering the arm. The alternation percentage was calculated by dividing the number of times the animal entered alternating arms by 14 (free-choice trials). An experimenter blinded to animal treatment scored the choice in each test and analyzed the data.

Cued and contextual fear-conditioning tests

Contextual fear memory is another form of hippocampus-dependent learning and memory, and cued fear memory is a hippocampus-independent learning and memory (Phillips and LeDoux, 1992). In this study, a modified cued and contextual fear conditioning test using weak foot shock conditioning paradigm (3 × 0.3 mA, 2 s shocks with 2 min inter-trial intervals) was used as previously described (Engstrom et al., 2017; Pan et al., 2012a). For the conditioning session, the mouse was placed into the foot shock context (10 inches × 10 inches × 16 inches arena with grid shock floor (Coulbourn Instruments, Holliston, MA)) and allowed to freely explore the arena for 2 min before the presentation of a 90 dB, 30s tone (conditioned stimulus, CS). During the last 2 s of the tone, a 0.3 mA foot shock (unconditioned stimulus, US) was delivered. This cycle was repeated two more times before the mouse was returned to its home cage. The CS and US were automatically delivered by the TruScan software (Coulbourn Instruments, Holliston, MA). The contextual fear memory test was then conducted 24 h after conditioning session. The mouse was placed back into the foot shock context for 2 min in the absence of tone or foot shock. For the cued test, which was performed 2 h after context test, the animal was placed into a novel context (new room; hexagonal

Plexiglas arena) and allowed to freely explore the new context for 2 min. The CS (tone) was then presented for 2 min. For the novel context test, which was performed 2 h after the cued test, the mouse was placed into another novel context (new room; rat cage) and allowed to freely explore for 2 min with no presentation of either tone or foot shock. In all three tests, persistent freezing behavior (four paws on the ground, no head or body movement besides breathing) was recorded by video camera and manually quantified during the 2 min scoring period by an experimenter blinded to animal treatment.

Olfactory habituation/dishabituation test

This test was conducted as previously described (Pan et al., 2012b; Wang et al., 2015; Zou et al., 2012). Briefly, mice were trained with mineral oil-laced cotton swabs for four of 60 s presentations with 2 min intervals between each presentation. This pre-training was to ensure that subsequent exposure to odorant laced cotton swabs did not induce a response due to the novelty of the cotton swab. Then, the olfactory habituation/dishabituation test was conducted by presenting isoamyl acetate, citralva, and vanillin sequentially with four 60 s presentations (with 2 min interval) for each odorant. During the test, the duration of the mouse's investigating (sniffing) of the cotton swab was recorded by video camera and scored by an experimenter blinded to animal treatment. The investigation of the swab was defined as the animal's nose approaching to and within 1 cm to the swab. A significant decrease in investigating time during subsequent presentation of the same odorant indicates odor habituation, and an

increase in the time spent in investigating the swab with a new odorant indicates dishabituation.

Threshold for odorant detection test

The threshold test was conducted as previously described (Pan et al., 2012b; Wang et al., 2015). Briefly, animals were presented with 2 cotton swabs simultaneously; one laced with increasing concentration of 1-octanol (50,100, or 200 μM) and another with mineral oil. The locations of the 2 cotton swabs were switched randomly between presentations to avoid spatial learning. Animals were presented with increasing concentrations of the odorant with one concentration for each day on consecutive days. Each presentation lasted for 3 min, and the total investigation time on each swab was recorded and scored by an experimenter blinded to animal treatment after the test. Results are presented as percentage of the time spent investigating the odorant over the total time spent in investigating the 2 swabs. An investigating duration > 50% indicates the animals detected the odorant.

Short-term olfactory memory

Short-term olfactory memory was assessed as previously described (Pan et al., 2012b; Wang et al., 2015). The animals were presented with a cotton swab laced with the same odorant during two 5 min sessions separated by different intervals (240, 300, and 360 min). A different odorant was used for each interval time point. To avoid cross interference of olfactory detection and memory, only one time interval was tested on each day. The investigating time on the swab was recorded by video camera and

scored by an experimenter blinded to animal treatment after the test. A significant decrease in investigation of the swab during the second presentation of the same odorant indicates olfactory memory for that odorant.

Sand-digging based odor cued associative olfactory learning test

We conducted this behavior test following the protocol as previously described (Wang et al., 2015; Zou et al., 2012). Before the test, mice were food restricted to maintain 85-90% of normal body weight for 5-6 days before the beginning and throughout the entire test. At the beginning, mice were pre-trained to learn to retrieve the food reward buried in sand. The pre-training session consisted of three consecutive days during which the food pellet was put on the top of sand (day 1), partially buried in sand (day 2), and deeply buried in sand (day 3) with 4 trials per block (1 min interval between each trial), 2 blocks each day and a 4 h interval between the 2 blocks. By the end of day 3, mice had learned to retrieve the food pellet within 50 s. The day after the pre-training session, mice were subjected to the olfactory discrimination test for 4 trials per block, 2 blocks per day with a 4 h interval for 10 days. During the test, both dishes were filled with sand with one scented with (+)-carvone and another with (-)-carvone. A food pellet was buried deeply in the sand with (+)-carvone. The two dishes were placed on each side twice per test block but no more than three consecutive times each day to avoid spatial cue. Scoring for correct or incorrect choice was based on the mouse’s first dig. If the mouse’s first choice was correct, it was allowed to finish the digging and to retrieve the food pellet as a reward. If the mouse made a wrong choice, it was allowed to finish the digging on that side but not allowed to self-correct. In this training, mice

were trained to learn to associate (+)-carvone with food reward. During the test, the experimenter was blinded to the animal treatment.

Blood Cd analysis

The Environmental Health Laboratory at the University of Washington measured blood Cd levels using inductively coupled plasma emission mass spectrometry. The experimenter who measured the blood Cd was blinded to the treatment of animals. The peak blood Cd analysis was conducted at sacrifice after the cessation of the Cd exposure from the Cd analysis group (n= 4 per treatment), using Agilent 7500 (Agilent Technologies, Santa Clara, CA) with a detection limit of < 0.5 μg/L. This equipment was since then replaced by Agilent 7900 (Agilent Technologies, Santa Clara, CA) which is more sensitive and has a detection limit of < 0.08 μg/L. The final blood Cd analysis was conducted at the end of the behavior tests from the neurobehavioral group (n = 7- 8 per treatment) using this newer equipment.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) and Excel (Microsoft, Redmond, WA). For behavioral tests, one-way ANOVA was used for the sand digging based test within the control or the Cd-treated group, respectively, to compare the % correct choices the animals made at different training days relative to their first training day. Student's two-tailed t test was used to analyze all other behavioral results. All data were expressed as mean ± SEM. n.s. not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

2.3 Results

Cd exposure results in peak blood Cd concentrations comparable to human environmental exposure.

Exposure to 10 mg/L $CdCl_2$ via drinking water has been commonly used to study the neurological effects of Cd in mice (Chen et al., 2013; Honda et al., 2013; Ishitobi et al., 2007). Mice can tolerate up to 100 mg/L of $CdCl_2$ in drinking water for 23 weeks with no weight changes (Blanco et al., 2010; Chakraborty et al., 2010; Honda et al., 2013; Hotz et al., 1999; Ikeda et al., 2004; Ishitobi et al., 2007; Jarup et al., 1993; Nakazato et al., 2008; Thijssen et al., 2007a; Thijssen et al., 2007b; Thijssen et al., 2007c). To assess Cd neurotoxicity through environmental exposure, we exposed 8-week old male C57BL/6 mice to 3 mg/L (3 ppm) Cd (in the form of $CdCl_2$) through drinking water to model environmental exposure via ingestion. We recorded body weight and water consumption every week during the entire exposure period and did not observe any effect of Cd on body weight (Fig. 2.1 A and B) or water consumption (Fig. 2.1 C and D). Mice were sacrificed after 5 or 13 weeks of exposure to measure the blood Cd concentrations. Cd exposure raised blood Cd concentrations in Cd-treated mice (Fig. 2.1 E). The peak blood Cd concentration in the 5-week exposure group (2.125 ± 0.47 μg/L) is similar to that of the 13-week exposure group (2.25 ± 0.48 μg/L), which suggests that the blood Cd concentrations reached a steady state by 5 weeks of exposure and remains consistent during the exposure.

3 mg/L Cd exposure via drinking water does not affect body weight, locomotor activity, or cause anxiety

To assess Cd neurotoxicity, we exposed 8-week old male C57BL/6 mice to 3 mg/L Cd through drinking water for 20 weeks to model sub-chronic environmental exposure via ingestion (Fig. 2.2 A). We recorded body weight and water consumption every week during the whole exposure period and did not observe any effect of Cd on body weight (Fig. 2.2 B) or water consumption (Fig. 2.2 C) between the two groups. All animals drank more water during weeks 14-17 when they were subjected to Morris water maze test at week 14-16, likely from the increased physical activity of swimming.

We conducted the open field test to assess the effect of Cd on locomotor activity and anxiety and did not find any significant differences in the open field activity between control and Cd-treated groups (Fig. 2.3). Both the control and Cd-treated mice traveled the same total distance with similar movement time and speed in the open field test (Fig. 2.3 A-C), suggesting that Cd exposure does not affect locomotor activity. Furthermore, both groups of mice had similar center entries (Fig. 2.3 D), spent similar amounts of time along the margins (Fig. 2.3 E) and center (Fig. 2.3 G), traveled similar distance along the margin (Fig. 2.3 F) and to the center area (Fig. 2.3 H), suggesting that Cd exposure at this dose does not cause overt anxiety.

The elevated plus maze test was also conducted to assess the effect of Cd on anxiety. There was no significant difference between control and Cd-treated mice on the percent of total time spent (Fig. 2.4 A) or total distance traveled (Fig. 2.4 B) in the open arms, the percent of open arm entries (Fig. 2.4 C), or total distance travelled in the

maze (Fig. 2.4 D). Together, the data from the open field test and elevated plus maze test indicate that Cd exposure did not induce overt anxiety in male C57BL/6 mice.

3 mg/L Cd-treated mice did not show deficits in the Morris Water Maze test

To determine the effect of Cd on the formation of spatial working memory, Cd-treated and control mice were subjected to 4 trials/day for 6 consecutive days in the Morris Water Maze test in which a hidden platform was placed in the same location at the virtual quadrant 3 (Q3). Both the control and Cd-treated mice learned the task and performed similarly over the 6 days of training, manifested as swimming a shorter distance to find the hidden platform (Fig. 2.5 A). In the probe test, mice in both groups spent similar time in the target quadrant Q3 searching for the hidden platform (Fig. 2.5 B).

The mice were subsequently subjected to a reversal-training paradigm in which the hidden platform was moved from Q3 to the opposite location in virtual quadrant 1 (Q1). The mice were trained using 4 trials per day for 10 days. Control and Cd-treated mice performed similarly in the reversal training session and the subsequent reversal probe test (Fig. 2.5 C and D). Two days after the reversal probe test, mice were subjected to a visible platform test in which mice of both groups had similar latency to reach the platform and swim speed (Fig. 2.5 E). These data indicate that the Cd-treated mice did not exhibit deficits in the Morris water maze test.

3 mg/L Cd exposure causes long-lasting impairment of hippocampus-dependent spatial memory in the novel object location (NOL) test

In order to assess the effect of Cd on other forms of hippocampus-dependent spatial memory, we conducted a 1 h NOL test (Fig. 2.6 A) before, during, and after Cd exposure in control and Cd exposure cohorts. At each time point tested (baseline through 9 weeks post Cd exposure), there was no difference in the exploration time of each object or each location (location A vs. B) in the training session (Fig. 2.6 B, D and F), suggesting that none of the mice exhibited a preference for either location or object. Before Cd exposure, both groups of mice spent significantly more time exploring the object in the novel location C vs. familiar location A in the test session (Fig. 2.6 C), indicating that they remembered the original object locations during training sessions. Six weeks into the Cd exposure, the control mice still spent significantly more time in exploring the object in the novel location, while the Cd-treated mice spent almost equal amount of time in exploring the two objects (Fig. 2.6 E), indicating that the Cd-treated mice did not discriminate between the old and new object locations, thus had impaired short-term spatial memory. The Cd-treated mice continued to exhibit a deficit in this form of spatial memory at 9 weeks after the cessation of Cd exposure (Fig. 2.6 G), suggesting that the memory impairment persists long after cessation of Cd exposure. We also calculated the discrimination ratio at different time points to compare spatial memory changes over time. There was no difference between control and Cd-treated mice before exposure. At 6 weeks and 7 weeks into Cd exposure and 9 weeks post Cd exposure, Cd-treated mice had a significantly lower discrimination ratio compared to controls (Fig. 2.6 H).

3 mg/L Cd exposure impairs hippocampus-dependent spatial memory in T-maze test

Rodents tend to alternate the arm they previously entered in a T-maze setting, which is partially due to the hippocampus-dependent spatial working memory (Engstrom et al., 2017; Gerlai, 1998; Spowart-Manning and van der Staay, 2004). Interestingly, the Cd-treated mice exhibited a statistically significant reduction in spontaneous alternation in the T-maze (Fig. 2.7). Together with the findings from the NOL test, these data further suggest that Cd exposure impairs hippocampus-dependent spatial working memory in male mice.

3 mg/L Cd exposure impairs contextual fear memory in mice

We conducted fear-conditioning tests to investigate the effect of Cd exposure on contextual fear memory, another form of hippocampus-dependent learning and memory, and on cued fear memory that is hippocampus-independent. We used a challenging training paradigm in which animals received 3 x 0.3 mA (2 s) foot shocks during the fear conditioning session (Fig. 2.8 A) because we previously reported that this form is more sensitive to changes in contextual fear memory (Engstrom et al., 2017; Pan et al., 2012a). Mice were assessed for the amount of time they froze in the testing arena 24 h after the foot shock training as a measurement of their fear memory. Cd-treated mice exhibited significantly reduced contextual fear memory compared to control mice (Fig. 2.8 B). However, there was no difference between control and Cd-treated mice in freezing behavior in the auditory-cued fear memory test or the novel context test. Therefore, Cd treatment at this dose did not affect the hippocampus-independent,

auditory-cued memory, or lead to generalized freezing behavior in mice. Collectively, these data suggest that 3 mg/L Cd exposure via drinking water in young adult mice attenuates hippocampus-dependent contextual fear memory.

3 mg/L Cd-treated mice show normal odor detection and odor detecting sensitivity

Since epidemiology studies suggested that occupational Cd exposure is associated with olfactory dysfunction in humans (Adams and Crabtree, 1961; Mascagni et al., 2003; Rose et al., 1992; Sulkowski et al., 2000), we investigated whether Cd exposure can affect olfactory behavior in animal models. We first conducted an olfactory habituation/dishabituation test and found no difference between control and Cd-treated mice (Fig. 2.9 A). These mice were then subjected to an odor threshold test to examine the effect of this Cd exposure level on their sensitivity for odor detection. In this test, both groups of mice spent more and similar amount of time sniffing the 1-octanol-laced cotton swab as its concentration increased (Fig. 2.9 B), suggesting that they have similar detection sensitivity toward 1-octanol. These data suggest that Cd exposure at this dose did not affect odorant detection or odor detection sensitivity.

3 mg/L Cd exposure impairs short-term olfactory memory

We conducted a cotton tip-based test to investigate the effect of Cd exposure on short-term olfactory memory. Mice were presented with the same odorant twice with a time interval between the two presentations (240, 300, or 360 min) on three separate days. Both control and Cd-treated mice spent significantly less time sniffing the odor-

laced cotton tips during the second presentation compared to the first presentation at the 240 min interval (Fig. 2.10), suggesting that they had memory for the odorant presented 240 min earlier. At the 300 min time interval, while the control mice still exhibited statistically significant olfactory memory, the Cd-treated mice did not. At the 360 min time point, both groups of mice had lost their memory for the odorant. These data suggest that Cd exposure at this dose in mice shortened their capacity to remember a specific odorant from 300 min in controls to 240 min.

3 mg/L Cd exposure impairs associative olfactory learning and memory

We subjected control and Cd-treated mice to a sand-digging odor-cued olfactory learning and memory test to study the effect of Cd exposure on associative olfactory memory. Mice were pre-trained for 3 days to associate food reward with sand digging, and both groups of mice learned the task by the end of the pre-training (Fig. 2.11 A). To assess associative olfactory learning and memory after pre-training, the mice had to use odor cues to discriminate between a pair of structurally similar odorants: (+)-carvone vs (-)-carvone in order to retrieve the food reward since the food pellet was only associated with (+)-carvone. Over the course of 10 d training and testing, the control mice gradually learned the task and retrieved the food reward correctly in about 80% of the time by the end of the 10-day training, whereas the Cd-treated mice did not, with the correct choice made only about 50% of the time in the Cd-treated group (Fig. 2.11 B). These data indicate that Cd exposure impairs odor-cued associative olfactory learning and memory in mice.

Blood Cd concentration at the end of the behavior tests

All behavior tests were completed by 60 weeks after the end of the 20-week Cd exposure and blood was collected from the animals at the end of the behavior tests to measure the final blood Cd levels. The final Cd concentration of Cd-treated mice was 0.18 ± 0.028 μg/L (Fig. 2.12). In the control group, the blood Cd ranged from below detection limit (< 0.08 μg/L, n=4) to 0.15 μg /L (n=3).

2.4 Discussion

In the present study, we exposed young adult male mice to Cd through drinking water that yielded peak blood Cd concentrations comparable to the higher range of Cd blood level in the general human population. We demonstrate that, although several neurobehavioral tests found no effect, this level of Cd exposure induces persistent impairments of several forms of hippocampus-dependent spatial working memory, contextual fear memory, and olfactory memory in mice. These data provide direct evidence that Cd exposure at the level of 3 mg/L in drinking water is sufficient to impair hippocampus-dependent learning and memory, as well as olfaction in an animal model. Together with previous epidemiology studies in humans, these results strongly suggest that Cd exposure can induce impairment of cognition and olfactory memory.

Cd is a heavy metal commonly found in Superfund hazardous waste sites. In addition to occupational exposure, humans can be exposed to Cd through ingestion of contaminated food and water, inhalation of tobacco smoke, or air pollution. In this study, we exposed young adult male mice to 3 mg/L Cd through drinking water to model environmental exposure through ingestion. This concentration is at least 3 times lower

than the 10-20 mg/L Cd in drinking water commonly used in previous *in vivo* studies (Gupta et al., 1995; Ramirez and Gimenez, 2003; Samuel et al., 2011; Shukla et al., 1996; Thijssen et al., 2007c). However, it is difficult for us to specify the dose per kg because 4-5 mice were housed in one cage and water consumption was *ad libitum*. Although we monitored water consumption every week, we do not know how much water each individual mouse drank every day. Assuming each mouse drank equal volume of water everyday, the estimated dose per kg the mice received at the initial exposure period was approximately 0.4-0.5 mg/kg per day. During the period of water maze test at week 14-16, all animals drank more water and at the peak of water consumption at week 15, the estimated dose was 0.6-0.7 mg/kg per day.

Animals were exposed to Cd for 20 weeks for behavior studies. We assessed hippocampus-dependent spatial memory in the novel object location assay at multiple time points before, during, and after Cd exposure to “probe” the onset and sustainability of Cd-induced spatial memory loss. The novel object location assay is reliable and noninvasive because novel objects are presented to mice for only 5 min during training and testing, and the memory only lasts for a few hours. Furthermore, the repeated tests reduce the number of animals needed to probe the progression of Cd toxicity. We conducted novel object location test at experimental week 4, 5, 6, 7. Once we confirmed that Cd exposure impaired memory in the novel object location test, we performed Morris water maze test at experimental week 14-16 but did not observe any differences between the groups. We confirmed that the memory for novel object location was still impaired at experimental week 19. Subsequently, we stopped Cd exposure at week 20 to model sub-chronic exposure.

The average peak blood Cd levels immediately after 5 weeks or 13 weeks exposure are very similar (2.125 ± 0.47 μg/L vs. 2.25 ± 0.48 μg/L), and only about 2-fold higher than the blood Cd concentration of current smokers in the U.S. (men: 0.58-0.94 μg/L; women: 0.69-1.17 μg/L) according to a National Health and Nutrition Examination survey (1999-2010) (Adams and Newcomb, 2014), but 2-fold lower than the standard trigger level of Cd (5 μg/L) for medical surveillance in Occupational Safety & Health Administration regulation. Furthermore, this blood Cd concentration can still be encountered among the general population around the world (Kim et al., 2015; Myong et al., 2014; Sakellari et al., 2016; Zhang et al., 2015). Therefore, the Cd exposure used in this study is relevant to Cd exposure in humans. Under this environmental relevant exposure level, we found statistically significant impairments of hippocampus-dependent and olfactory memory in Cd-treated mice.

Sixty weeks after the 20-week Cd exposure, the blood Cd concentration dropped from the peak level to 0.18 ± 0.028 μg/L, a level that is slightly higher than that in control mice (ranging from < 0.08 to 0.15 μg/L). Importantly, the final Cd concentration of 0.18 μg/L in Cd-treated mice is at the lower spectrum of the blood Cd concentrations in the general population in the U.S. (men: 0.19-0.32 μg/L; women: 0.22-0.39 μg/L) (Adams and Newcomb, 2014), and much lower than that in current smokers in the U.S. (Adams and Newcomb, 2014). These results suggest that Cd impairments of cognition and olfaction are irreversible, persisting long after the initial peak exposure, even when the blood Cd concentration has returned to almost normal levels.

Cadmium is toxic to multiple peripheral organs, however, its neurotoxic effect has not been fully established. Epidemiological studies have reported associations between

elevated Cd exposure and reading difficulties, behavioral problems, reduced attention, memory and lower cognitive scores in adult humans (Emsley et al., 2000; Gao et al., 2008; Hart et al., 1989; Struempler et al., 1985; Viaene et al., 2000). An epidemiology study involving 5662 U.S. adults, age 20-59, reported that higher cumulative Cd exposure was associated with decreased attention and perception in those adults whose Cd exposure is primarily through diet with no smoking or work-based Cd exposure (Ciesielski et al., 2013a). Importantly, this association was observed at 0.19-0.82 μg/L Cd in urine, levels common in U.S. adults and previously considered to be without adverse effects. Another recent study involving 2282 representative U.S. children between 6-15 years of age found that children who have higher urinary Cd concentrations have increased risk of learning disabilities. This association was observed at exposure levels that are common among U.S. children and were originally thought to be safe (Ciesielski et al., 2012). Several other studies in children have also shown associations between higher Cd levels and mental retardation, decreased verbal IQ, and learning disabilities (Capel et al., 1981; Ely et al., 1981; Jiang et al., 1990; Marlowe et al., 1983; Pihl and Parkes, 1977; Thatcher et al., 1982). These findings are very interesting and animal studies should help to establish a direct correlation between Cd exposure and cognitive impairment, as well as putative cellular/molecular mechanisms that could be responsible for such effects.

Several previous animal studies have also reported memory deficits associated with Cd exposure. Lehotzky et al. (1990) found that prenatal exposure to Cd significantly retarded the acquisition of the conditioned escape response in rats. Cd exposure also decreases the step-down latency in inhibitory avoidance task in rats (Abdalla et al.,

2014; da Costa et al., 2017; Goncalves et al., 2010; Goncalves et al., 2012). However, the effects of Cd on learning and memory in animal models are still not fully understood, especially effects on hippocampus-dependent learning and memory. In this study, we examined the effects of Cd on several forms of learning and memory, both hippocampus-dependent and independent. Cd exposure did not affect cued fear conditioning, a form of hippocampus-independent memory formation. Although we did not find any difference between control and Cd-treated mice in the performance of the Morris Water Maze test, Cd exposure impaired several other forms of hippocampus-dependent memory formation including contextual fear memory as well as spatial working memory in the NOL test and T-maze test. The Cd-treated mice developed memory loss in the NOL test starting at 6 weeks into Cd exposure. Furthermore, this memory deficit was persistent through 9 weeks after the cessation of Cd exposure. Because the T-maze and fear conditioning tests were also conducted more than 9 weeks after the cessation of Cd exposure, our data suggest that Cd exposure in young adult mice can cause persistent impairment of hippocampus-dependent learning and memory long after exposure has ended.

Occupational inhalation exposure to Cd has been associated with olfactory dysfunction in humans (Adams and Crabtree, 1961; Mascagni et al., 2003; Rose et al., 1992; Sulkowski et al., 2000). Although strongly suggestive, these studies cannot rule out confounding exposures such as nickel dust. Animal studies showed that acute intranasal administration of Cd led to partial destruction of the olfactory epithelium and impaired odor detection (Bondier et al., 2008; Czarnecki et al., 2011; Czarnecki et al., 2012). These studies suggest that occupational or acute and relatively high-dose Cd

exposure may impair olfaction. However, there has been no previous reports as to whether Cd exposure through ingestion of food or water at environmentally relevant blood Cd concentrations can also impair olfaction. In this study, we present evidence that Cd exposure did not affect odorant detection or odor detection sensitivity. However, Cd-treated mice showed impaired short-term olfactory memory toward previously exposed odors when compared to control mice. Furthermore, we also demonstrated that in the odor-cued associative learning test, Cd-treated mice did not learn the task of associating food reward with a specific odorant as effciently as control mice did. These data provide the first evidence that Cd exposure through ingestion impairs olfaction in mice.

Our findings indicate that Cd exposure impairs hippocampus-dependent and olfactory memories in adult mice, but the underlying mechanism is still unknown. Accumulating evidences suggest that Cd neurotoxicity may due to the induction of reactive oxygen species (ROS), which leads to oxidative stress in the brain (Chen et al., 2008; Chen et al., 2011). In animals, Cd exposure can significantly increase the level of lipid peroxidation in parietal cortex, striatum, and cerebellum (Mendez-Armenta et al., 2003), and the injury in cerebral microvessels induced by Cd is thought to be associated with oxidative stress (Shukla et al., 1996). *In vitro* studies also found that Cd induced ROS, which leads to apoptosis in SH-SY5Y and PC12 cells (Chen et al., 2008). Cd can disrupt intracellular free calcium homeostasis, leading to apoptosis in a variety of neuronal cells, such as cerebral cortical neurons and primary murine neurons(Wang and Du, 2013). Cd can also disturb the homeostasis of Cu, Zn, and Co, and then impairs different types of cells in central nervous system (Gupta and Shukla 1996; Jin

et al., 1998; Wang and Du, 2013). Recently, new approaches to the study of adult neurogenesis have unveiled new potential mechanism of Cd neurotoxicity. A growing number of studies have found that adult neurogenesis plays an important role in certain forms of hippocampus-dependent learning and memory, and olfactory function (Clelland et al., 2009; Deng et al., 2009; Pan et al., 2012a; Pan et al., 2012b; Pan et al., 2012c; Wang et al., 2015; Wang et al., 2014; Zou et al., 2012). We have previously reported that lead can impair hippocampus-dependent learning and memory through affecting adult hippocampal neurogenesis (Engstrom et al., 2015; Engstrom et al., 2017). Recently, we also found that Cd impairs the survival and proliferation of adult subventricular neural stem cells *in vitro* (Wang et al., 2017). Thus, we hypothesize that Cd inhibit adult neurogenesis in the hippocampus and olfactory bulb, contributing to the persistent impairments of learning and memory as well as olfaction.

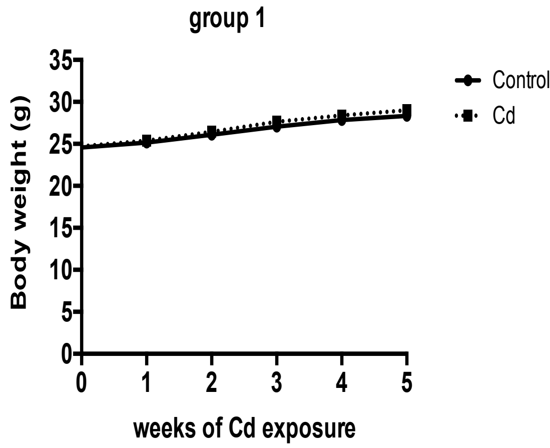
2.5 Conclusion

In summary, our study found that Cd exposure in young adult male mice through drinking water yields peak blood Cd concentration comparable to the higher range of Cd blood level in the general human population. Although a number of neurobehavioral tests were found to be normal, this exposure was sufficient to induce persistent impairments of several forms of hippocampus-dependent learning and memory, as well as olfactory memory in mice. To our knowledge, these findings provide some of the first direct evidence that Cd exposure can induce impairments of hippocampus-dependent and olfactory memories in animal models. These results support the findings of epidemiology studies that low-level environmental Cd exposure is associated with

impairments of cognition in humans (Ciesielski et al., 2013b; Ciesielski et al., 2012), and provide new insights into our understanding of Cd neurotoxicity.

2.6 Figures and Tables

A



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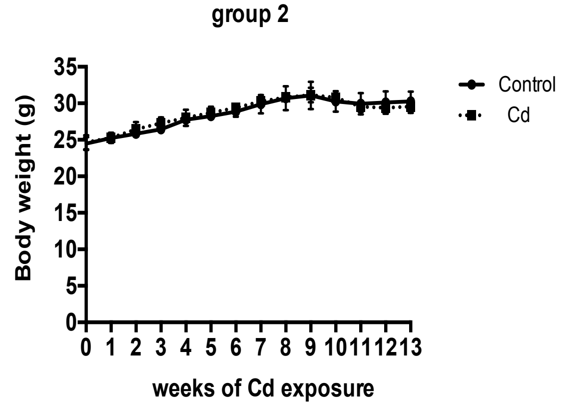

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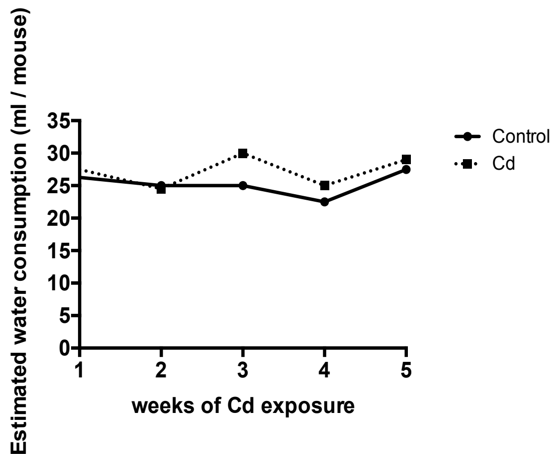

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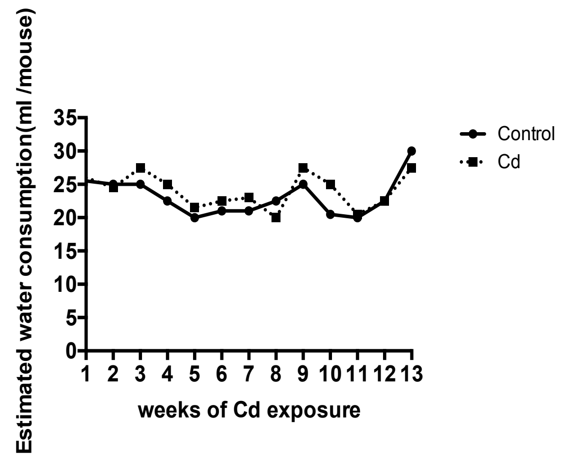


E

	In vivo Cd exposure	
	Control	Cd (3mg/L)
Cd in blood (µg/L)	< 0.5	2.125 ± 0.47 (5 weeks) 2.25 ± 0.48 (13 weeks)

Figure 2.1 Cd exposure increases blood Cd concentration in mice. (A) 5 weeks of Cd exposure did not affect the body weight of mice. (B) 13 weeks of Cd exposure did not affect the body weight of mice. (C) 5 weeks Cd exposure group had similar water consumption with control group. (D) 13 weeks Cd exposure group had similar water consumption with control group. (E) Peak blood Cd concentrations of control and Cd-treated mice. Data are presented as mean ± SEM with n = 4 in each group.

A

Age (weeks): 8 — 28 — 88

Experimental week 0 — 20 — 80

NOL (week 4-7)

Morris water Maze (week 14-16)

NOL (week19)

0 or 3mg/L Cd^{2+} in drinking water

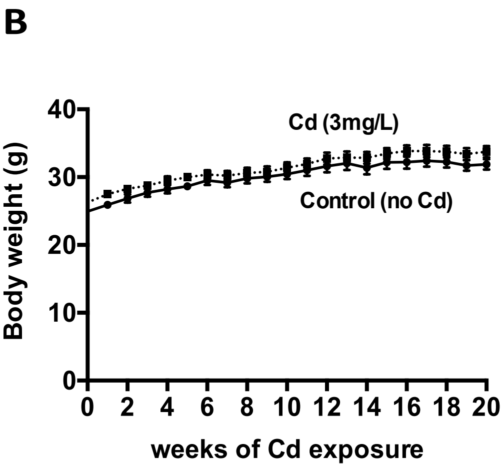
Cd exposure stop

Blood collection

Baseline behavior tests:
- Open field
- Novel object location (NOL)

Post-Cd behavior tests:
- Open field (week 21)
- Novel object location (week 29)
- T-maze (week 30)
- Cued and contextual fear conditioning (week 34)
- Olfaction test (week 46 – 68)
- Elevated plus maze (week 75)

B



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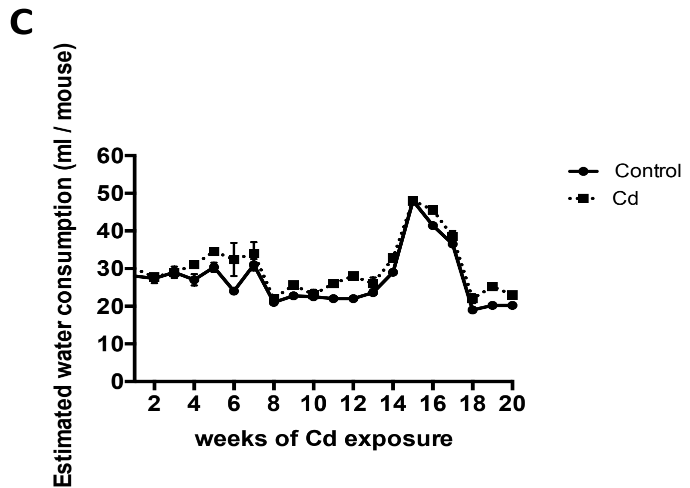

Figure 2.2. Cd exposure does not affect the body weight in neurobehavioral group. (A) Experiment design and timeline for behavior test. (B) The Cd-treated mice did not exhibit a significant difference in body weight during the whole exposure period when compared with control group. (C) The Cd-treated mice did not exhibit a significant difference in water consumption during the whole exposure period when compared with control group. Data are presented as mean ± SEM with n = 9-10 in each group.

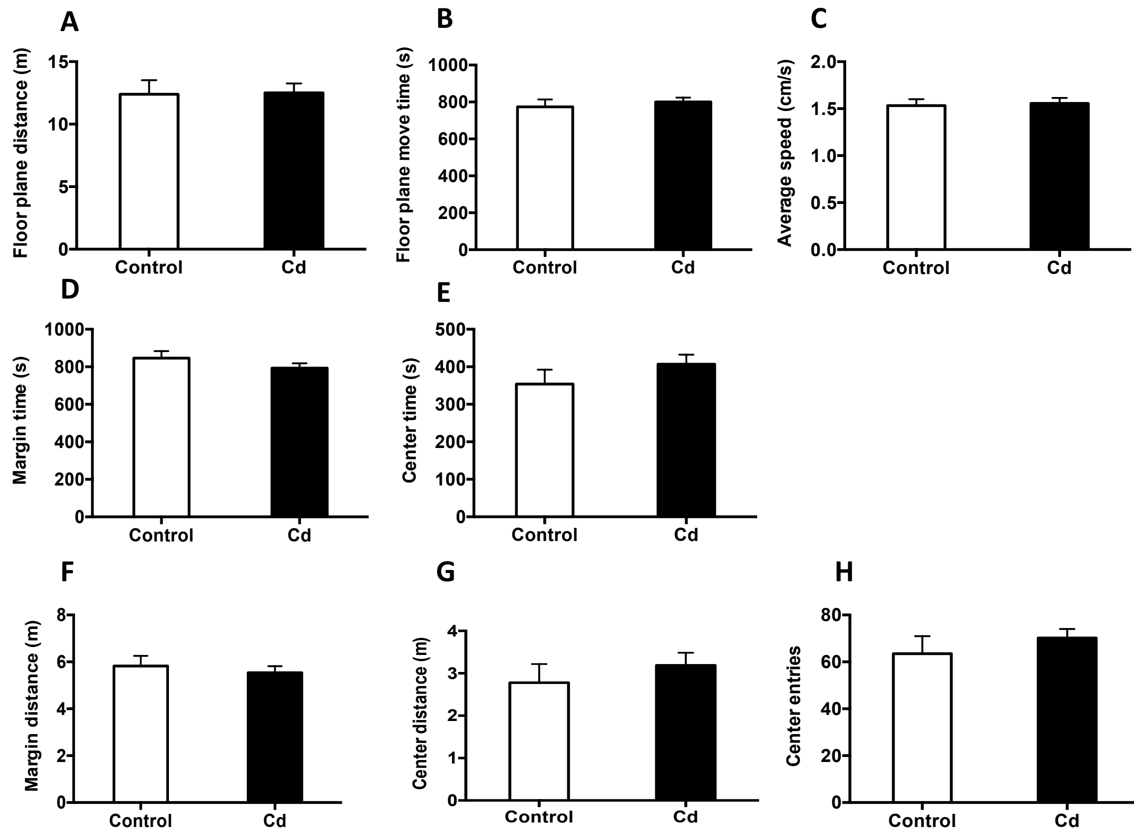

Figure 2.3. Cd exposure does not affect the locomotor activity or anxiety of mice in open field test. For locomotor activity, there was no significant difference between control and Cd-treated mice in the (A) total distance traveled, (B) movement time, or (C) speed. For anxiety, there was no significant difference between control and Cd-treated mice in (D) the number of center entries and the time spent in (E) margin or (G) center. Both groups traveled a similar distance (F) along the margin and (H) in the center area. Data are presented as mean ± SEM. n = 8-10 mice/group.

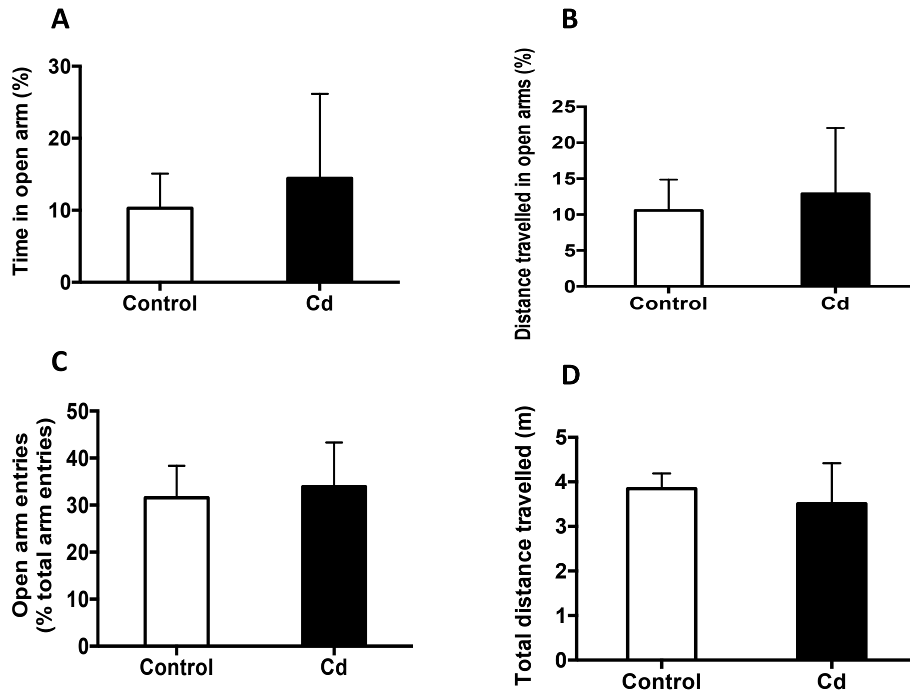

Figure 2.4. Cd exposure does not cause anxiety in elevated plus maze test. Both the control and Cd-treated mice spent (A) a similar amount of time and traveled (B) a similar distance in the open arms of the maze. The control and Cd exposure group mice also had similar (C) open arm entries and (D) total distance travelled in the maze. Data are presented as mean ± SEM. n = 8-10 mice/group.

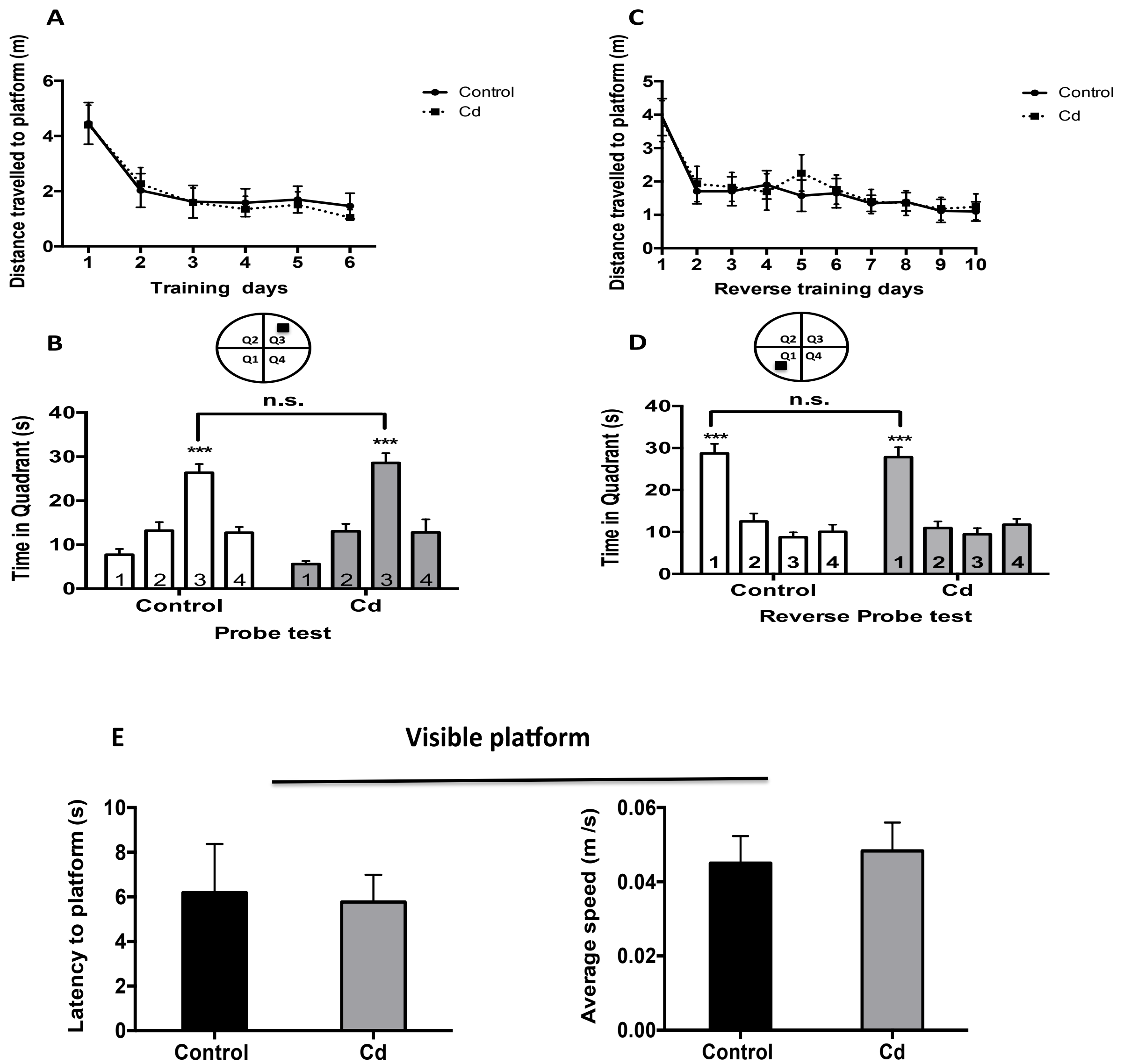

Figure 2.5. Cd exposure does not affect the performance of mice in the Morris Water Maze test. (A) During a 6-d training period, Cd-treated mice swam similar distances as control mice to locate the hidden platform. (B) In the probe test, both control and Cd-treated mice spent significantly more time in the target quadrant (Q3) than any other quadrant. (C) Mice were subjected to the reversal training after the probe test. During the 10 d reversal training, both the control and Cd-treated mice swam similar distances to reach the hidden platform. (D) In the

reversal probe test, both control and Cd-treated mice spent significantly more time in the new target quadrant (Q1) than any other quadrant. (E) Control and Cd group performed equally well in the visible platform test; they showed similar latency to acquisition of the visible platform and swimming speed. Data are presented as mean ± SEM. n = 9-10 mice /group. n.s., not significant; ***$p<0.001$.

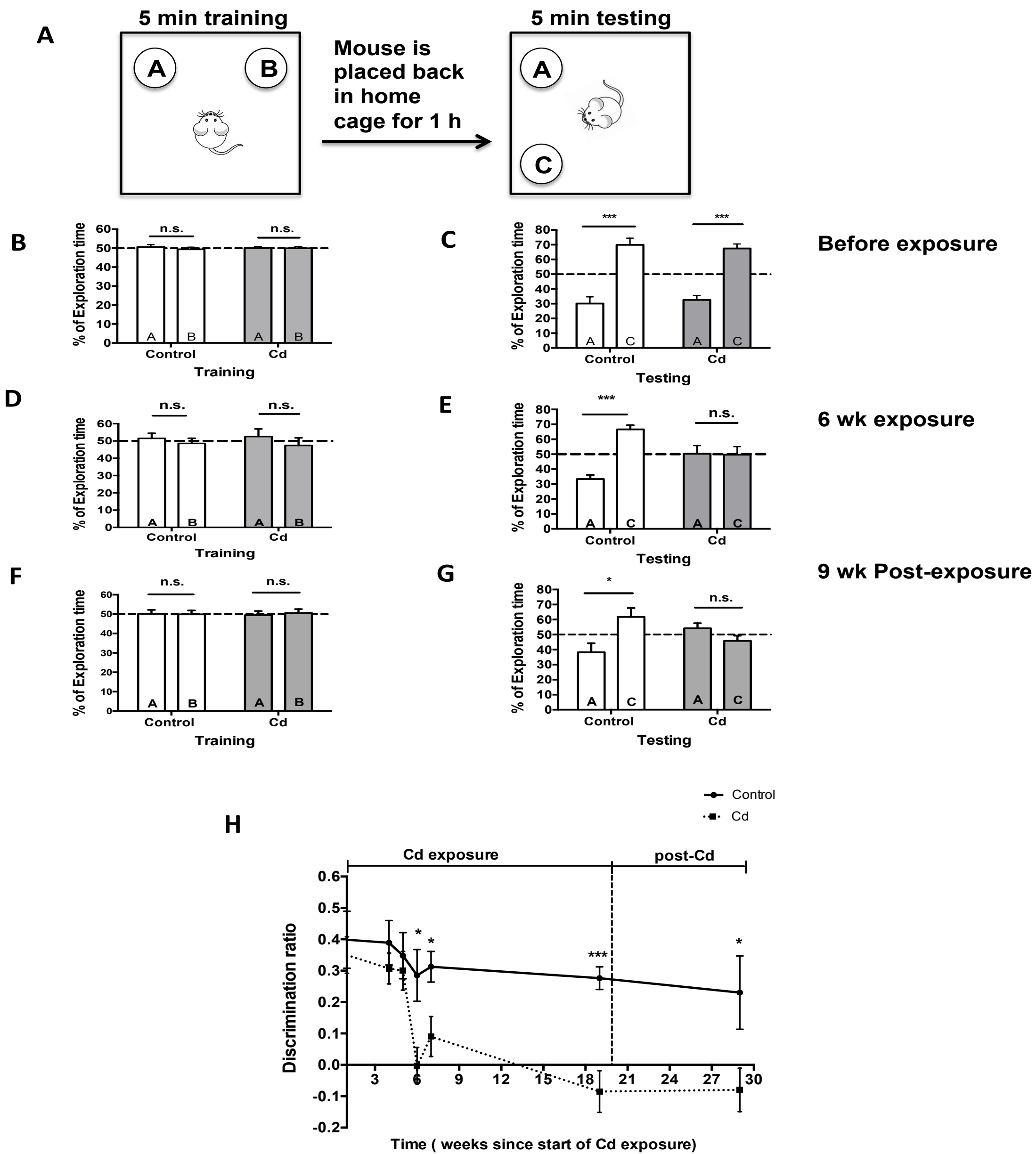

Figure 2.6. Cd exposure impairs short-term spatial memory in the Novel Object Location test (NOL). (A) Schematic of 1h Novel Object Location test. (B, D and F) All animals spent equal amount of time in two objects in training session. (C) Both the control and Cd-treated mice exhibited short-term spatial memory before Cd exposure. (E) At 6 weeks into Cd exposure, the Cd-treated mice did not discriminate between the old vs. novel object locations. (G) Cd-treated

mice continued to exhibit spatial working memory deficits at 9 weeks post-Cd exposure while the control mice still had intact spatial memory. (H) Discrimination ratio. Data are presented as mean ± SEM. n = 8-10 mice /group. n.s., not significant; *p<0.05; ***p<0.001.

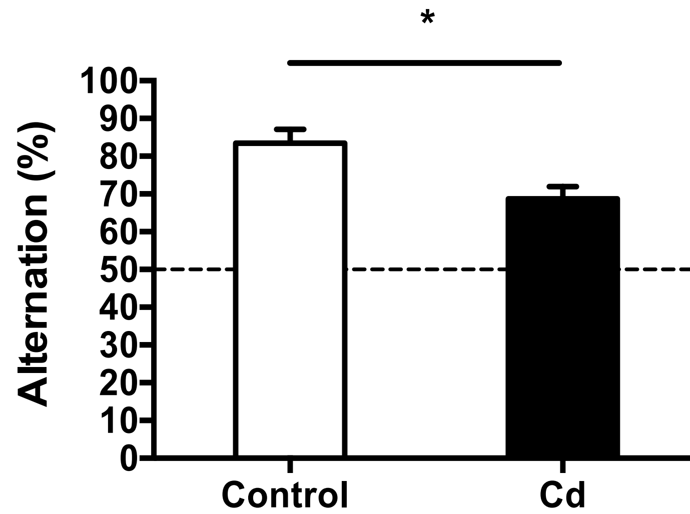

Figure 2.7. Cd exposure impairs spontaneous alternation in T-maze test. Data are presented as mean ± SEM. n = 9-10 mice/group. *p<0.05.

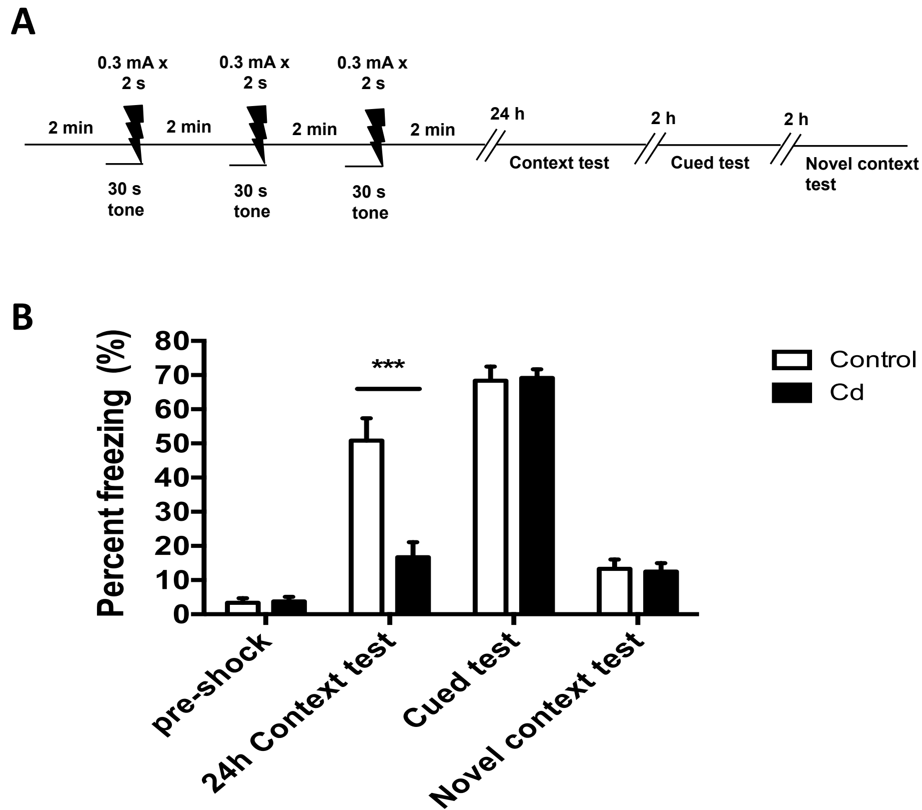

Figure 2.8. Cd exposure impairs contextual fear memory in mice. (A) Schematic of cued and contextual fear conditioning test. (B) Both control and Cd-treated mice had low baseline freezing behavior (pre-shock). In the context test, there was a significant effect of Cd on freezing behavior in Cd-treated mice. Auditory-cued fear memory was not affected (Cued test). Both groups exhibited low freezing behavior when placed in a novel, non-shock context (Novel context test). Data are presented as mean ± SEM. n = 9-10 mice/group. ***p<0.001.

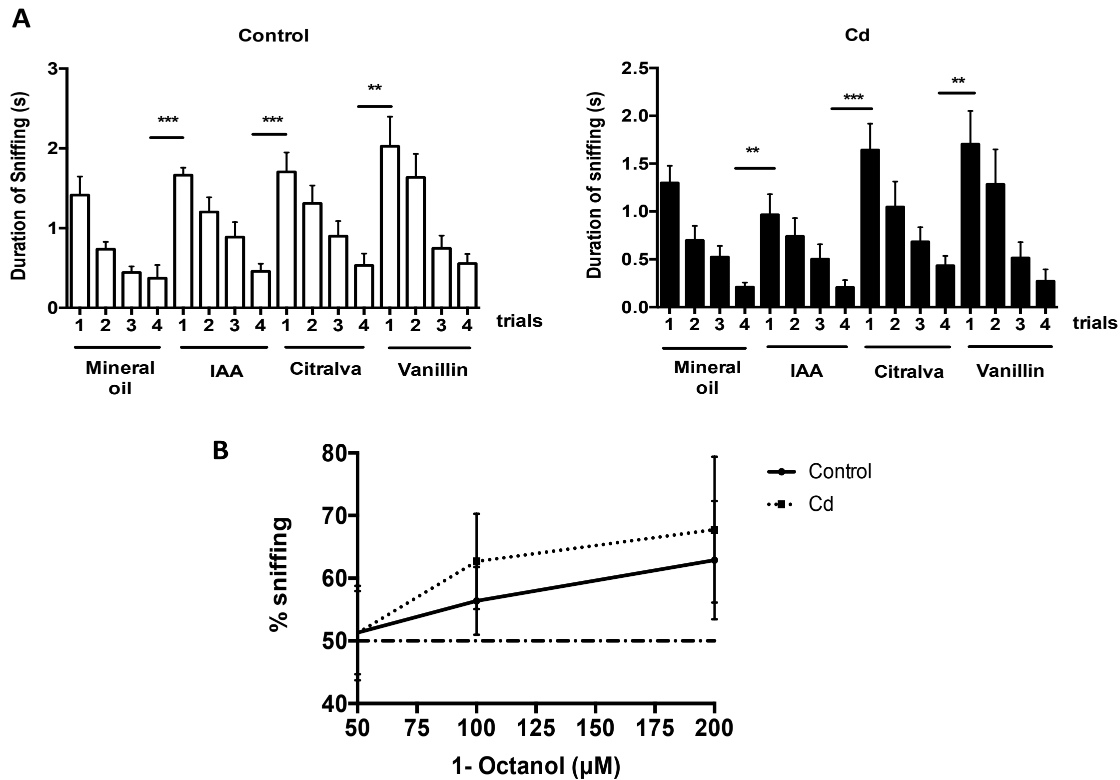

Figure 2.9. Cd exposure does not impair odor detection or odor detecting sensitivity. (A) Control and Cd-treated mice had comparable olfactory detection to discrete odorants in habituation/dishabituation assays. Mice were pretrained with four presentations of mineral oil-soaked cotton swabs, and then exposed to three other odorants: IAA, Citralva, and Vanillin, each with four presentations. Stepwise decrease in the duration of sniffing during the presentations of the same odor followed by increased interest of the new odorant suggests normal olfactory habituation/dishabituation. (B) Cd-treated mice had similar odor detecting sensitivity as control mice. Mice were presented with two cotton swabs, one laced with mineral oil and the other with increasing concentrations of 1-octanol (50 - 200 μM). A > 50% sniffing duration indicates odorant detection. Data are presented as mean ± SEM. n = 9-10 mice/group.

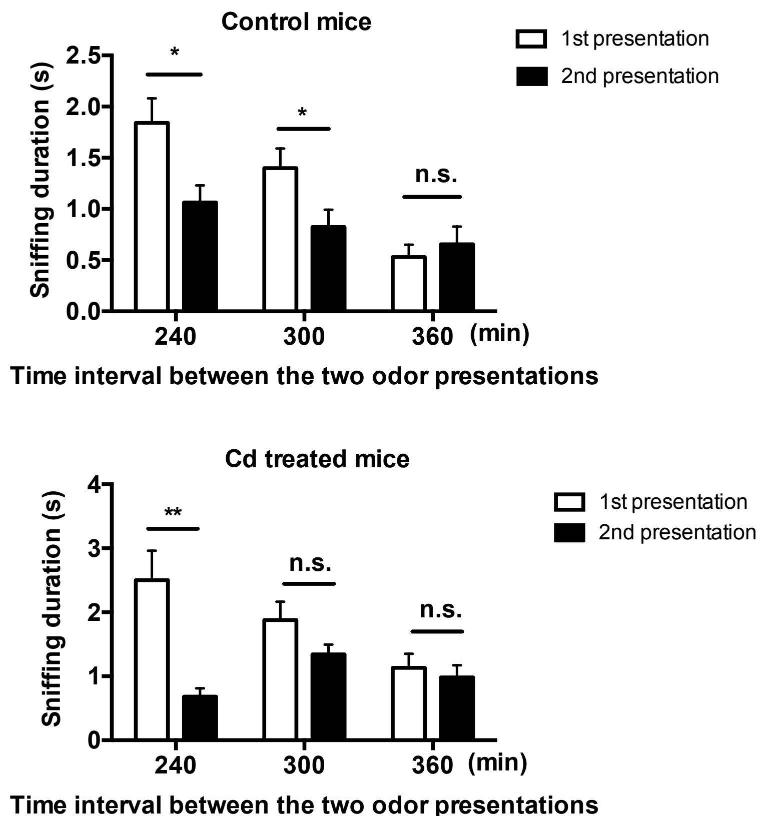

Figure 2.10. Cd exposure impairs short-term olfactory memory in mice. Both control and Cd-treated mice were presented with cotton swabs laced with the same odorant twice with time intervals as indicated. A decrease in investigation during the second presentation of the same odorant suggests olfactory memory of the odorant. Data are presented as mean ± SEM. n = 9-10 mice/group. n.s., not significant; *p<0.05; **p<0.01.

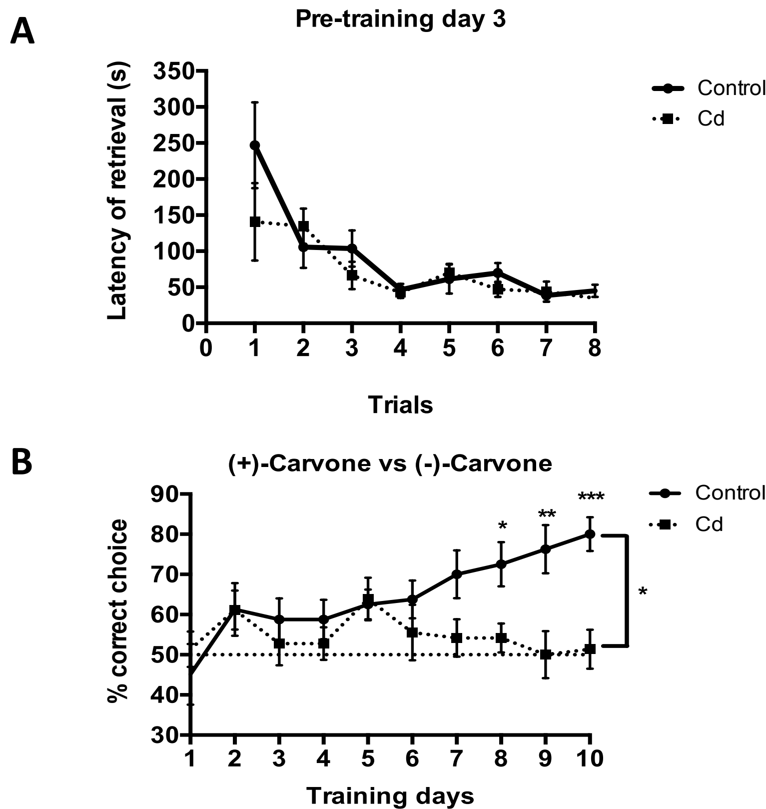

Figure 2.11. Cd exposure impairs odor-cued associative learning and memory in mice. (A) On the third day of pre-training, control and Cd-treated mice learned to retrieve food reward that was deeply buried in the sand. (B) Control mice made significantly more correct choices on training day 7, 8, 9, and 10 when compared with training day 1, but Cd-treated mice did not. Control mice performed significantly better after 10 days training ($F(9,90) = 3.325$, $p = 0.0015$), but the Cd treated mice did not ($F(9,80) = 0.7676$, $p = 0.6464$). Data are presented as mean ± SEM. n = 9-10 mice/group. n.s., not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

	In vivo Cd exposure	
	Control	**Cd** (3mg/L for 20 weeks)
Cd in blood(μg/L)	< 0.08 (detection limit) (n = 4) 0.15 ± 0.04 (n = 3)	0.18 ± 0.028 (n = 7) (60 weeks post Cd exposure)

Figure. 2.12. Blood Cd concentrations at the end of the behavior tests. At 60 weeks after we stopped the 20-week Cd exposure, all behavior tests were completed and the blood of these mice was collected to measure the final blood Cd concentrations. Data are presented as mean ± SEM with n = 7-8 in control and Cd exposure group.

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Chapter 3: Cadmium impairs the survival and proliferation of cultured adult subventricular (SVZ) neural stem cells through activation of the JNK and p38 MAP kinases

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3.1 Introduction

Cadmium (Cd), a naturally occurring heavy metal, is widely used in industrial and household products. According to the U.S. Geological Survey in 2016, approximately 24,200 metric tons of Cd was produced in 2015 worldwide. Cd is also considered one of the top 20 hazardous substances (Liao and Freedman, 1998), ranking number 7 on the Superfund hazardous waste priority list. Cd is released to the environment from refining of metals, burning of fossil fuels and wastes, smelting, and the use of phosphate fertilizer (Waisberg et al., 2003; Wang and Du, 2013). Because of its high rate of soil-to-plant transfer, Cd is a contaminant found in a variety of human foods. Ingestion of foods containing Cd and cigarette smoking are two main routes of Cd exposure in the general population (Clemens 2006; Saldivar et al., 1991; Satarug et al., 2010; Stohs et al., 1997). Since Cd accumulates in the kidney and the bone, it has a long half-life (about 10-30 years) in the human body (Waisberg et al., 2003). Chronic exposure to Cd causes toxic effects in various human organs, including kidney (Torra et al., 1995), liver (Goering et al., 1993; Torra et al., 1995), lung (Manca et al., 1994; Shukla et al., 2000), testis (Mao et al., 2012; Oliveira et al., 2012) and bone (Chen et al., 2009; Staessen et al., 1999; Wallin et al., 2013).

Other studies suggest that Cd is also a neurotoxicant. In zebrafish, Cd affects the commitment of neural progenitor cells during embryonic brain development (Chow et al., 2008). Cd induces apoptosis and/or necrotic cell death in neuronal cells including PC-12, SH-SY5Y cells and primary rat cerebral cortical neurons (Chen et al., 2008a; Chen et al., 2008b; Lopez et al., 2003; Yuan et al., 2013). Cd also induces extensive hemorrhages in the cerebral cortex and cerebellum in rats and rabbits (Gabbiani et al., 1967; Wong and Klaassen, 1982). Several human epidemiology studies have reported that Cd exposure is associated with attention deficits, olfactory and cognitive dysfunction (Ciesielski et al., 2013; Ciesielski et al., 2012; Friberg, 1948; Mascagni et al., 2003; Rose et al., 1992). However, our understanding of the molecular and cellular mechanisms for Cd neurotoxicity is limited.

Adult neurogenesis is a process that generates functional neurons from adult neuronal progenitor/stem cells (aNPCs). Under normal physiological conditions, adult neurogenesis is restricted to two specific regions in adult brain: subventricular zone (SVZ) and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus. Adult-born neurons from SVZ-aNPCs are critical for olfaction (Bond et al., 2015; Wang et al., 2015; Zou et al., 2012). The process of adult neurogenesis can be modulated by many external influences. For example, drug abuse, stress, and irradiation can reduce adult neurogenesis, while exercise and environmental enrichment increase it (Kempermann et al., 1997; Mak et al., 2007; Mirescu et al., 2006; van Praag et al., 1999). We reported that a hydroxylated metabolite of PBDE-47, 6-OH-PBDE-47, and lead (Pb), impairs SVZ and SGZ adult neurogenesis *in vitro* respectively (Engstrom et al., 2015; Li et al., 2013). Several studies also suggest that manganese (Mn^{2+}) can also affect adult

neurogenesis *in vivo* (Fu et al., 2016; Kikuchihara et al., 2015). Since Cd is a potential neurotoxicant and its exposure is associated with impairment of olfaction, we examined whether Cd is toxic to SVZ-aNPCs and began to elucidate the underlying signaling mechanisms, using primary cultured SVZ-aNPCs as an *in vitro* model.

3.2 Materials and Methods

Materials

The preparation, use, and disposal of hazardous agents were carried out as recommended by the Environmental Health and Safety Office at the University of Washington. Cadmium chloride (Cat. 202908, Sigma-Aldrich, St. Louis, MO) was dissolved in deionized distilled water (H_2O) to make a 1mM (Cd^{2+}) stock solution and stored at -20°C. The JNK (Cat. SP600125, Calbiochem, Billerica, MA) and p38 (Cat. SB202190, Calbiochem, Billerica, MA) inhibitors were dissolved in dimethyl sulfoxide (DMSO) to yield 3 mM stock solutions and stored at -20°C. 5-bromo-2'-deoxyuridine (BrdU) (Cat.B9285, Sigma-Aldrich, St. Louis, MO) was stored as a 65 mM stock solution. The primary antibodies and dilutions used in immunocytochemistry were rat anti-BrdU (1:500, AbD Serotec, Raleigh, NC), rabbit anti-active caspase-3 (1:200, Cell Signal Technology), and mouse anti-SOX2 (1:500, R&D Systems). Goat anti-mouse, goat anti-rat, and goat-anti rabbit Alexa Fluor-conjugated secondary antibodies as well as Hoechst 33342 were from Invitrogen (Carlsbad, CA). For Western blot analysis, the following rabbit primary antibodies (1: 1000, Cell Signaling, Beverly, MA) were used: monoclonal anti-phospho JNK (Cat. 4668), monoclonal anti-JNK (Cat. 9258), polyclonal anti-phospho-p38 (Cat. 9211), and monoclonal anti-GAPDH (Cat. 2118). Horseradish

peroxidase-conjugated secondary antibodies were purchased 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 involving animal use. The primary aNPCs were prepared as previously described (Guo et al., 2012; Pan et al., 2013a; Pan et al., 2013b; Pan et al., 2012) from the SVZ of the lateral ventricles from 9.5 week-old male C57BL/6J mice. The solutions and media used during the SVZ-aNPCs isolation were filter sterilized. Briefly, the whole brain from three to four adult male mice was harvested following cervical dislocation and placed in HBSS (Invitrogen). Each brain was then sliced into 1 mm sections by using an adult mouse brain matrix (Kent Scientific, Torrington, CT), and the SVZ tissue was isolated from these sections via microdissection under a dissection microscope. The SVZ tissue was placed in Solution A (1X HBSS (Invitrogen, Carlsbad, CA) with 30 mM Glucose, 2mM pH 7.4 HEPES (Invitrogen), and 26 mM $NaCO_3$) and spun at 1,000 rpm for 10 min. The pelleted tissue was then resuspended and dissociated with a combination of mechanical and enzymatic digestion (MACS Neural Tissue Dissociation Kit, Miltenyi Biotec, San Diego, CA). To stop the digestion, DMEM/F-12 medium (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen, Carlsbad, CA) was added, and the SVZ tissue was then filtered through a 70μm cell strainer (Fisher Scientific, Waltham, MA) and spun down at 1,000 rpm for 3 min. The pellet was then 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 Science, Marlborough, MA) solution (1:10 Percoll in PBS), followed by spins at 1,000 rpm for 3 and 15 min, respectively. Then, 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), 10 ng/ml basic fibroblast growth factor (bFGF; EMD Chemicals), and 20 ng/ml of epidermal growth factor (EGF; EMD Chemicals) and followed by 5 min spins at 1,500 rpm. The cells were then seeded in a petri dish with initial proliferation medium and cultured at 37°C and 6.5% CO_2. Growth factors (EGF and bFGF) were refreshed every 3 d unless noted otherwise. Primary neurospheres formed after 7-14 d, then, the neurospheres were collected, enzymatically and mechanically dissociated, and resuspended in growth media (Advanced DMEM/F-12, 1X N2 Supplement (Invitrogen), 1X B27 Supplement without Vitamin A (Invitrogen); 2 mM L-Glutamine; 100 U/ml Penicillin/streptomycin (Invitrogen), 2 µg/ml Heparin sodium salt (Gilbert et al., 2001), 20 ng/ml EGF, and 10 ng/ml bFGF). The neurospheres were maintained in petri dishes in the growth media and passaged ≤ 11 times.

Drug treatment

For experiments, the SVZ neurospheres were dissociated in 0.125% trypsin-EDTA (Invitrogen) for 5 min, followed by incubation with 0.014% trypsin inhibitor (Gilbert, et al., 2001) for 5 min, and then plated as a monolayer culture on poly-L-ornithine (15 µg/ml) and fibronectin (1 mg/ml) (BD Biosciences, San Jose, CA) double-coated ACLAR coverslips or culture plates. Cells were seeded at a cell density of 7.5×10^3 cells per

well (48-well plate) in growth media and allowed to attach overnight. The next day, the media was changed to B27-free growth media and the cells were treated with agents for 0-96 h as described in the figure and figure legends. Cadmium chloride ($CdCl_2$) was dissolved in H_2O while the JNK and p38 inhibitors were dissolved in DMSO, so either an equal volume of H_2O or an equal concentration of DMSO was used as respective vehicle controls. To assess cell proliferation, BrdU was added to each well at final concentration of 10 μM for 2 h before the end of treatment.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Engstrom et al., 2015; Li et al., 2013). Briefly, 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 20 min at room temperature (RT). The fixed cells were then washed for 3 x 10 min in PBS, permeabilized by 1 x 5 min in 1% SDS in PBS, and washed 3 x 5 min in PBS. After that, the cells were blocked with 5% BSA in PBST (0.1% Triton-X 100 in PBS) for 30 min at RT, and 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, 3 x 15 min in 0.1 M borate buffer (pH 8.5). Following incubation with primary antibodies, the cells were washed with PBST for 3 x 10 min and then incubated with secondary antibodies for 2 h at RT. After that, cells were washed 3 x 10 min with PBST, incubated with 2.5 μg/ml Hoechst 33342 for 20 min, washed once again with PBST, and then mounted onto slides using anti-fade Aqua Poly/Mount (Polysciences, Warrington, PA) solution.

Imaging and quantification of immunostained cells

All images were captured using a fluorescence microscope (Zeiss, Germany) equipped with a camera, and a 10x or 20x objective (Zeiss, Germany). Images were adjusted for color, brightness, and contrast uniformly with ImageJ (NIH). A cell with nuclear condensation or fragmentation was scored as apoptotic. A cell was scored as marker positive if the cell had a uniformly stained Hoechst$^+$ nucleus as well as marker expression in the cell body. At least 250 cells per coverslip per treatment were quantified, and the experimenter was blinded to treatment.

Western blot analysis

The cells were seeded at 5 x 10^5 cells per well in poly-L-ornithine and fibronectin-coated 12-well plates for 24 h. Then, cells were 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 by using the BCA protein assay (Thermo Scientific, Waltham, MA). Samples containing 10 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) using a ChemiDoc XRS Imaging System (Bio-Rad, Hercules, CA). ImageJ (NIH) was used for the densitometry analysis and determination of fold induction normalized to a loading control (total unphosphorylated protein or GAPDH).

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) or Excel (Microsoft, Redmond, WA). All of the immunocytochemical data are from at least three independent experiments with triplicates for each data point ($n \geq 9$ for each data point). The Western blot data are representative data from three independent experiments. 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 Tukey post-hoc analysis ($\alpha = 0.05$) was used to analyze all of the drug treatment data (MAPK inhibitors). Two-way ANOVA with a Sidak post-hoc analysis ($\alpha = 0.05$) was used to compare JNK3 WT and KO groups. Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3 Results

Isolated SVZ-aNPCs retain stem cell properties

After isolation from the SVZ of the lateral ventricles of adult male C57Bl/6J mice, the SVZ-aNPCs were maintained as neurospheres and used for experiment within 11 passages. After dissociation and plating as a monolayer in the growth media, more than 98.2% of cells expressed SOX2 (Fig. 3.1), a marker of the neural stem cells, even at passage 11. This result suggests that the SVZ-aNPCs used in this study retain their stem cell characteristics.

Cd significantly decreases the total cell number and induces apoptosis in SVZ-aNPCs

To determine whether sub-micromolar concentrations of Cd exposure can induce toxic effects on SVZ-aNPCs, cells were treated with 0 to 0.45 μM $CdCl_2$ (50 ppb Cd^{2+}) for 96 h *in vitro*. Cd significantly decreased the total cell number, starting at 0.09 μM $CdCl_2$ (10 ppb Cd^{2+}), in a dose-dependent manner (Fig. 3.2 A-D). In addition, Cd treatment induced apoptosis, quantified by nuclear condensation and/or fragmentation, beginning at 0.09 μM $CdCl_2$, (Fig. 3.2 E). Furthermore, Cd significantly increased the number of cells positively immunostained for cleaved caspase-3, indicating caspase-3 activation (Fig. 3.2 F, G). These data suggest that Cd causes cell loss and induces apoptosis in SVZ-aNPCs.

Cd inhibits the proliferation of SVZ–aNPCs

To determine if Cd interferes with SVZ-aNPC cell proliferation, we pulsed the cells with BrdU during the final 2 h of a 24 h Cd treatment to label actively replicating cells going through S phase. Starting at 0.09 μM $CdCl_2$, we found a significant decrease of the percentage of $BrdU^+$ cells in a dose-dependent manner after a 24 h-treatment (Fig. 3.3 A-D). These data indicate that Cd inhibits proliferation of SVZ-aNPCs *in vitro*. Combined with data in Fig. 3.2, these results suggest that Cd decreases the total cell number through apoptosis and inhibition of proliferation.

Activation of the JNK signaling pathway contributes to Cd-induced cytotoxicity in SVZ-aNPCs

The c-Jun NH2-terminal kinase (JNK) is a member of the mitogen activated protein kinase (MAPK) family. JNK activation can stimulate proliferation or induce apoptosis (Klintworth et al., 2007; Lin, 2003; Newhouse et al., 2004; Xia et al., 1995). To elucidate the underlying signaling mechanisms of Cd-induced cytotoxicity on SVZ-aNPCs, we examined whether Cd activates the JNK signaling pathway. SVZ-aNPCs were treated with 0.45 μM $CdCl_2$ for 0-6 h, and the cell lysates were subjected to Western blot analysis. Treatment with Cd for 0.5, 1 and 2 h induced phosphorylation of JNK, indicative of JNK signaling activation (Fig. 3.4 A and B). Pre-incubation with a pan-JNK inhibitor SP600125 (0.5 μM) for 1h significantly reversed the cell number loss and apoptosis induced by Cd treatment (0.45 μM $CdCl_2$ for 96h) (Fig. 3.4 C and D). These data suggest that activation of the JNK signaling pathway plays an important role in Cd-induced toxic effects on SVZ-aNPCs.

Deletion of the neural specific JNK3 isoform of the JNKs attenuates Cd-induced apoptosis and cell loss of SVZ-aNPCs

To further examine the role that the JNK signaling pathway plays in Cd-induced cytotoxicity, we isolated SVZ-aNPCs from adult JNK3 KO or their wild type (WT) littermate mice, treated them with 0-0.18 μM $CdCl_2$ (20 ppb Cd^{2+}) for 96 h. We found that Cd induced apoptosis and caused cell loss in WT SVZ-aNPCs at 0.09 μM and 0.18 μM, respectively. In contrast, Cd treatment had no effect on apoptosis or cell number in JNK3 KO SVZ-aNPCs (Fig. 3.5 A and B). The effect of Cd is significantly different

between the WT and JNK3 KO groups (P = 0.0011 for apoptosis; P=0.0046 for total cell number, two-way ANOVA.). This result indicates that the JNK3 isoform contributes to the cytotoxicity of Cd in SVZ-aNPCs.

Activation of the p38 signaling pathway also contributes to Cd-induced cytotoxicity in SVZ-aNPCs

p38 kinase is another member of the MAP kinases. It is important for control of cellular responses to environmental stimuli (Johnson and Lapadat, 2002; Namgung and Xia, 2001; Newhouse et al., 2004; Xia et al., 1995). Here, we also examined if the p38 signaling pathway is involved in Cd-induced cytotoxicity. By Western blot analysis, we found that Cd exposure significantly increased p38 phosphorylation after 0.5, 1 and 2 h treatment (Fig. 3.6 A and B). Furthermore, pre-treatment with 0.5 μM p38 inhibitor (SP202190) for 1h almost completely blocked the toxic effects of Cd on apoptosis and total cell number (Fig. 3.6 C and D). These results suggest that the activation of p38 also contributes to Cd-induced toxic effects on SVZ-aNPCs.

3.4 Discussion

The goal of this study was to examine the potential toxicity of Cd on SVZ adult neurogenesis and investigate underlying signaling mechanisms. To address this issue, we used aNPCs isolated from adult mouse SVZ as a model system. We discovered that Cd significantly decreases the total cell number, inhibits proliferation, activates caspase-3, and induces apoptosis in a dose-dependent manner. Thus, Cd exposure impairs several aspects of SVZ adult neurogenesis, including cell proliferation and survival.

JNK and p38 MAPK are two members of the MAPK family and have been implicated in toxicant-induced apoptosis in various cells, including neuronal cells (Chen et al., 2008a; Chen et al., 2008b; Choi et al., 2010; Giordano et al., 2008; Kim et al., 2005; Klintworth et al., 2007; Namgung and Xia, 2001; Newhouse et al., 2004; Xia et al., 1995). We found that Cd significantly increased JNK and p38 phosphorylation, indicative of their activation. Although activation of JNK or p38 signaling can stimulate cell proliferation in some cells (Davis, 2000; Kyriakis and Avruch, 2012; Zhang et al., 2011), it is unlikely that their activation plays an important role here in terms of cell proliferation since Cd inhibits, rather than stimulate SVZ-aNPC proliferaiton. However, pharmacological inhibition of JNK or p38 prevented cell loss and apoptosis induced by Cd treatment, suggesting that JNK and p38 activation contributes to the cytotoxicity of Cd in SVZ-aNPCs. These results are consistent with other reports that Cd induces apoptosis in neuronal cells through activating the JNK signaling pathway (Chen et al., 2008a; Chen et al., 2008b).

There are three JNK isoforms encoded by different genes in the JNK family, JNK1, JNK2, and JNK3. Among them, JNK1 and JNK2 are widely present in a variety of tissues, whereas JNK3 is selectively expressed in the nervous system and to a much lesser extent in the testis and heart (Gupta et al., 1996; Martin et al., 1996; Mohit et al., 1995). Previous studies have found that JNK3 mediates arsenic-induced apoptosis of rat cerebellar neurons (Namgung and Xia, 2001), as well as paraquat- and rotenone-induced dopaminergic neuron death (Choi et al., 2010). Using SVZ-aNPCs isolated from JNK3-WT and JNK3-KO littermates, we discovered that deletion of the neural-

specific JNK3 attenuates Cd-induced apoptosis and cell loss. This suggests a critical role for the JNK3 isoform in Cd-induced SVZ-aNPCs death.

High-level occupational Cd exposure is associated with neurobehavioral defects including loss of olfaction in workers (Mascagni et al., 2003; Rose et al., 1992; Wang and Du, 2013). Two recent epidemiological studies have found that Cd exposure at levels that had previously been considered to be without adverse effect and common in the USA is also associated with impairment of cognition both in adults and children (Ciesielski et al., 2013; Ciesielski et al., 2012). In our present study, we investigated whether low-level Cd exposure can affect SVZ adult neurogenesis. Compared to Cd concentrations used in other *in vitro* studies (10-25 μM) (Chen et al., 2008a; Chen et al., 2008b), the concentrations of Cd^{2+} we used (0.03 μM-0.45 μM) were much lower. Nevertheless, we observed significant Cd toxicity in SVZ-aNPCs at a relatively low dose (0.09 μM $CdCl_2$, which is equivalent to 10 ppb Cd^{2+}). This concentration is close to the US EPA's maximum allowable contaminant level of Cd in drinking water (5 ppb). Thus, our findings suggest that Cd may exert adverse effects on SVZ adult neurogenesis under environmentally relevant exposure levels.

3.5 Conclusion

In conclusion, we provide evidence using an *in vitro* model system that low-level Cd exposure impairs SVZ adult neurogenesis. Specifically, Cd induces apoptosis and inhibits proliferation at environmentally relevant concentrations. We also identify activation of the p38 and JNK3 MAP kinases as critical signaling mechanisms underlying Cd cytotoxicity. Since adult neurogenesis in the SVZ is critical for normal

olfactory function (Bond et al., 2015; Wang et al., 2015; Zou et al., 2012), inhibition of adult neurogenesis may contribute to Cd impairment of olfaction.

3.6 Figures and Tables

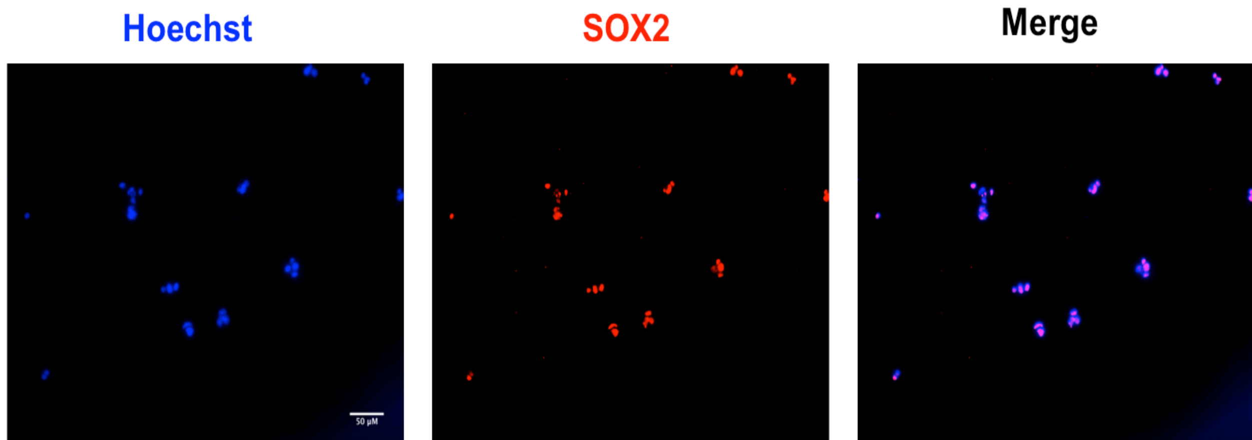
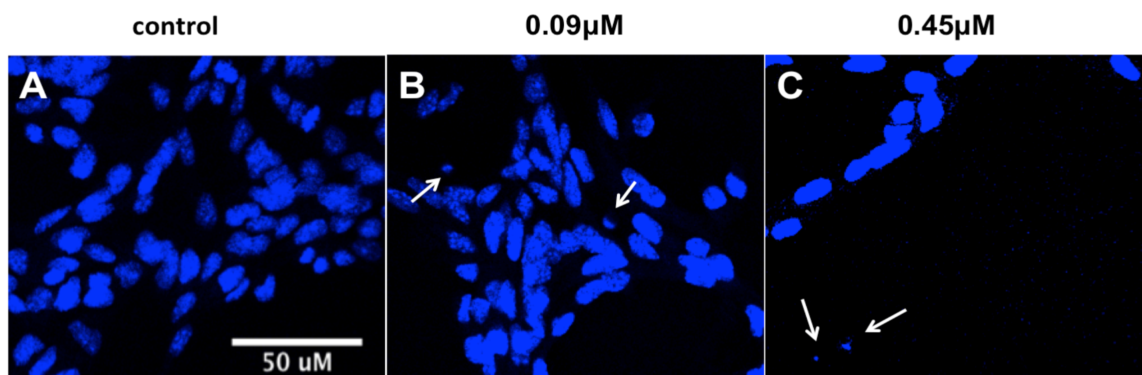


Figure 3.1. The SVZ-aNPCs retain their stem cell characteristics in vitro. At passage 11, the SVZ-aNPCs were fixed and immunostained for SOX2 (red), a marker for neural stem cells. Hoechst stained nuclei (blue) were used to identify all nuclei. Scale bar, 50 μm.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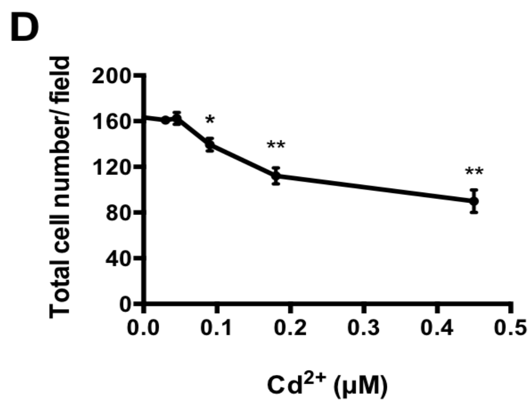
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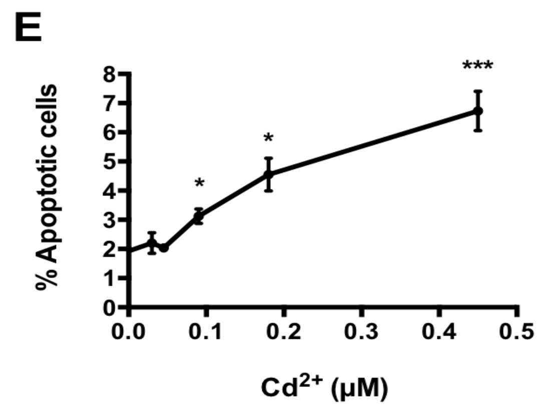
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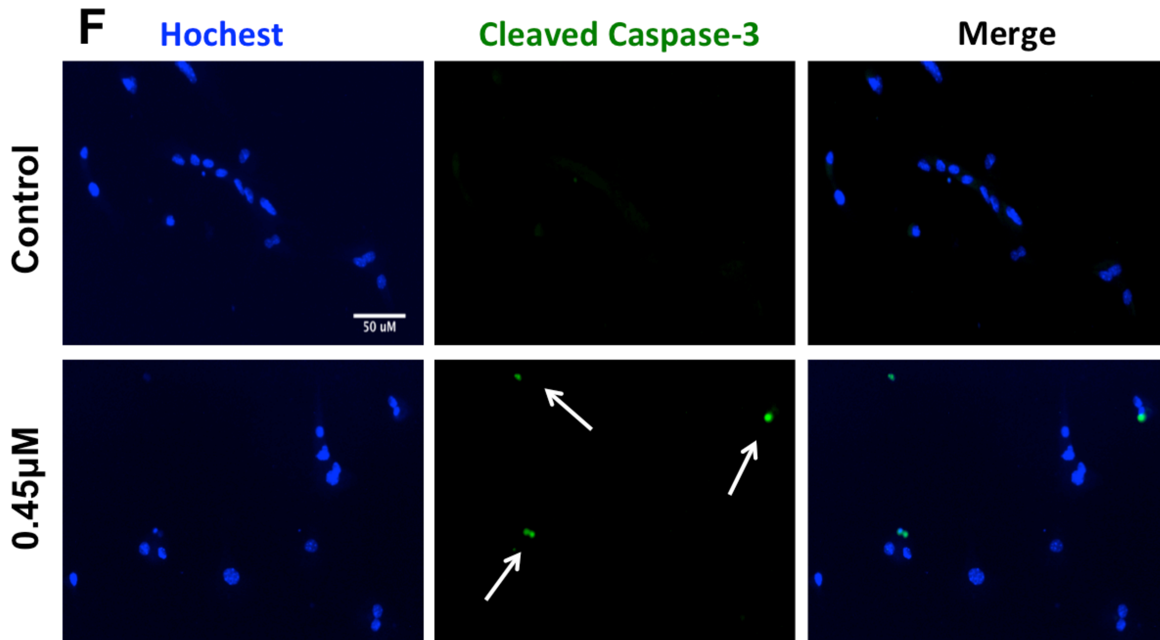

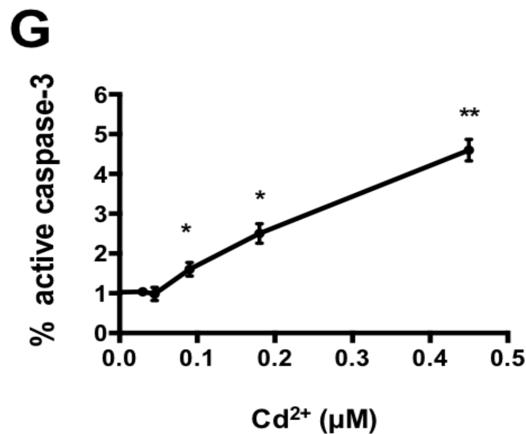

Figure 3.2. Cd decreases the total cell number and increases apoptosis in SVZ-aNPCs. (A-C) Representative Hoechst nuclei staining from (A) Control, (B) 0.09 μM, and (C) 0.45 μM $CdCl_2$-treated SVZ-aNPCs after 96 h exposure. Quantification of (D) the total cell number and (E) the percent apoptotic cells with nuclear condensation and/or fragmentation after 96 h treatment with $CdCl_2$. Arrows point to apoptotic cells with condensed and/or fragmented nuclei. (F, G) Cd activates caspase-3 in SVZ-aNPCs. (F) Representative Hoechst nuclei (blue) and

active caspase-3 (green) co-staining from Control and 0.45 μM $CdCl_2$-treated SVZ-aNPCs after 96 h exposure. (G) Quantification of the percent active caspase-3^+ cells after 96 h treatment with Cd. Arrows point to cells with cleaved Caspase-3. Hoechst: nuclei staining. n=3 independent experiments for a total of 9 coverslips per data point. Scale bar, 50 μm. Data represent mean ± SEM., n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

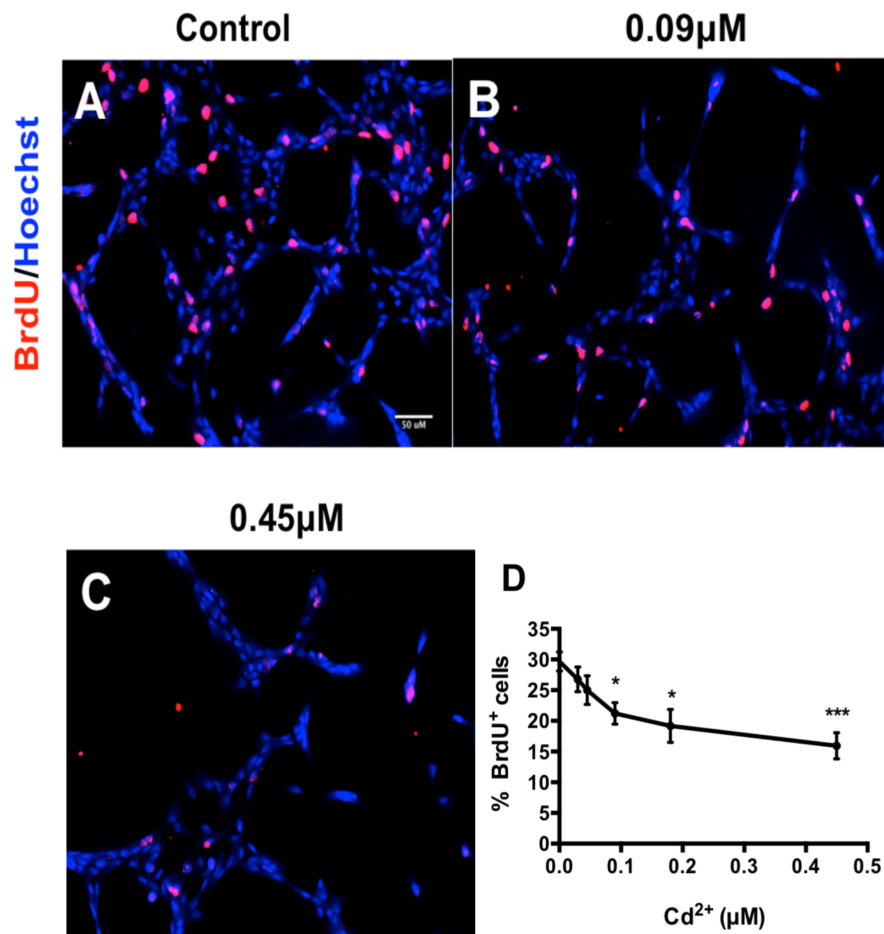

Figure 3.3. Cd reduces proliferation in SVZ-aNPCs. Representative BrdU (red) and Hoechst (blue) co-staining from (A) Control, (B) 0.09 μM, and (C) 0.45 μM $CdCl_2$-treated SVZ-aNPCs after 24h exposure. (D) Quantification of the percent $BrdU^+$ cells after 24 h treatment with Cd. BrdU: a marker for cells in S phase of the cell cycle. n=3 independent experiments for a total of 9 coverslips per data point. Scale bar, 50 μm. Data represent mean ± SEM., n.s., not significant; *p < 0.05; ***p<0.001.

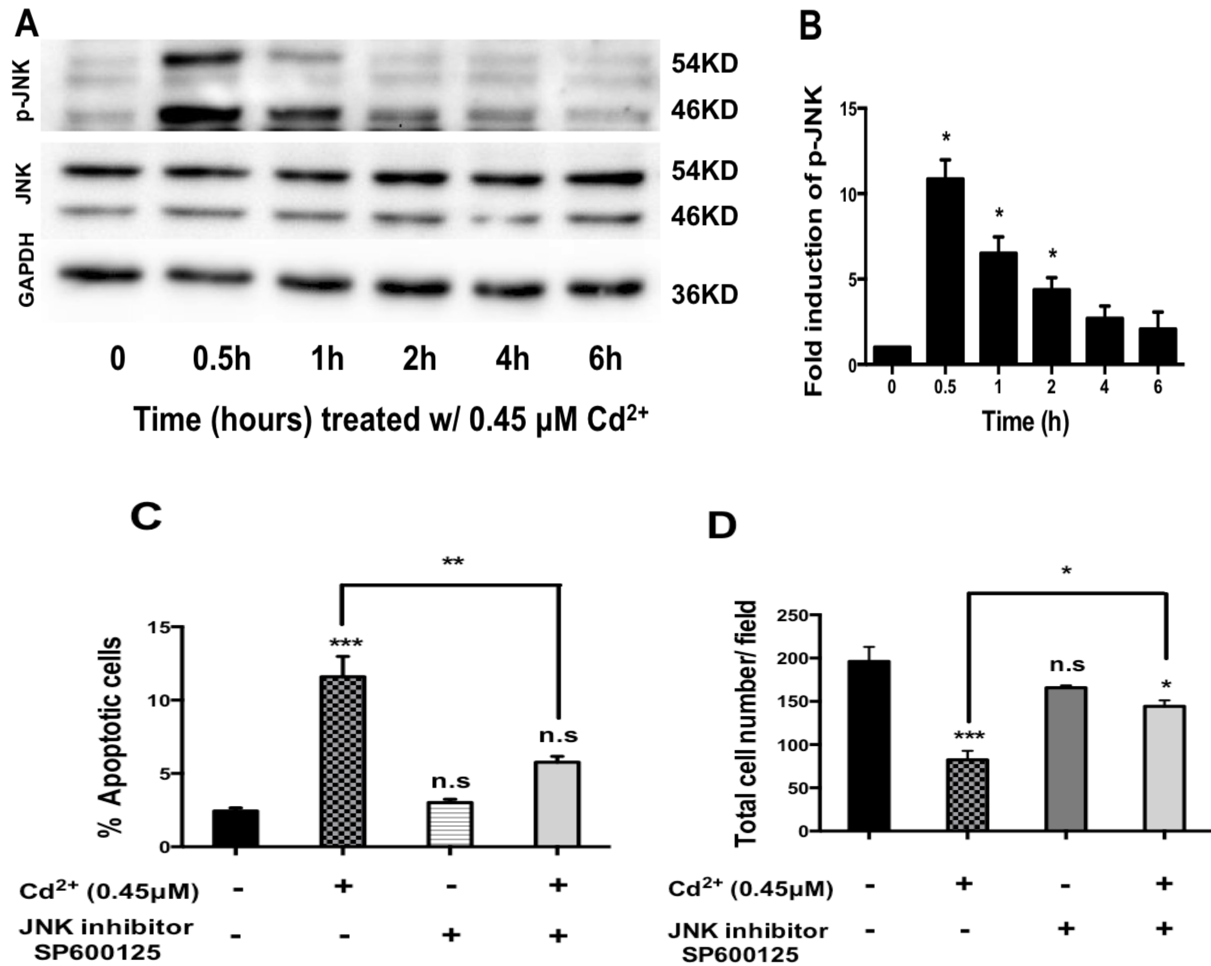

Figure 3.4. Activation of the JNK signaling pathway contributes to Cd-induced cytotoxicity in SVZ-aNPCs. (A and B), SVZ-aNPCs were treated with 0.45 μM $CdCl_2$ for the indicated amount of time. The cell lysates were subjected to Western blot analysis for (A) phosphorylated-JNK, total JNK, and the protein loading control GAPDH, and (B) the fold induction of p-JNK (normalized to total JNK) in Cd-treated cells compared to controls. (C-D) SVZ-aNPCs were pretreated with 0.5 μM of the pan-JNK inhibitor (SP600125) for 1h and then treated with 0.45 μM $CdCl_2$ for an additional 96h. Quantification of (C) the percent apoptotic cells and (D) the total cell number. Data represent mean ± SEM., n.s., not significant; *p < 0.05; **p< 0.01; ***p<0.001.

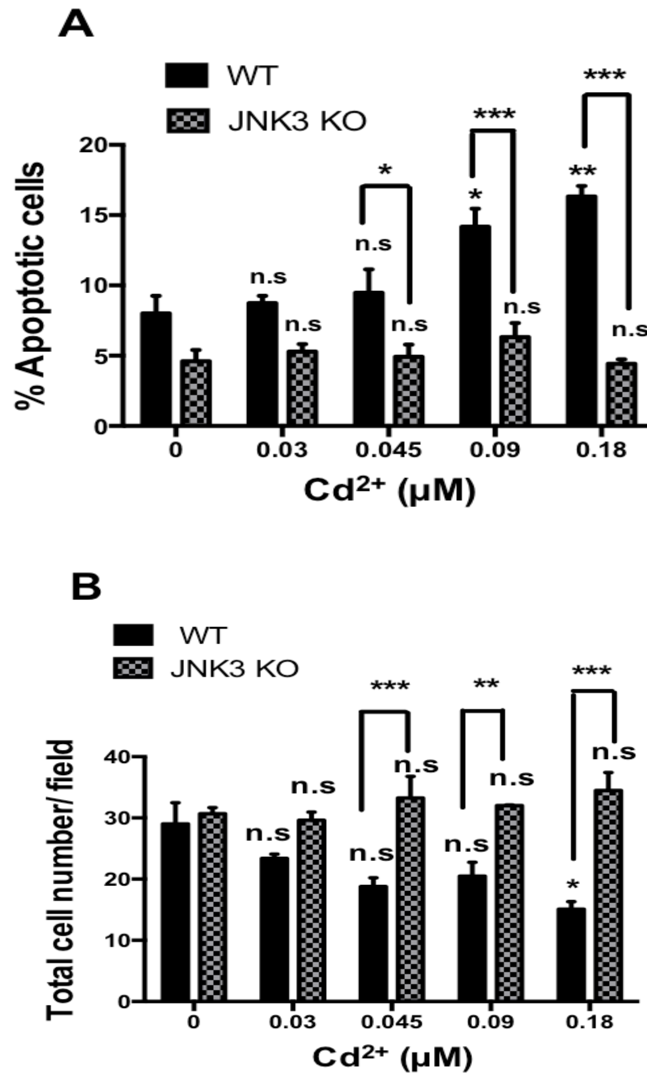

Figure 3.5. Deletion of the neural specific JNK3 isoform attenuates Cd-induced apoptosis and cell loss of SVZ-aNPSCs. SVZ-aNPCs isolated from WT and JNK3 KO littermate mice were treated for 96h with several concentrations of Cd^{2+} as indicated. Quantification of (A) the percent apoptotic cells and (B) the total cell number. Data represent mean ± SEM., n.s., not significant; *p < 0.05; **p< 0.01; ***p<0.001.

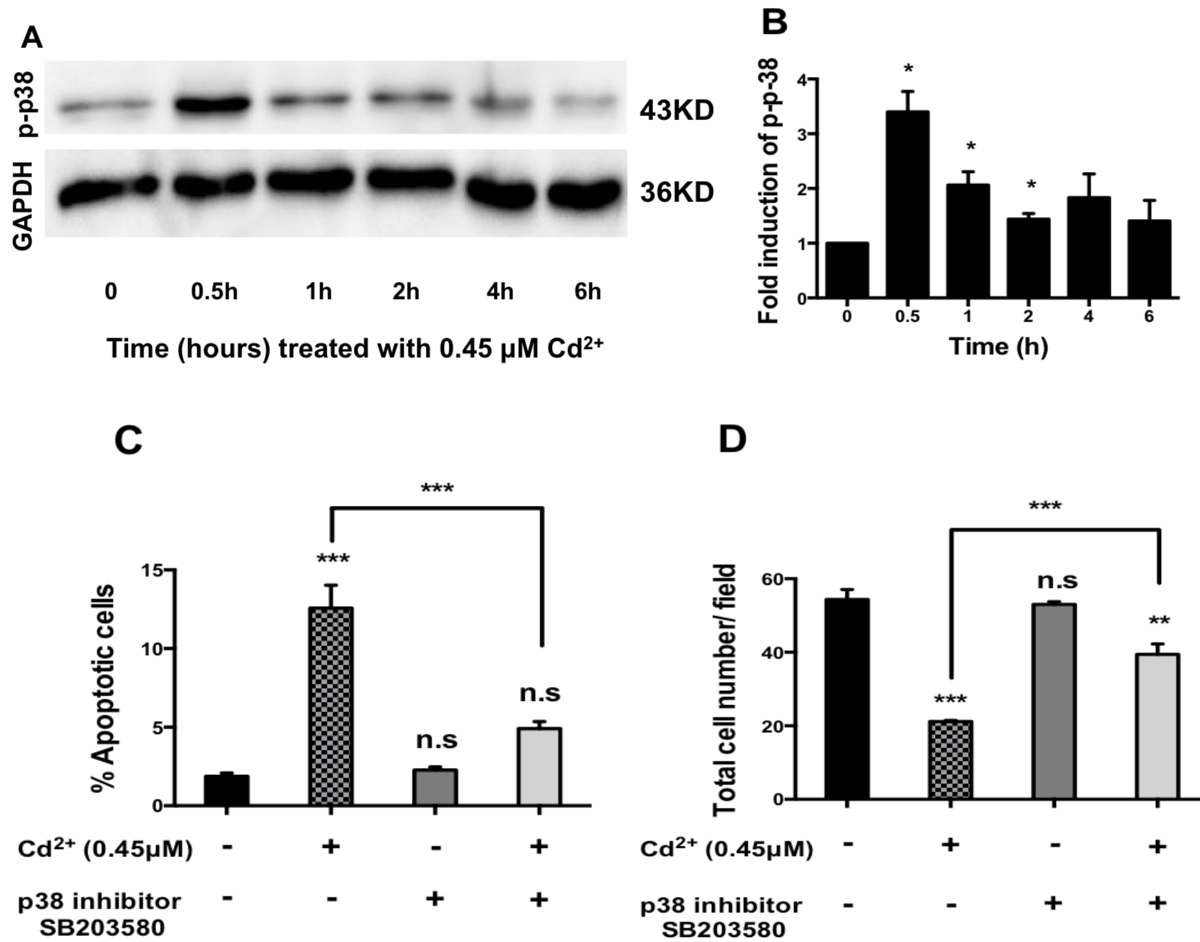

Figure 3.6. Activation of the p38 signaling pathway contributes to Cd-induced cytotoxicity in SVZ-aNPCs. (A and B) SVZ-aNPCs were treated with 0.45 μM $CdCl_2$ for the indicated amount of time. The cell lysates were subjected to Western blot analysis for (A) phosphorylated-p38, and (B) the fold induction of p-38 (normalized to GAPDH) in Cd-treated cells compared to controls. (C-D) SVZ-aNPCs were pretreated with 0.5 μM of the pan-p38 inhibitor (SP202190) for 1h and then treated with 0.45 μM $CdCl_2$ for an additional 96h. Quantification of (C) the percent apoptotic cells and (D) the total cell number. Data represent mean ± SEM., n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

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Chapter 4: Cadmium impairs the survival, proliferation, and differentiation of cultured adult subgranular (SGZ) neural stem cells through activation of the JNK and p38 MAP kinases

4.1 Introduction

Cadmium (Cd) is a ubiquitous environmental pollutant and a major public health concern. Cd is considered as one of the top 20 hazardous pollutants, ranking No. 7 on U.S Environmental Protection Agency (EPA)’s superfund hazardous waste priority list (Liao and Freedman, 1998). Cd is mainly released into the environment from anthropogenic sources, such as smelting, refining of metals, application of phosphate fertilizers, and burning of fossil fuels (Faroon et al., 2012; Waisberg et al., 2003). Because of its high soil-plant transfer rate, the diet is the main source of Cd exposure for general people. The food contains high concentrations of Cd includes leafy vegetables, peanuts, rice, shellfish, and organ meets (Clemens, 2006; Faroon et al., 2012; Satarug et al., 2010). In addition, smoking is another major source of Cd because tobacco leaves naturally accumulates high levels of Cd (Saldivar et al., 1991; Stohs et al., 1997). Since the half-life of Cd in human body is about 10-30 years, Cd accumulates in human bodies and then induces toxic effects in various organs, such as kidney (Edwards and Prozialeck, 2009; Johri et al., 2010; Torra et al., 1995), bone (Chen et al., 2009; Kjellstrom, 1992; Wilson et al., 1996), liver (Dudley et al., 1982; Goering et al., 1993; Kim et al., 2018; Liu et al., 2009), lung (Lag et al., 2010; Manca

et al., 1994; Takenaka et al., 2004; Yang et al., 1997), cardiovascular system (Messner and Bernhard, 2010; Nai et al., 2015), and GI tract (Tinkov et al., 2018).

Increasing studies suggest that Cd is a potential neurotoxicant. *In vitro* studies have shown that Cd exposure can induce cell death in different neural cells, including SH-SY5Y cells, PC-12 cells, and rat cerebral cortical neurons (Chen et al., 2008a; Chen et al., 2008b; Lopez et al., 2003; Yuan et al., 2013). Our study also found that Cd exposure induced apoptosis and inhibited proliferation in SVZ adult neural progenitor/stem cells (Wang et al., 2017). Animal study also reported that Cd induced hemorrhages in the cerebral cortex and cerebellum, affects functions of neurotransmitter, and a serious of behavior deficits (Ali et al., 1986; Gabbiani et al., 1967; Goncalves et al., 2010; Goncalves et al., 2012; Maodaa et al., 2016; Wong and Klaassen, 1982). Several epidemiological studies have suggested that Cd exposure is associated with impairments of cognition in humans, including both adult and children (Ciesielski et al., 2013; Ciesielski et al., 2012). Our recent study also found that chronic Cd exposure yielding blood levels of Cd close to what has been measured in the general population impaired hippocampus-dependent learning and memory in adult mice, providing the direct experimental evidence that Cd exposure can affect cognitive functions in animals (Wang et al., 2018). However, the potential mechanisms of Cd neurotoxicity on hippocampus-dependent memory are still unknown.

Adult hippocampal neurogenesis is the process that generates new functional neurons from adult neural progenitor/stem cells (aNPCs) in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Ming and Song, 2005). These adult-born neurons have been implicated in certain forms of hippocampus-dependent learning and

memory formation (Clelland et al., 2009; Deng et al., 2010; Deng et al., 2009; Pan et al., 2012a; Pan et al., 2012b; Wang et al., 2014). Importantly, adult hippocampal neurogenesis can be modulated by various external influences. For example, exercise and environmental enrichment increase adult neurogenesis (Kempermann et al., 1997; van Praag et al., 1999), while stress, drug abuse, and lead exposure inhibit adult neurogenesis (Eisch et al., 2000; Engstrom et al., 2015; Mirescu and Gould, 2006). Since Cd exposure impairs hippocampus-dependent learning and memory, which can be modulated by adult hippocampal neurogenesis, it is important to investigate the effects of Cd on adult hippocampus neurogenesis. In this study, by using primary cultured aNPCs from the SGZ of adult mice as an *in vitro* model, we examined whether Cd exposure can affect adult hippocampal neurogenesis, providing important information to elucidate the underlying molecular mechanisms.

4.2 Materials and Methods

Reagents

The preparation, use, and disposal of hazardous agents were carried out as recommended by the Environmental Health and Safety Office at the University of Washington. Cadmium chloride (Cat. 202908, Sigma-Aldrich, St. Louis, MO) was dissolved in deionized distilled water (H_2O) to make a 1mM (Cd^{2+}) stock solution and stored at -20°C. The JNK (Cat. SP600125, Calbiochem, Billerica, MA) and p38 (Cat. SB202190, Calbiochem, Billerica, MA) inhibitors were dissolved in dimethyl sulfoxide (DMSO) to yield 3 mM stock solutions and stored at -20°C. 5-bromo-2'-deoxyuridine (BrdU) (Cat.B9285, Sigma-Aldrich, St. Louis, MO) was stored as a 65 mM stock solution.

The primary antibodies and dilutions used in immunocytochemistry were rat anti-BrdU (1:500, AbD Serotec, Raleigh, NC), rabbit anti-active caspase-3 (1:200, Cell Signal Technology), and mouse anti-SOX2 (1:500, R&D Systems). Goat anti-rat, goat anti-rabbit, and goat-anti mouse Alexa Fluor-conjugated secondary antibodies, and Hoechst 33342 were purchased from Invitrogen (Carlsbad, CA). For Western blot analysis, the following rabbit primary antibodies (1: 1000, Cell Signaling, Beverly, MA) were used: monoclonal anti-phospho JNK (Cat. 4668), monoclonal anti-JNK (Cat. 9258), polyclonal anti-phospho-p38 (Cat. 9211s), polyclonal anti- p38 (Cat. 9212s) and monoclonal anti-GAPDH (Cat. 2118). Horseradish peroxidase-conjugated secondary antibodies were purchased from EMD Millipore (Billerica, MA).

Cell culture

The University of Washington Institutional Animal Care and Use Committee approved all experimental animal use procedures. The primary aNPCs were prepared as previously described (Guo et al., 2012; Pan et al., 2013a; Pan et al., 2013b; Pan et al., 2012c) from the SGZ of the dentate gyrus in the hippocampus from 6.5-9 week-old male C57BL/6J mice. The solutions and media used during the SGZ-aNPCs isolation were filter sterilized. Briefly, the whole brain from four to five adult male mice was harvested following cervical dislocation and placed in HBSS (Invitrogen, Carlsbad, CA). Each brain was then sliced into 1 mm sections by using an adult mouse brain matrix (Kent Scientific, Torrington, CT), and the SGZ tissue was isolated from these sections via microdissection under a dissection microscope. The SGZ tissue was then placed in Solution A (1X HBSS (Invitrogen) with 30 mM Glucose, 2mM pH 7.4 HEPES

(Invitrogen), and 26 mM $NaCO_3$) and spun at 1,000 rpm for 10 min. The pelleted tissue was suspended and dissociated with a combination of mechanical and enzymatic digestion (MACS Neural Tissue Dissociation Kit, Miltenyi Biotec, San Diego, CA). To stop the digestion, DMEM/F-12 medium (Invitrogen) with 10% FBS (Invitrogen) was added, and the SVZ tissue was filtered through a 70μm cell strainer (Fisher Scientific, Waltham, MA) and spun down at 1,000 rpm for 3 min. The pellet was then 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 Science, Marlborough, MA) solution (1:10 Percoll in PBS), followed by spins at 1,000 rpm for 3 and 15 min, respectively. Then, 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), 10 ng/ml basic fibroblast growth factor (bFGF; EMD Chemicals), and 20 ng/ml of epidermal growth factor (EGF; EMD Chemicals) and followed by 5 min spins at 1,500 rpm. The cells were then seeded in a petri dish with initial proliferation medium and cultured at 37°C and 6.5% CO_2. Growth factors (EGF and bFGF) were refreshed every 3 d unless noted otherwise. Primary neurospheres formed after 7-14 d. Then, the neurospheres were collected, enzymatically and mechanically dissociated, and resuspended in growth media (Advanced DMEM/F-12, 1X N2 Supplement (Invitrogen), 1X B27 Supplement without Vitamin A (Invitrogen); 2 mM L-Glutamine; 100 U/ml Penicillin/streptomycin (Invitrogen), 2 μg/ml Heparin sodium salt, 20 ng/ml EGF, and 10 ng/ml bFGF). The neurospheres were maintained in petri dishes in the growth media and passaged ≤ 10 times.

Chemical treatments

For experiments, the SGZ neurospheres were dissociated in 0.125% trypsin-EDTA (Invitrogen) for 5 min, followed by incubation with 0.014% trypsin inhibitor (Gilbert, et al. 2001) for 5 min, and then plated as a monolayer culture on poly-L-ornithine- (15 µg/ml) and fibronectin (1 mg/ml) (BD Biosciences, San Jose, CA) double-coated ACLAR coverslips or culture plates. Cells were seeded at a cell density of 7.5×10^3 cells per well (48-well plate) in growth media overnight. The following day, the media was changed to B27-free growth media and the cells were treated with agents for 0-96 h as described in the figure and figure legends. Cadmium chloride ($CdCl_2$) was dissolved in H_2O while the JNK and p38 inhibitors were dissolved in DMSO, so either an equal volume of H_2O or an equal concentration of DMSO was used as respective vehicle controls. To assess cell proliferation, BrdU was added to each well at final concentration of 10 µM for 2 h before the end of treatment.

To examine differentiation, the cells were seeded at density of 2×10^4 cells per well overnight. The next day, the media was replaced with growth factor free media supplemented with 1 mg/ml bovine serum albumin (BSA) (Equitech Bio, Kerrville, TX), and then cells were treated with Cd or vehicle control for 5 days.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Wang, et al., 2017). Briefly, the cells were fixed by removing half of the media from each well and replacing with an equal volume of 8% paraformaldehyde (PFA)/8% sucrose in PBS for 20 min at room temperature (RT). The fixed cells were then washed for 3 x 10 min in

PBS, permeabilized by 1 x 5 min in 1% SDS in PBS and washed 3 x 5 min in PBS. After that, the cells were blocked with 5% BSA in PBST (0.1% Triton-X 100 in PBS) for 30 min at RT and 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, 3 x 15 min in 0.1 M borate buffer (pH 8.5). Following incubation with primary antibodies, the cells were washed with PBST for 3 x 10 min and then incubated with secondary antibodies for 2 h at RT. Then, cells were washed 3 x 10 min with PBST, incubated with 2.5 µg/ml Hoechst 33342 for 15 min, washed once again with PBST, and then mounted onto slides using anti-fade Aqua Poly/Mount (Polysciences, Warrington, PA) solution.

Imaging and quantification of immunostained cells

All images were captured by using a fluorescence microscope (Zeiss, Germany) equipped with a camera, and a 10x or 20x objective (Zeiss, Germany). Images were adjusted for color, brightness, and contrast uniformly with ImageJ (NIH). A cell with nuclear condensation or fragmentation was scored as apoptotic. A cell was scored as marker positive if the cell had a uniformly stained $Hoechst^+$ nucleus as well as marker expression in the cell body. At least 500 cells per coverslip per treatment were quantified, and the experimenter was blinded to treatment.

Western blot analysis

The cells were seeded at 1 x 10^6 cells per well in poly-L-ornithine and fibronectin-coated 12-well plates for 24 h. Then, cells were 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 by using the BCA protein assay (Thermo Scientific, Waltham, MA). Samples containing 10-15 μg proteins were separated by gel electrophoresis on a 12.5% SDS-PAGE gel and transferred to a PVDF membrane (EMD Millipore, Burlington, MA). Following antibody incubation, the protein of interest was detected with ECL prime (GE Healthcare, Marlborough, MA) using a ChemiDoc XRS Imaging System (Bio-Rad, Hercules, CA). ImageJ (NIH) was used for the densitometry analysis and determination of fold induction normalized to a loading control.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) or Excel (Microsoft, Redmond, WA). All of the immunocytochemical data are from at least three independent experiments with triplicates for each data point ($n \geq 9$ for each data point). The Western blot data are representative data from three independent experiments. 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 Tukey post-hoc analysis ($\alpha = 0.05$) was used to analyze all of the drug treatment data (MAPK inhibitors). Data represent mean ± SEM., n.s. not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.3 Results

Isolated SGZ-aNPCs maintains stem cell properties

The SGZ-aNPCs were isolated from the SGZ of dentate gyrus of adult male C57BL/6J mice and maintained as neurospheres for experiment within 11 passages. After dissociation and plating as a monolayer in the growth media, more than 97% of cells expressed SOX2 (Fig. 4.1), a marker of the neural stem cells, even at passage 11. This result suggests that the SGZ-aNPCs used in this study retain their stem cell characteristics.

Cd decreases the total cell number and induces apoptosis in SGZ-aNPCs

To examine if sub-micromolar concentrations of Cd exposure induces toxic effects on SGZ-aNPCs, cells were treated with 0 to 0.45 μM $CdCl_2$ (50 ppb Cd^{2+}) for 96 h. Cd significantly decreased the total cell number in a dose-dependent manner, starting at 0.09 μM $CdCl_2$ (10 ppb Cd^{2+}) (Fig. 4.2 A, D, G, J). Cd also induced apoptosis, quantified by nuclear condensation and/or fragmentation, beginning at 0.09 μM $CdCl_2$, (Fig. 4.2 K). In addition, starting at 0.09 μM, Cd significantly increased activation of caspase-3 (Fig. 4.2 B, C, E, F, H, I, L). These data indicate that Cd treatment causes cell loss and induces apoptosis in SGZ-aNPCs.

Cd inhibits the proliferation of SGZ-aNPCs

To determine if Cd interferes with the proliferation of SGZ-aNPCs, we pulsed the cells with BrdU during the final 2 h of a 24 h Cd treatment to label actively replicating cells in S phase. We found a significant decrease of the percentage of $BrdU^+$ cells,

starting at 0.09 μM $CdCl_2$ in a dose-dependent manner after a 24 h-treatment (Fig. 4.3 A-D). These data suggest that Cd inhibits proliferation of SGZ-aNPCs *in vitro*. With data in Fig. 4.2, these results further suggest that Cd exposure decreases the total cell number through both apoptosis and inhibition of proliferation.

Cd inhibits spontaneous neuronal differentiation of SGZ-aNPCs

We also investigated if Cd decreases spontaneous neuronal differentiation of SGZ-aNPCs. The cells were cultured in EGF/bFGF-free media containing vehicle and Cd for 5 d to allow spontaneous neuronal differentiation. We examined SGZ-aNPCs differentiation by immunostaining for β-III tubulin, which is a marker of immature neurons. We found that starting at 0.09 μM, Cd treatment significantly decreased the percentage of β-III tubulin$^+$ cells (Fig. 4.4 A-J), which suggests that Cd exposure inhibits spontaneous neuronal differentiation of SGZ-aNPCs.

Activation of the JNK signaling pathway contributes to Cd cytotoxicity in SGZ-aNPCs

The c-Jun NH2-terminal kinase (JNK) is a member of the mitogen activated protein kinase (MAPK) family. Its activation can induce apoptosis (Klintworth et al., 2007; Lin, 2003; Newhouse et al., 2004; Xia et al., 1995). To elucidate the underlying signaling mechanisms of Cd cytotoxicity in SGZ-aNPCs, we investigated if the JNK signaling is activated by Cd treatment. Cells were initially treated with 0.45 μM Cd for 0-6 h, the cell lysates were subjected to western blot analysis. Treatment with Cd for 0.5 h and 1 h induced significant increase of phosphorylation of JNK, indicating JNK signaling

activation (Fig. 4.5 A and B). Then, we treated SGZ-aNPCs with 0 - 0.45 μM Cd for 1 h, and found starting at 0.09 μM, Cd treatment induced activation of JNK (Fig. 4.5 C and D). In addition, pre-treatment with a pan-JNK inhibitor SP600125 (0.5 μM) for 1h significantly reversed the apoptosis and cell number loss induced by Cd (0.45 μM $CdCl_2$ for 96h) (Fig. 4.5 E and F). These data indicate that activation of the JNK signaling pathway is involved in Cd-induced toxic effects in SGZ-aNPCs.

Activation of the p38 signaling pathway contributes to Cd cytotoxicity in SGZ-aNPCs

p38 kinase is also a member of the MAP kinases family. It is important for cellular responses to environmental stimuli (Johnson and Lapadat, 2002; Namgung and Xia, 2001; Newhouse et al., 2004; Xia et al., 1995). Here, we further investigated if the p38 signaling pathway is important for Cd-induced cytotoxicity. We treated SGZ-aNPCs with 0.45 μM Cd for 0-6 h and found 0.5 h and 1 h Cd exposure significantly increased the phosphorylation of p38 (Fig. 4.6 A and B). We also found that 1 h Cd treatment significantly increased p38 phosphorylation at 0.09 μM, 0.18 μM, and 0.45 μM (Fig. 4.6 C and D). Furthermore, pre-treatment with 0.5 μM p38 inhibitor (SP202190) for 1h almost significantly decreased the cytotoxicity of Cd on apoptosis and total cell number loss (Fig. 4.6 E and F). These results suggest that p38 signaling pathway also contributes to Cd-induced toxic effects in SGZ–aNPCs.

4.4 Discussion

The neurtoxicity of Cd, especially on cognition, is not fully understood. Recently, two epidemiological studies reported association between exposures to environmentally relevant blood levels of Cd and impairment of cognition, both in adults and children in USA (Ciesielski et al., 2013; Ciesielski et al., 2012). In their studies, they found higher cumulative Cd exposure is associated with decreased performance in task requiring attention and perception in adult, and increased risk of learning ability in children. In our recent study, we discovered low-level Cd exposure (3 mg/L) through drinking water impairs certain forms of hippocampus-dependent learning and memory, including spatial working memory and contextual fear memory (Wang et al., 2018). However, the research about underlying mechanisms of Cd toxicity in hippocampus-dependent learning and memory is still lacking. Since adult hippocampal neurogenesis plays an important role in the regulation of cognitive function, the goal of our study was to investigate the potential toxic effects of Cd on adult hippocampal neurogenesis and investigate underlying signaling mechanisms, by using aNPCs prepared from adult mouse SGZ as a model system.

Compared to other *in vitro* studies that typically use concentrations in the 10-25 μM range (Chen et al., 2008a; Chen et al., 2008b), the concentrations of Cd used in our study (0.03 μM-0.45 μM) were much lower. Nevertheless, we observed significant Cd cytoxicity in SGZ-aNPCs at a relatively low dose (0.09 μM $CdCl_2$, equivalent to 10 ppb or 10 μg/L Cd). This Cd exposure is close to the blood Cd concentrations of smokers in general population (0.5 – 1.5 μg/L). Thus, our findings suggest that Cd may exert toxic effects on adult hippocampal neurogenesis at environmentally relevant exposure levels.

Our data demonstrate that Cd treatment impairs several important processes in adult hippocampal neurogenesis. Under proliferation conditions (with EGF and bFGF in medium), Cd significantly decreased the total cell number, and induced apoptosis at concentration as low as 0.09 μM. At this exposure level, Cd also decreased the percentage of $BrdU^+$ cells, which suggest that Cd treatment inhibited cell proliferation. In differentiation conditions (without EGF and bFGF in medium), Cd impaired SGZ aNPCs spontaneous neuronal differentiation, starting at 0.09 μM. These data provide the first evidence that Cd exposure impairs several aspects of SGZ adult neurogenesis *in vitro*, including cell proliferation, differentiation, and survival.

The underlying signaling mechanisms for Cd toxicity in SGZ-aNPCs have not been well studied. JNK and p38 MAPK are two key members of the MAPK family and have been implicated in toxicant-induced apoptosis in various neuronal cells, such as dopaminergic neuron and cerebellar granule neurons (Chen et al., 2008a; Chen et al., 2008b; Choi et al., 2010; Giordano et al., 2008; Kim et al., 2005; Klintworth et al., 2007; Namgung and Xia, 2001; Newhouse et al., 2004; Xia et al., 1995). We found that Cd significantly increased JNK and p38 phosphorylation, indicative of their activation. Although activation of JNK or p38 signaling can increase proliferation in some cells (Davis, 2000; Kyriakis and Avruch, 2012; Zhang et al., 2011), it is unlikely that their activation plays an important role here in terms of cell proliferation since Cd inhibits, rather than stimulate proliferation of SGZ-aNPC. In addition, pharmacological inhibition of JNK or p38 reduced Cd-induced cell loss and apoptosis, suggesting that JNK and p38 activation are involved in the cytotoxicity of Cd in SGZ-aNPCs. These

results are consistent with other reports that Cd induces apoptosis in neuronal cells through activating the JNK signaling pathway (Chen et al., 2008a; Chen et al., 2008b).

4.5 Conclusion

In conclusion, we have demonstrated that sub-micromolar Cd exposure impairs SGZ adult neurogenesis *in vitro*. Specifically, Cd induced apoptosis, inhibited proliferation, and decreased spontaneous neuronal differentiation at environmentally relevant concentrations. We also identify activation of the JNK and p38 MAP kinases as critical signaling mechanisms underlying Cd cytotoxicity. Since the adult hippocampus is critical for normal function of hippocampus-dependent learning and memory (Bond et al., 2015; Wang et al., 2015; Zou et al., 2012), inhibition of SGZ adult neurogenesis may contribute to Cd neurotoxicity of cognition.

4.6 Figures and Tables

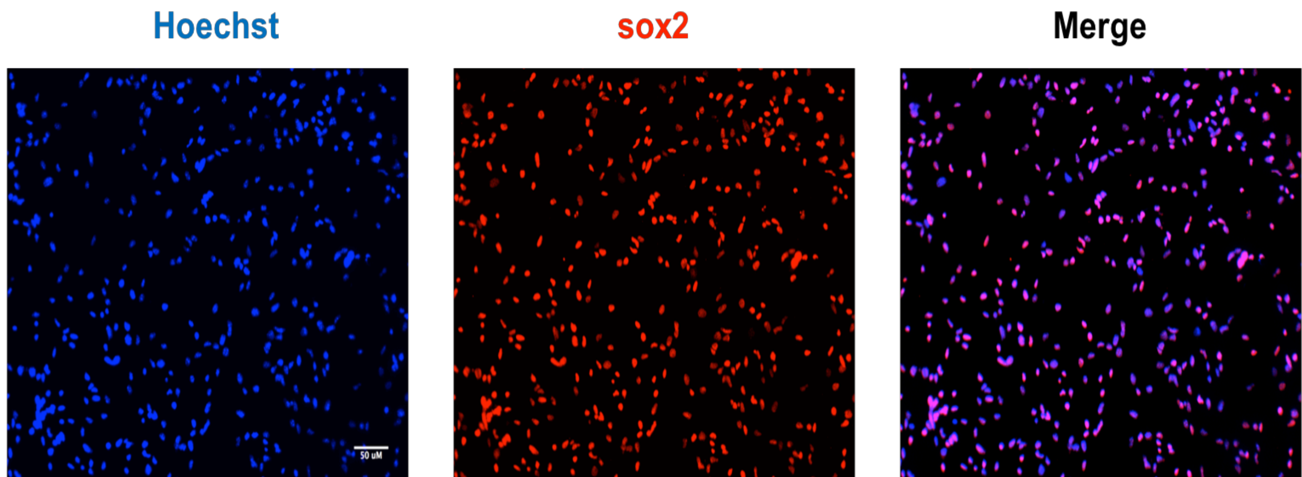


Figure 4.1. The SGZ-aNPCs maintain stem cell characteristics in vitro. At passage 11, the SGZ-aNPCs were fixed and immunostained for SOX2 (red), a marker for neural stem cells. Hoechst stained nuclei were used to identify nuclei. Scale bar, 50 µm.

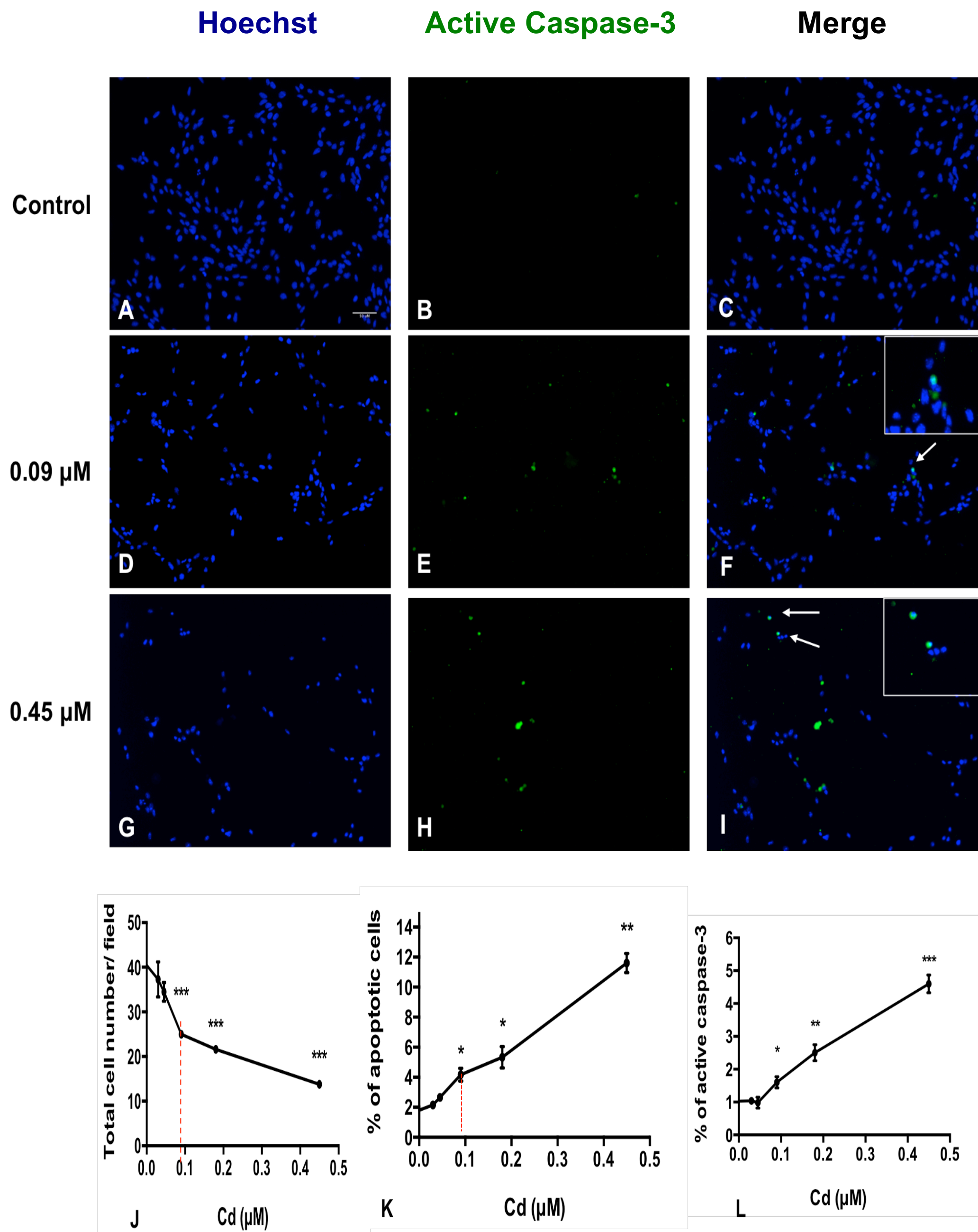


Figure 4.2. Cd decreases the total cell number and increases apoptosis in SGZ-aNPCs. (A, D, G) Representative Hoechst nuclei staining from (A) Control, (D) 0.09 μM, and (G) 0.45 μM Cd-treated SGZ-aNPCs after 96 h exposure. Quantification of (J) the total cell number and (K) the percent of apoptotic cells with nuclear condensation and/or fragmentation after 96 h

treatment with Cd. (B, E, H) Representative cleaved caspas-3 staining from (B) Control, (E) 0.09 μM, and (H) 0.45 μM Cd-treated SGZ-aNPCs after 96 h exposure. Quantification of (L) the percent of active caspase-3 (C, F, I) Representative Hoechst (blue) and active caspase-3 (green) co-staining from (C) Control, (F) 0.09 μM, and (I) 0.45 μM Cd-treated SGZ-aNPCs after 96 h exposure. Arrows point to cells with cleaved Caspase-3. Hoechst: nuclei staining. n=3 independent experiments for a total of 9 coverslips per data point. Scale bar, 50 μm. Data represent mean ± SEM., n.s., not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

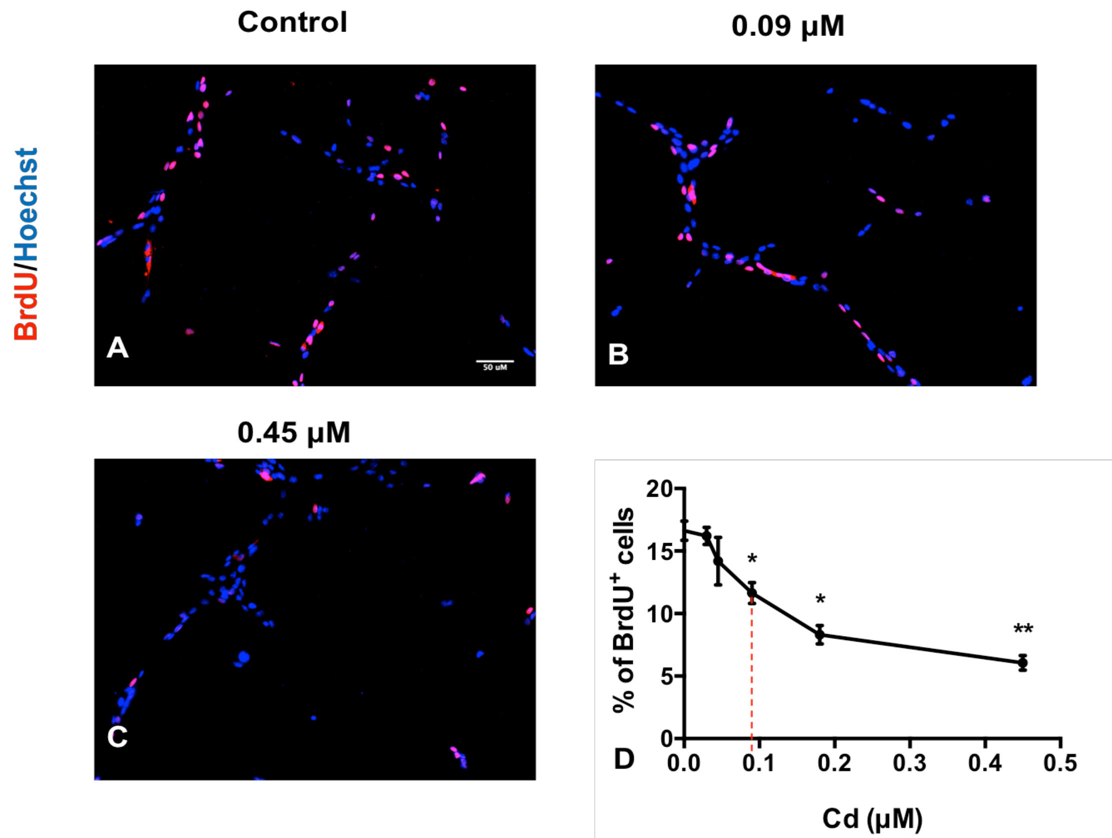
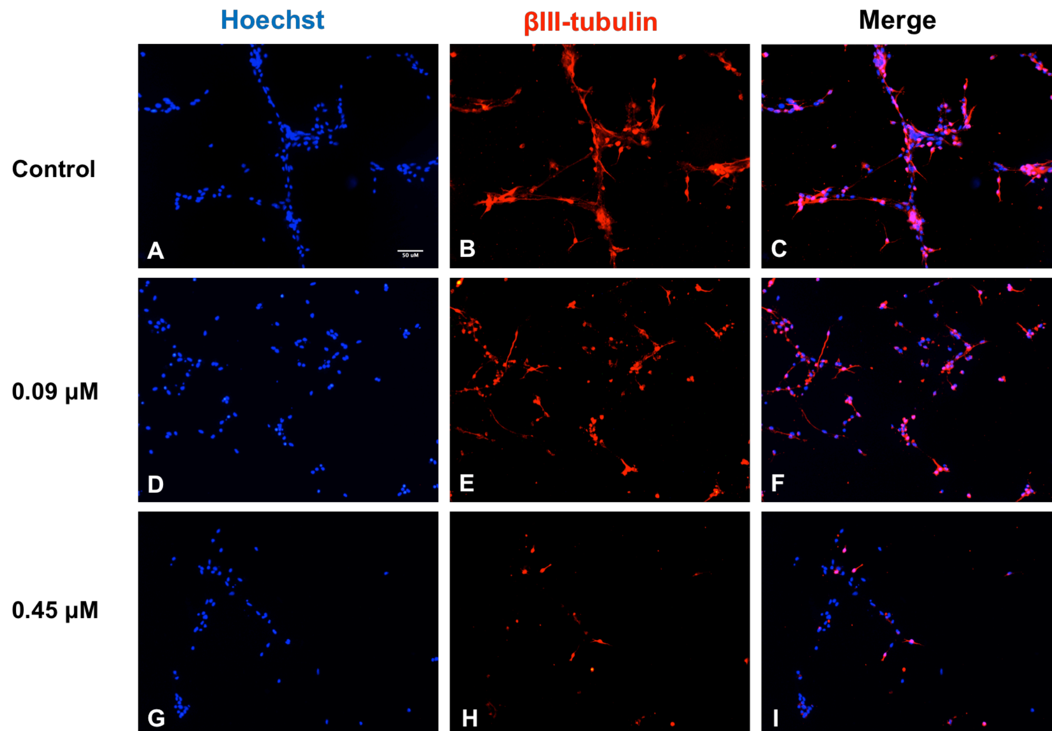


Figure 4.3. Cd inhibits proliferation in SGZ-aNPCs. Representative Hoechst (blue) and BrdU (red) co-staining from (A) Control, (B) 0.09 μM, and (C) 0.45 μM $CdCl_2$-treated SGZ-aNPCs after 24h exposure. (D) Quantification of the percent $BrdU^+$ cells after 24 h treatment with Cd. BrdU: a marker for cells in S phase of the cell cycle. n=3 independent experiments for a total of 9 coverslips per data point. Scale bar, 50 μm. Data represent mean ± SEM., n.s., not significant; *$p < 0.05$; **$p<0.01$.



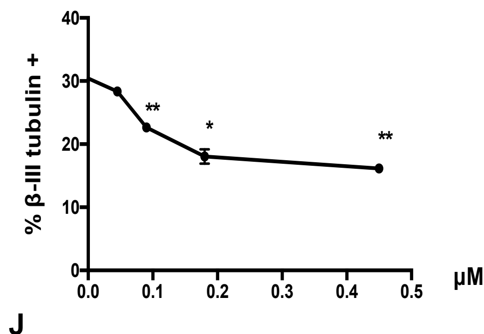


Figure 4.4. Cd decreases spontaneous neuronal differentiation of SGZ-aNPCs. (A, D, G) Representative Hoechst (blue) staining from (A) Control, (D) 0.09 μM, and (G) 0.45 μM Cd-treated SGZ-aNPCs after 5 d exposure. (B, E, H) Representative β-III tubulin staining from (B) Control, (E) 0.09 μM, and (H) 0.45 μM Cd-treated SGZ-aNPCs after 5 d exposure. (C, F, I) Representative Hoechst nuclei (blue) and β-III tubulin (red) co-staining from (C) Control, (F) 0.09 μM, and (I) 0.45 μM Cd-treated SGZ-aNPCs after 5 d exposure. Quantification of (J) the percent of β-III tubulin^{+} cells after 5 h Cd treatment. Scale bar, 50 μm n.s., not significant; *$p < 0.05$; **$p<0.01$.

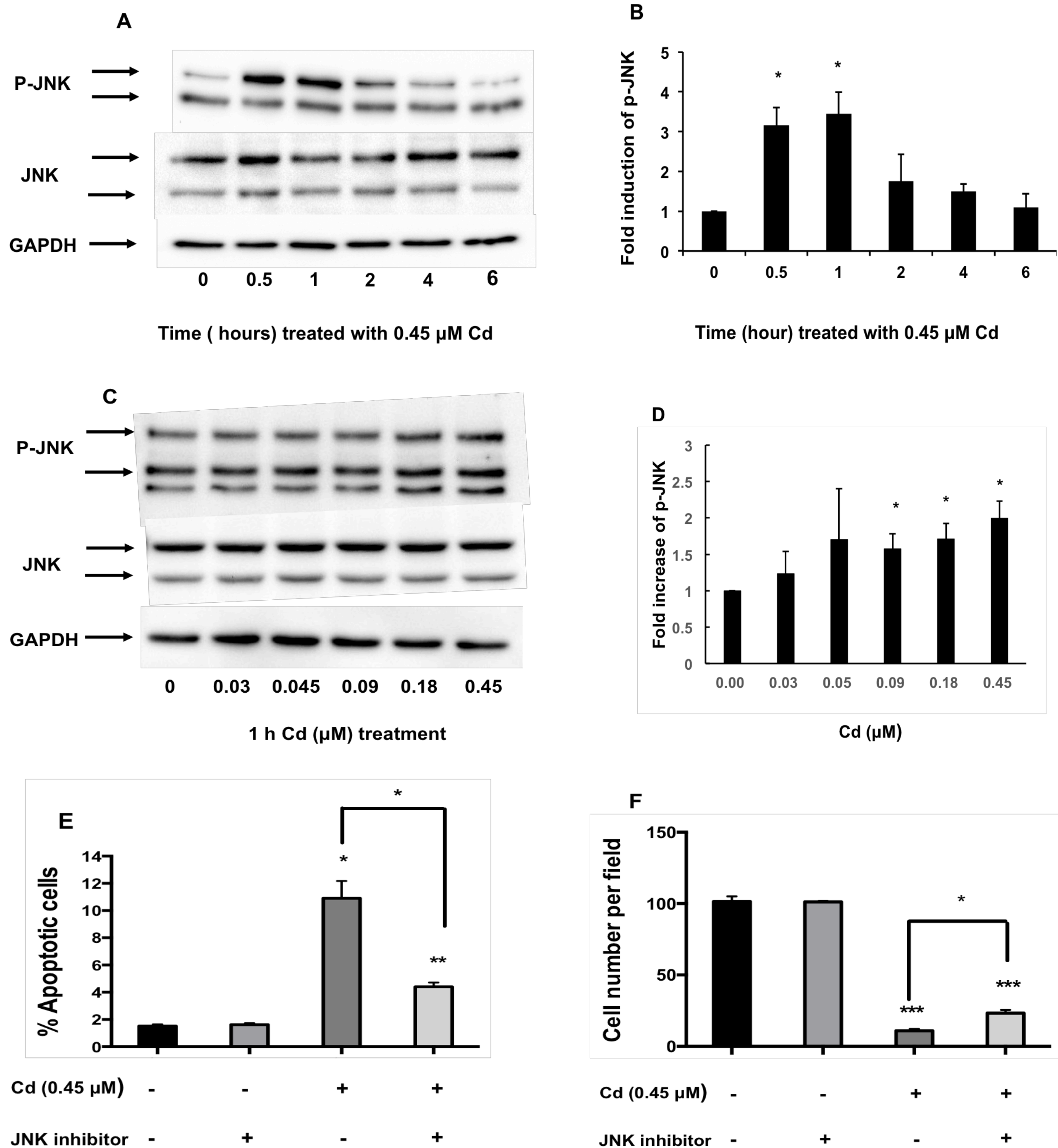


Figure 4.5. Activation of the JNK signaling pathway contributes to Cd-induced toxicity in SGZ-aNPCs. (A and B), SGZ-aNPCs were treated with 0.45 μM Cd for 0-6 h. The cell lysates were subjected to Western blot analysis for (A) phosphorylated-JNK, total JNK, and the protein loading control GAPDH, and (B) the fold induction of p-JNK (normalized to total JNK) in Cd-

treated cells compared to controls. (C and D), SGZ-aNPCs were treated with 0-0.45 μM Cd for 1 h. The cell lysates were subjected to Western blot analysis for (A) phosphorylated-JNK, total JNK, and the protein loading control GAPDH, and (B) the fold induction of p-JNK (normalized to total JNK) in Cd-treated cells compared to controls. (E-F) SGZ-aNPCs were pre-treated with 0.5 μM of the pan-JNK inhibitor (SP600125) for 1 h and then treated with 0.45 μM Cd for an additional 96 h. Quantification of (E) the percent apoptotic cells and (F) the total cell number. Data represent mean ± SEM., n.s., not significant; *$p < 0.05$; **$p<0.01$; ***$p<0.001$.

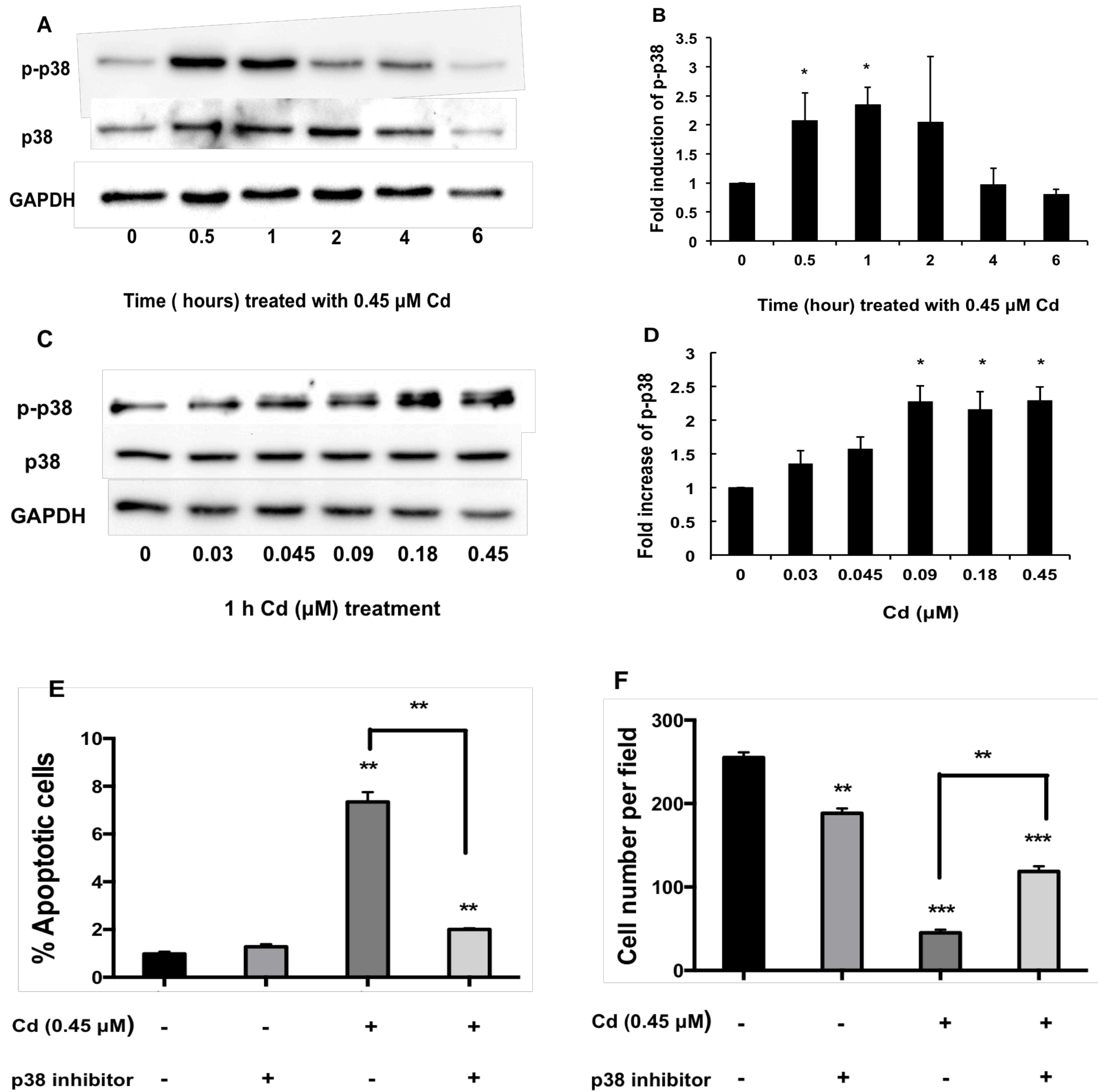


Figure 4.6. Activation of the p38 signaling pathway contributes to Cd-induced toxicity in SGZ-aNPCs. (A and B), SGZ-aNPCs were treated with 0.45 μM Cd for 0-6 h. The cell lysates were subjected to Western blot analysis for (A) phosphorylated-p38, total p38, and the protein loading control GAPDH, and (B) the fold induction of p-p38 (normalized to total p38) in Cd-treated cells compared to controls. (C and D) SGZ-aNPCs were treated with 0-0.45 μM Cd for the 1 h. The cell lysates were subjected to Western blot analysis for (C) phosphorylated-p38,

p38 and the protein loading control GAPDH, and (D) the fold induction of p-38 (normalized to p38) in Cd-treated cells compared to controls. (E-F) SGZ-aNPCs were pretreated with 0.5 μM of the pan-p38 inhibitor (SP202190) for 1 h and then treated with 0.45 μM Cd for an additional 96h. Quantification of (E) the percent apoptotic cells and (F) the total cell number. Data represent mean ± SEM., n.s., not significant; $*p < 0.05$; $**p<0.01$; $***p<0.001$.

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Chapter 5: Cadmium exposure impairs adult neurogenesis *in vivo*

5.1 Introduction

Adult neurogenesis is a dynamic process by which new neurons are generated from adult neural progenitor/stem cells (aNPCs). Adult neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) along the lateral ventricles in adult mammalian brains (Altman and Das, 1965; Alvarez-Buylla et al., 1988). Adult-born neurons generated in the SGZ adult neurogenesis continuously integrate into the hippocampal circuits (Ming and Song, 2011; Ming and Song, 2005) and contribute to hippocampus-dependent learning and memory (Clelland et al., 2009; Deng et al., 2009; Pan et al., 2012b; Pan et al., 2013; Wang et al., 2014). Meanwhile, steadily dividing aNPCs in the SVZ migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB) and differentiate into local interneurons there (Whitman and Greer, 2009). Several studies suggest that adult neurogenesis in the SVZ is involved in some forms of olfactory function in mice, such as odor discrimination, odor-detection sensitivity, and short-term olfactory memory (Breton-Provencher and Saghatelyan, 2012; Kageyama et al., 2012; Moreno et al., 2009; Pan et al., 2012a; Wang et al., 2015). A variety of external influences have been found to modulate adult neurogenesis (Engstrom et al., 2015; Li et al., 2013; Ming and Song, 2011). However, the information about the effects of neurotoxicants, such as heavy metals, on adult neurogenesis is very limited.

Cadmium (Cd), a biologically nonessential heavy metals, is considered as one of the top 20 hazardous substances. Cd is released into the environment from smelting, burning of fossil fuels and wastes, refining of metals (Satarug et al., 2010). Cd has a long half-life in the human body (10-30 years), thus chronic Cd exposure can accumulate in tissues and produce toxic effects in several organs, including kidney, liver, and bone (Chen et al., 2009; Dudley et al., 1982; Goering et al., 1993; Johri et al., 2010; Kjellstrom, 1992; Torra et al., 1995).

In recent years several studies have suggested that Cd may also be a neurotoxicant. For example, blood levels of Cd have been associated with the dysfunction of cognition and olfactory functions (Ciesielski et al., 2013; Ciesielski et al., 2012; Mascagni et al., 2003; Rose et al., 1992; Wang et al., 2018). Adult neurogenesis plays an important role in hippocampus-dependent learning and memory, as well as olfactory functions (Clelland et al., 2009; Deng et al., 2009; Pan et al., 2012a; Pan et al., 2012b; Wang et al., 2014). Here we hypothesize that cognitive and olfactory deficits induced by Cd exposure are due, in part, to the negative effects of Cd on adult neurogenesis in the SGZ and SVZ. Our previous studies have found that Cd impairs adult neurogenesis *in vitro* (Wang et al., 2017*).* Thus, in this study, we used adolescent and adult Cd exposure of male C57BL/6 mice as an *in vivo* model, to directly examine the effects of Cd exposure on adult neurogenesis.

5.2 Materials and Methods

Animals and treatments

Three-week-old and six-week-old male C57BL/6J mice were purchased from Charles River Laboratories and housed (4-5 animals per cage) in standard conditions (12 h light/dark cycle) with food and water provided *ad libitum.* The mice received normal drinking water or drinking water with 3 mg/L Cd (in the form of $CdCl_2$) starting at 4 and 8 weeks of age. Animal drinking water with $CdCl_2$ (Cat. 202908, Sigma-Aldrich, St. Louis, MO) was prepared from a stock solution and replaced every week. Water consumption was monitored every week during the entire exposure period. For the cellular adult neurogenesis study group (n = 4-5 in control or Cd-treated group), the exposure time was 13 weeks. At the end of the study, the mice were sacrificed by decapitation and blood was collected for blood Cd concentration analysis. The preparation, use and disposal of hazardous reagents were conducted according to the guidelines set forth by Environmental Health and Safety Office at the University of Washington. All animal care and treatments were approved by the University of Washington Institutional Animal Care and Use Committee.

BrdU administration

5-bromo-2'-deoxyuridine (BrdU) was purchased from Sigma (Cat. B9285) and dissolved in sterile saline and administered at 100 mg/kg by intraperitoneal injection 3 times per day for 2 days. Mice were killed at 5 weeks after the last BrdU injection to identify BrdU-labeled adult-born cells.

Immunohistochemistry

Mice were anesthetized (ketamine/xylazine) and then killed by decapitation. One brain hemisphere was post-fixed in 4% PFA in PBS overnight, and followed by 30% (w/v) sucrose /PBS (PH7.4) at 4 °C for 2-3 days. Coronal brain sections (30 μM) were used for immunohistochemistry using a free-floating method or by direct staining on Superfrost plus slides (VWR). The following primary antibodies were used for immunohistochemistry: rat monoclonal anti-BrdU (1: 500; AbD Serotec), mouse monoclonal anti-NeuN (1 :1000; Millipore), rabbit polyclonal anti-Ki67 (1:200; Leica), mouse monoclonal anti-GFAP (1:1000; Millipore). Goat anti-mouse, goat anti-rat, and goat anti-rabbit Alexa Fluor-conjugated secondary antibodies, as well as Hoechst 33342 were from Invitrogen. All antibodies (primary and secondary) were diliuted in the appropriate blocking buffer (10% goat serum and 1% BSA).

Quantification and imaging of immunostained cells

Immunostained cells were quantified as previously described (Pan et al., 2012a; Pan et al., 2012b; Wang et al., 2015; Wang et al., 2014). Every eighth serial section containing the SGZ or SVZ and every sixth section containing the olfactory bulb was immunostained for each marker (or combination of markers). For SGZ and SVZ, The markers were quantified by the experimenter blinded to treatment . This number was multiplied by 8 or 6 in order to get the estimation of the total number of marker positive cells. For the OB, stereology software (MBF Bioscience) was used to count cells as previously described (Wang et al., 2015; Zou et al., 2012). The colocalization of markers was defined as overlapping fluorescent signals in a single cell using a Z-serious stack. All the positive cells from at least 9-10 coronal sections per mouse were

quantified for each immuno marker. All images were captured with an Olympus Fluoview-1000 laser scanning confocal microscope with these lenses: numerical aperture (NA) 0.75, 10X, 20X. Optical Z-sections were collected and processed using ImageJ software (NIH). Images were uniformly adjusted for color, brightness, and contrast with ImageJ software (NIH).

Blood Cd analysis

The Environmental Health Laboratory at the University of Washington measured blood Cd levels using inductively coupled plasma mass spectrometry. The experimenter who measured the blood Cd was blinded to the treatment of animals. The blood Cd analysis was conducted at sacrifice after the cessation of the Cd exposure from the Cd analysis group (n= 4 per treatment), using Agilent 7500 (Agilent Technologies, Santa Clara, CA) with a detection limit of < 0.5 μg/L.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) and Excel (Microsoft Redmond, WA). Student's two-tailed t test was used to analyze the results. All data were expressed as mean ± SEM. n.s. not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

5.3 Results

Adolescent and adult Cd exposure results in blood Cd concentration comparable to human environmental exposure

To assess Cd neurotoxicity on adult neurogenesis, we exposed 4-week old and 8-week old male C57BL/6 mice to 3 mg/L (3 ppm) Cd (in the form of $CdCl_2$) through drinking water to model environmental exposure via ingestion for 13 weeks (Fig. 5.1 A and B). The 3 mg/L Cd concentration we chose is at least 3 times lower than 10-20 mg/L Cd, a concentration commonly used in previous studies (Gupta et al., 1995; Ramirez and Gimenez, 2003; Samuel et al., 2011; Shukla et al., 1996; Thijssen et al., 2007). We recorded body weight every week throughout the exposure period and did not observe any effect of Cd on body weight (data not shown). Mice were sacrificed after 13 weeks of Cd exposure to measure the blood Cd conccentrations. Cd exposure raised blood Cd concentrations approximately 4-fold above background (Fig. 5.1 C). The peak blood Cd concentration in the adolescent group was 1.25 ± 0.144 μg/L while the blood Cd concentration in the adult exposure group was 2.25 ± 0.48 μg/L (detection limit was 0.5 μg/L). There was no statistically significant age-related difference in blood Cd levels between the two groups, although the mean Cd level in adolescent mice was 45% lower than the mean level in adults.

Adolescent Cd exposure decreases the number of adult-born cells and adult-born mature neurons in the DG

In order to investigate the effect of Cd on adult-born cell survival in the DG, we treated adolescent mice with BrdU 3 times per day (i.p.) for 2 days, 5 weeks before sacrifice. We found that at 5 weeks after BrdU injection, Cd-treated mice had significantly fewer total adult-born cells ($BrdU^+$) compared to control mice (Fig. 5.2 A and B). Furthermore, we also found that there was a significant decrease in the total

number of adult-born neurons ($BrdU^+$ and $NeuN^+$) in the Cd-treated mice (Fig. 5.2 C). Meanwhile, the percent of neuronal differentiation in Cd-treated mice is lower than control mice (Fig. 5.2 D). In addition, we also found that Cd exposure does not increase the number of $GFAP^+$ cells in SGZ and SVZ areas (data not shown), which indicates that at this exposure level, Cd does not induce inflammation in mice brains.

Adult Cd exposure decreases the number of adult-born cells and adult-born mature neurons in the DG

In order to examine the effect of Cd on adult-born cell survival in the DG of adult mice, we dosed 8 week-old mice with BrdU 3 times per day for 2 days, 5 weeks before sacrifice. We found that at 5 weeks after BrdU injection, Cd-treated mice had significantly fewer total adult-born cells ($BrdU^+$) compared to control mice (Fig. 5.3 A and B). Furthermore, we also found that there was a significant decrease in the total number of adult-born neurons ($BrdU^+$ and $NeuN^+$) in the Cd-treated group (Fig.5.3 C). Meanwhile, the percent of neuronal differentiation in Cd-treated adult mice is also lower than control mice (Fig. 5.3 D). This result suggest that adult Cd exposure impairs adult hippocampal neurogenesis.

Adult Cd exposure decreases the number of adult-born cells and adult-born interneurons in the OB

In the adult exposure group, we found that at 5 weeks after BrdU injection, Cd-treated mice had significantly lower number of adult-born cells ($BrdU^+$) in OB (Fig. 5.4 A and B). Furthermore, we also found that there was a significant decrease of the total

number of adult-born interneurons ($BrdU^+$ and $NeuN^+$) in the Cd-treated mice (Fig. 5.4 C). However, there is no significant difference between the two groups in the percent of neuronal differentiation (Fig. 5.4 D). This result suggest that adult Cd exposure impairs adult-born cells survival in the OB.

Adult Cd exposure dose not affect proliferation in the SGZ and SVZ

To determine if adult Cd exposure impair cell proliferation in the SGZ and SVZ, we counted the $Ki67^+$ cells in both areas, and found there was no significant difference between control and Cd-treated mice in the total number of $Ki67^+$ cells in the SGZ (Fig. 5.5 A) and SVZ (Fig. 5.5 B). These data suggest at this exposure level, Cd does not affect cell proliferation in the SGZ and SVZ.

5.4 Discussion

Adult neurogenesis plays important roles in hippocampus-dependent learning and memory, as well as olfactory function. Our previous *in vivo* studies have shown that Cd exposure impairs cognition and olfactory memory in mice (Wang et al., 2018) and previous *in vitro* studies demostrated that Cd exposure can affect adult neurogenesis *in vitro* (Wang et al., 2017). Here, we used adolescent and adult Cd exposure of male C57BL/6 mice as a model to assess whether Cd impairs adult neurogenesis *in vivo.* Our data show that adolescent and adult Cd exposure is sufficient to impair adult neurogenesis through impairing the survival of adult-born cells and perturbing the neuronal differentiation of adult-born cells in the SGZ and the SVZ/OB.

In the adolescent exposure group, 3 mg/L Cd exposure for 13 weeks decreased the number of adult-born cells and adult-born neurons , and impaired the neuronal differentiation of aNPCs in the SGZ. However, Cd exposure did not induce an increase of $GFAP^+$ (a marker for inflammation) cells in brain (Kassubek et al., 2017), which suggests that at this exposure level, Cd does not induce inflammation in adolescent mice brains. In the adult Cd exposure group, we also observed significant effects of Cd on adult neurogenesis, including fewer adult-born cells and adult-born neurons in the SGZ, as well as fewer adult-born cells and adult-born interneurons in the OB. Meanwhile, we also found that Cd exposure impaired neuronal differentiation of aNPCs in the SGZ. However, we did not observe significant differences between control and Cd-treated mice in $Ki67^+$ cells in the SGZ and SVZ, which indicates that adult Cd exposure did not affect the cell proliferation.

Cd is a common heavy metal pollutant around the world. In addition to occupational exposure, humans can be exposed to Cd through ingestion and inhalation. In this study, we exposed adolescent and adult male mice to 3 mg/L Cd through drinking water to model environmental relevant exposure through ingestion. The average blood Cd levels immediately after 13 weeks exposure (1.25 ± 0.14 μg/L in adolescent group, and 2.25 ± 0.48 μg/L in adult exposure group) are close to the blood Cd concentrations of current smokers in the US (men: 0.58-0.94 μg/L; women: 0.69-1.17 μg/L) according to a National Health and Nutrition Examination survey (1999-2010) (Adams and Newcomb, 2014), and lower than the standard trigger level of Cd (5 μg/L) for medical surveillance in Occupational Safety & Health Administration regulation. Furthermore, the blood Cd concentration of our study (1.25-2.25 μg/L) has been

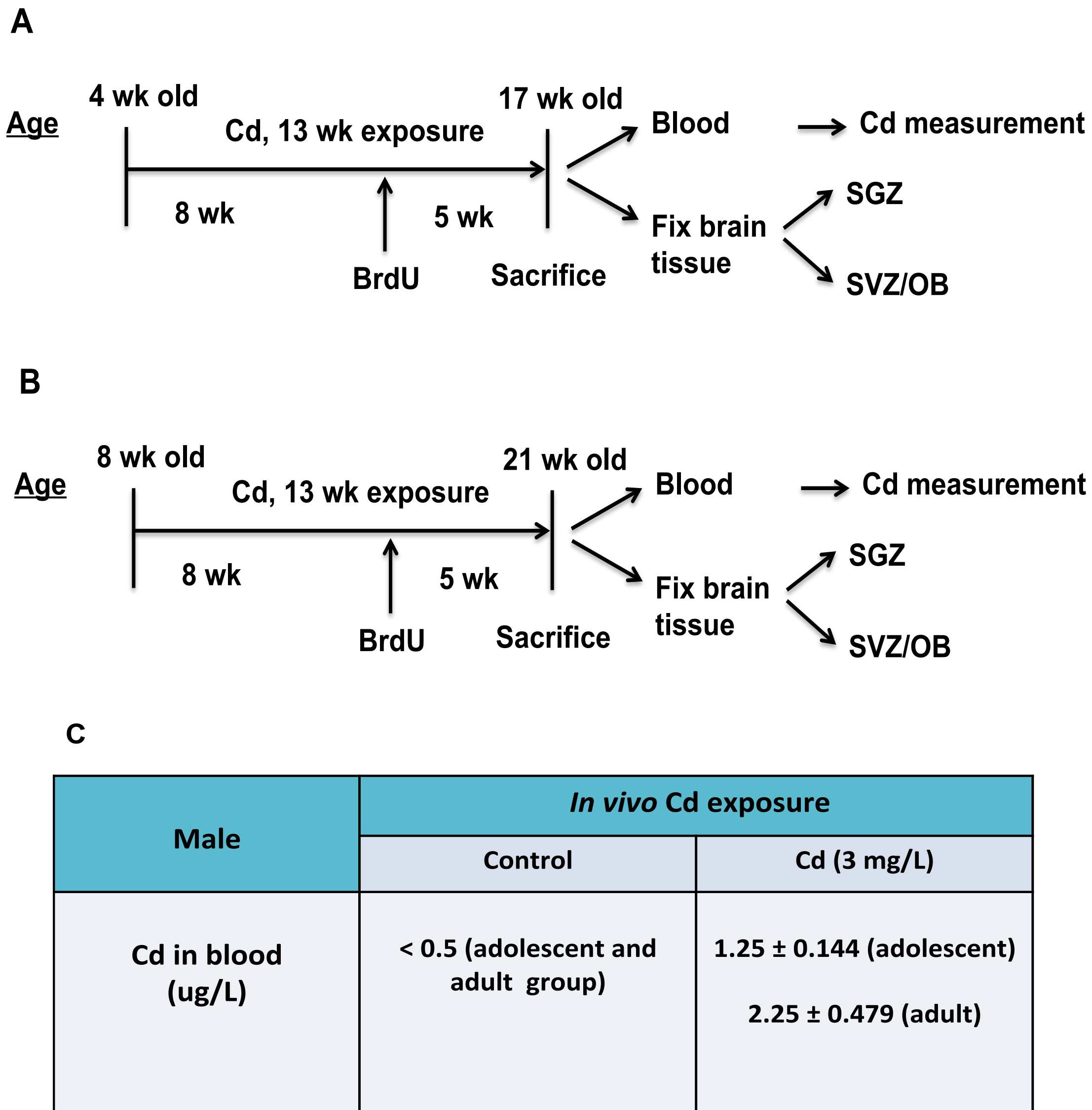
reported to occur in non-occupationally-exposed around the world (Kim et al., 2015; Myong et al., 2014; Sakellari et al., 2016; Zhang et al., 2015). Therefore, the Cd exposure level used in this study is relevant to environmental Cd exposure in humans.

Under these Cd exposure levels, we have found that Cd impairs the survival of adult-born cells and adult-born neurons , as well as neuronal differentiation in adolescent and adult exposure group. However, Cd exposure does not affect cell proliferation in adult exposure group. We do not know, however, if adolescent Cd exposure impairs cell proliferation or Cd exposure impairs the dendritic complexity of immature neurons in both groups. In order to get a full picture of Cd effects on adult neurogenesis, additional characterization of the adult neurogenesis status is needed.

5.5 Conclusion

In summary, our study found that Cd exposure in adolescent and adult mice through drinking water, which yields environmentally relevant blood Cd concentrations, is sufficient to impair several forms of adult neurogenesis in the SGZ and SVZ/OB. These results are in consistent with our previous findings that Cd exposure impairs adult neurogenesis *in vitro*, and induces impairments of cognition and olfactory functions in mice. These data together suggest that Cd may induce cognitive and olfactory deficits by affecting adult neurogenesis.

5.6 Figures and Tables



Male	*In vivo* Cd exposure	
	Control	Cd (3 mg/L)
Cd in blood (ug/L)	< 0.5 (adolescent and adult group)	1.25 ± 0.144 (adolescent) 2.25 ± 0.479 (adult)

Figure 5.1. Cd exposure increases blood Cd concentration in mice. (A) Experiment design and timeline for adolescent exposure group. (B) Experiment design and timeline for adult exposure group. (C) Blood Cd concentrations of control and Cd-treated mice in adolescent and adult exposure groups. Data are presneted as mean ± SEM with n = 4-5 in each goup.

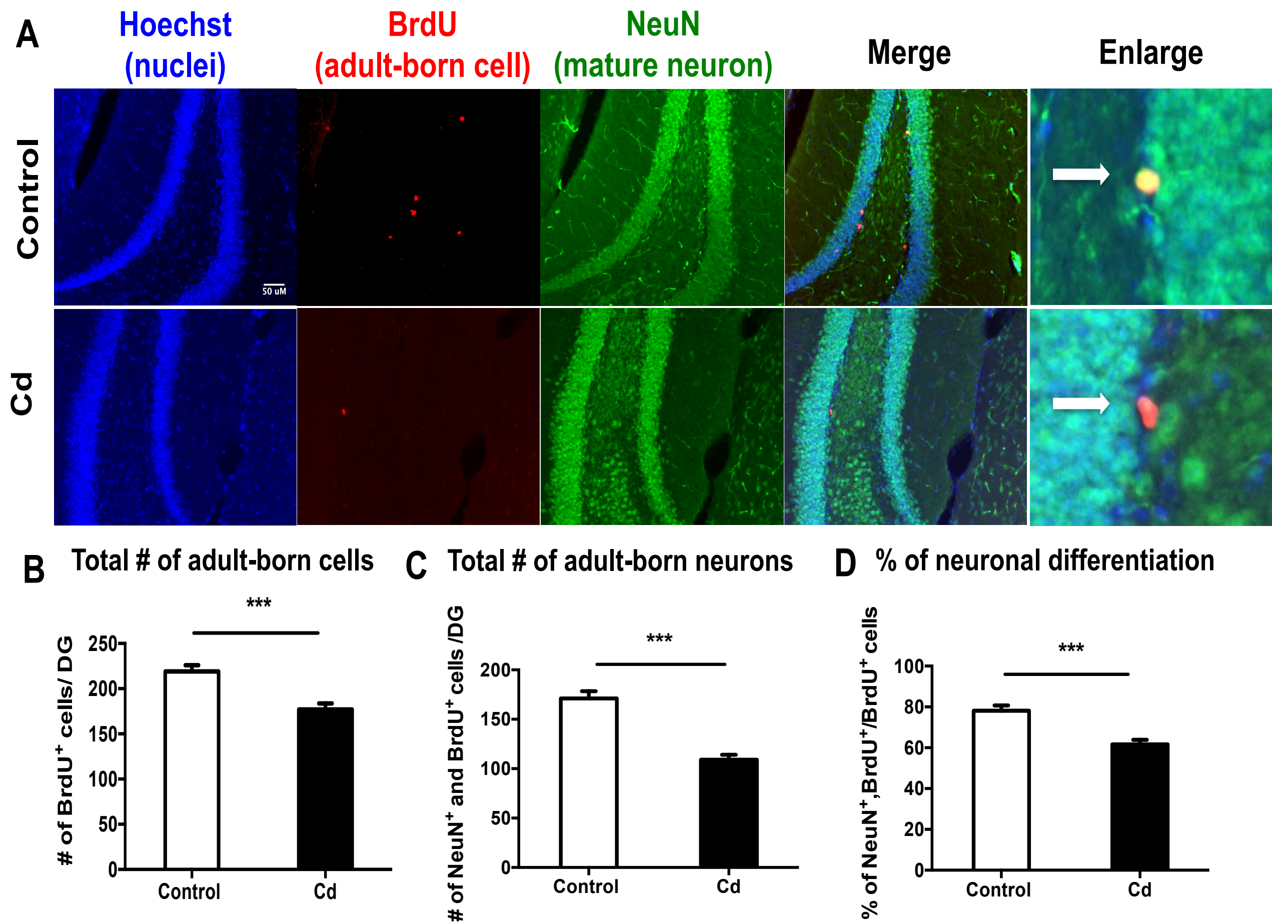


Figure 5.2. Adolescent Cd exposure impairs SGZ adult neurogenesis. (A) Representative Hoechst (blue), BrdU (red), and NeuN (green) co-staining of SGZ from control and Cd treted mice after 13 weeks Cd exposure. (B) Quantification of the total number of BrdU^{+} cells in SGZ of control and Cd treated mice. (C) Quantification of the total number of BrdU^{+} and NenN^{+} cells in SGZ of control and Cd-treated mice. (D) Quantification of the percent of BrdU^{+} and NenN^{+} cells / BrdU^{+} cells in SGZ of control and Cd-treated mice. Data are presneted as mean ± SEM with n = 4-5 in each goup. ***p<0.001.

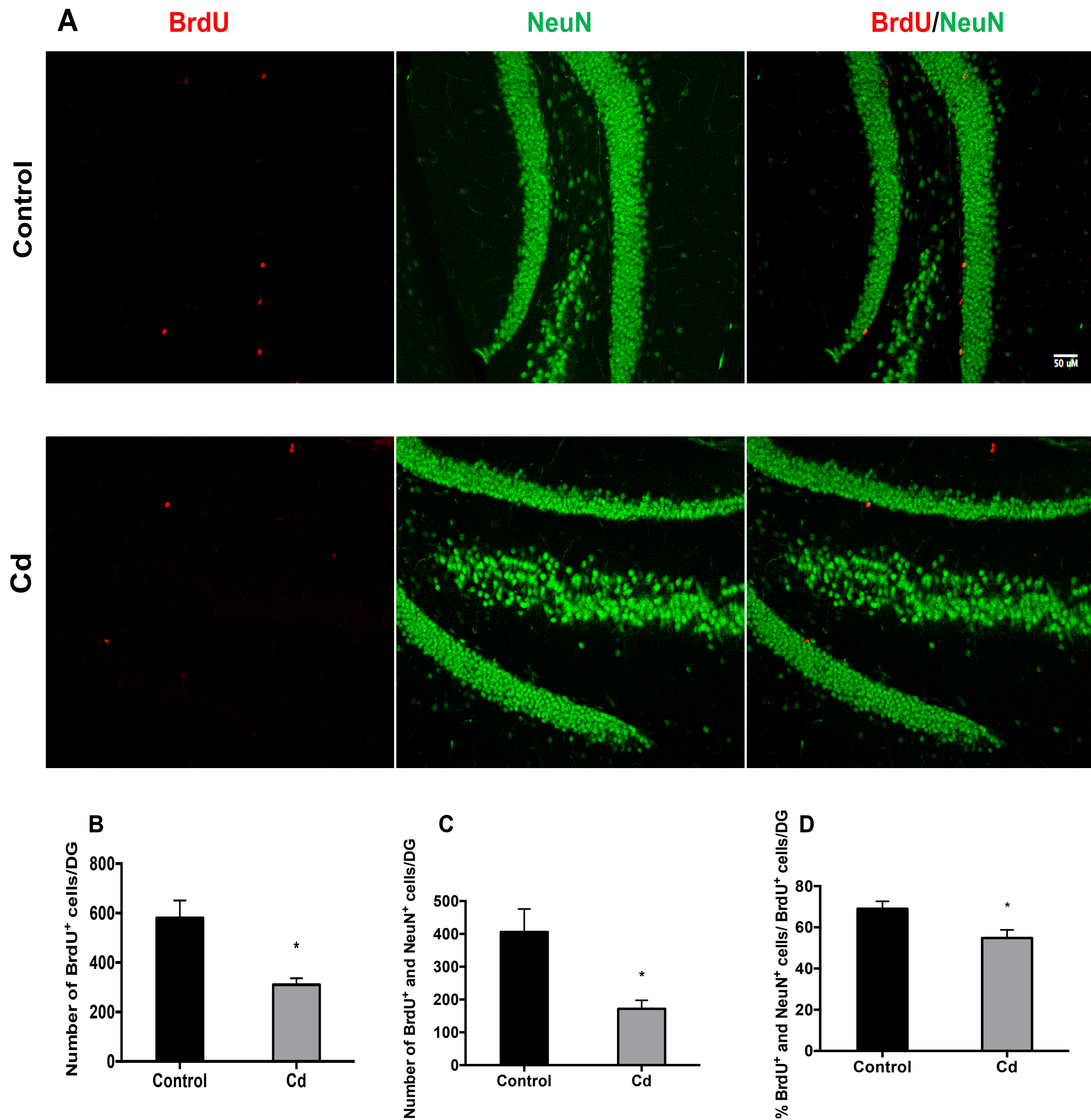
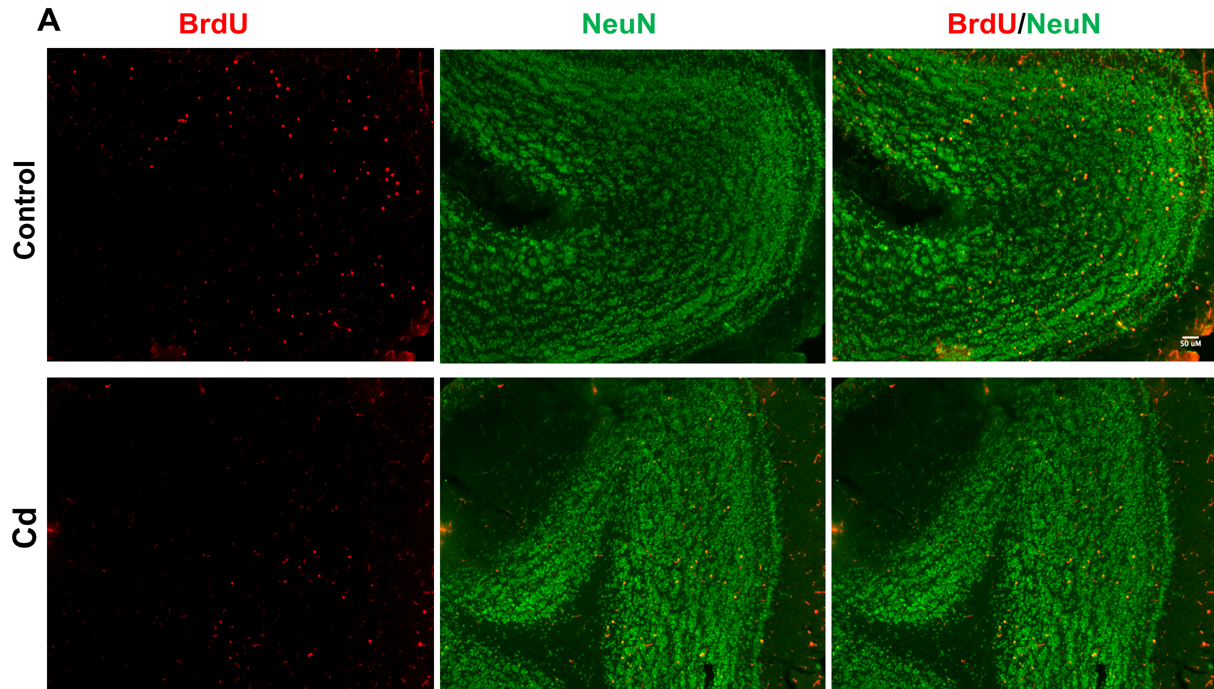


Figure 5.3. Adult Cd exposure impairs survival and neuronal differentiation of aNPCs in the SGZ. (A) Representative BrdU (red), and NeuN (green) co-staining of the SGZ from control and Cd treted mice after 13 weeks Cd exposure. (B) Quantification of the total number of BrdU$^+$ cells in SGZ of control and Cd treated mice. (C) Quantification of the total number of BrdU$^+$ and

NenN$^+$ cells in SGZ of control and Cd-treated mice. (D) Quantification of the percent of BrdU$^+$ and NenN$^+$ cells / BrdU$^+$ cells in SGZ of control and Cd-treated mice. Data are presneted as mean ± SEM with n = 4-5 in each goup. *$p<0.05$.



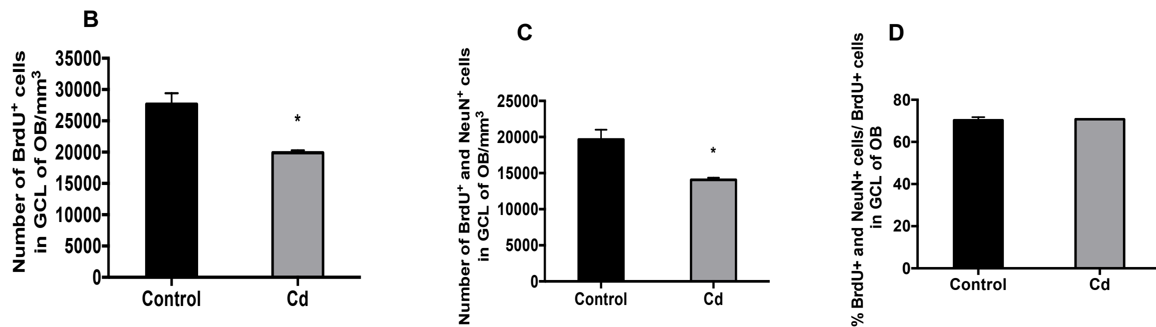
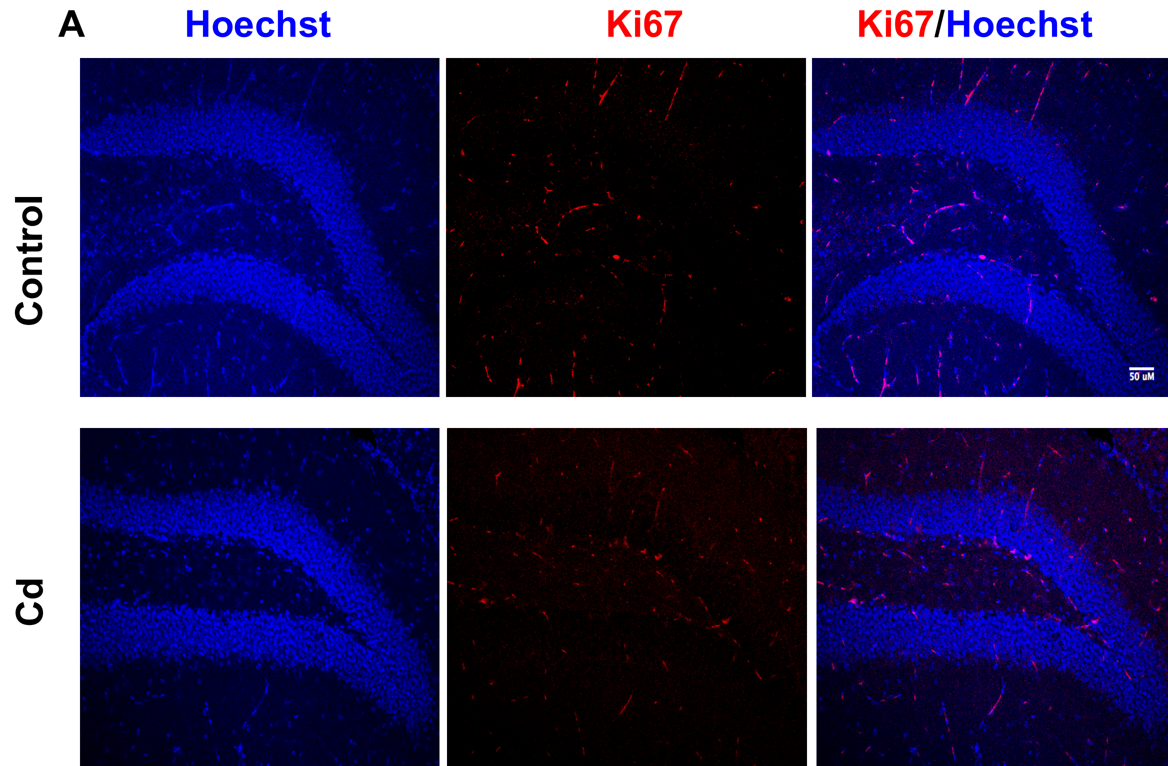
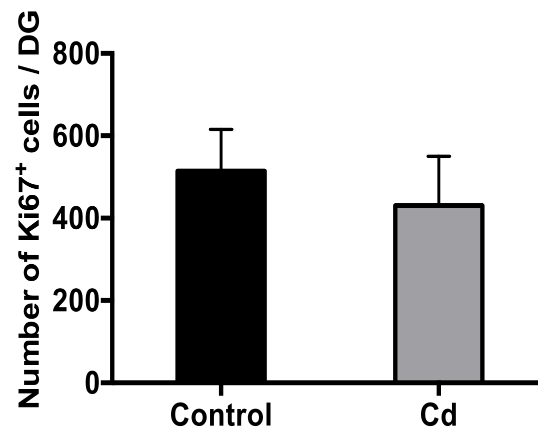
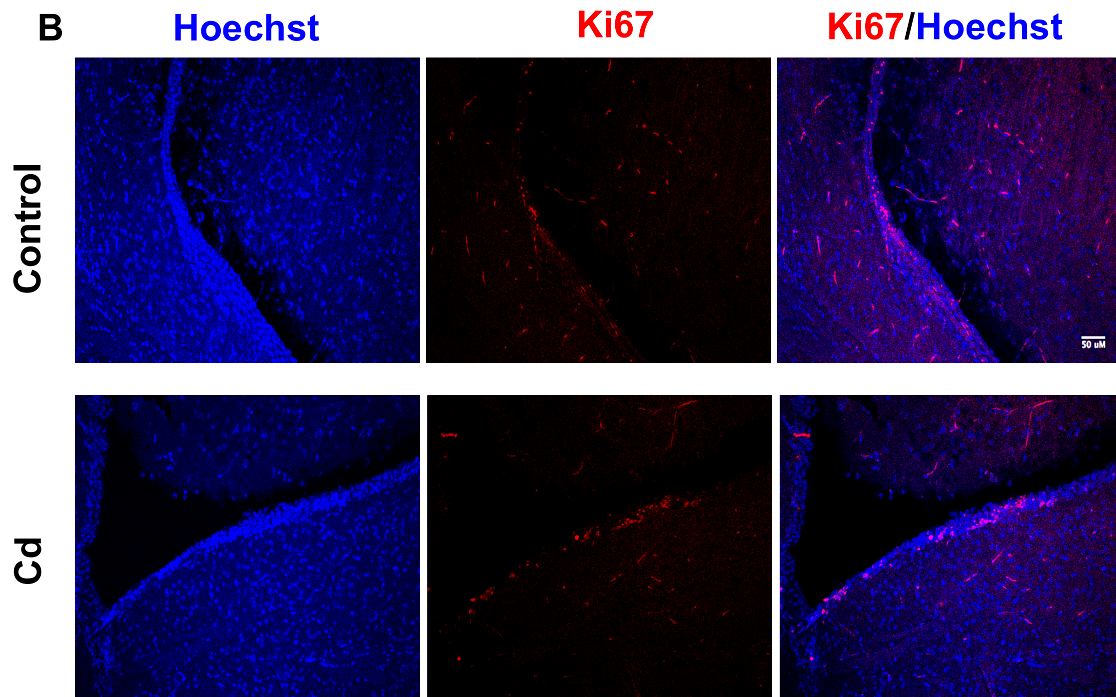


Figure 5.4. Adult Cd exposure impairs survival of aNPCs in the olfactory bulb (OB). (A) Representative BrdU (red), and NeuN (green) co-staining of OB from control and Cd treted mice after 13 weeks Cd exposure. (B) Quantification of the total number of $BrdU^+$ cells in GCL of OB from control and Cd treated mice. (C) Quantification of the total number of $BrdU^+$ and $NenN^+$ cells in GCL of OB from control and Cd treated mice (D) Quantification of the percent of $BrdU^+$ and $NenN^+$ cells / $BrdU^+$ cells in GCL of OB from control and Cd treated mice. Data are presneted as mean ± SEM with n = 4-5 in each goup. *$p<0.05$.


A
Hoechst
Ki67
Ki67/Hoechst
Control
Cd
50 uM


Number of Ki67+ cells / DG
800
600
400
200
0
Control
Cd



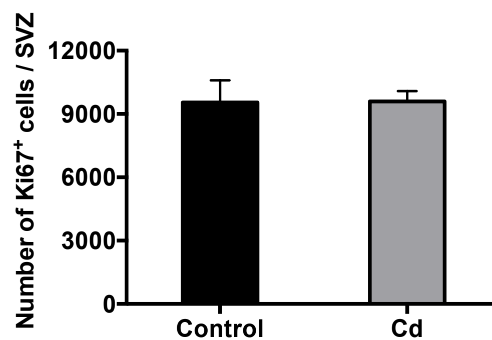


Figure 5.5. Adult Cd exposure does not affect cell proliferation in the SGZ and SVZ. (A) Representative Hoechst (blue), and Ki67 (red) co-staining of the SGZ in control and Cd treted mice after 13 weeks Cd exposure. (B) Quantification of the total number of $Ki67^{+}$ cells in the SGZ of control and Cd treated mice. (C) Representative Hoechst (blue), and Ki67 (red) co-staining of the SVZ in control and Cd treted mice after 13 weeks Cd exposure. (D) Quantification of the total number of $Ki67^{+}$ cells in the SVZ of control and Cd treated mice.. Data are presneted as mean ± SEM with n = 4-5 in each goup.

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Chapter 6: Genetic and conditional stimulation of adult neurogenesis rescues cadmium-induced impairments of hippocampus-dependent memory and olfactory memory in mice

6.1 Introduction

Cadmium (Cd) is a toxic heavy metal that can cause toxicity in many organs, including kidney (Torra et al., 1995), liver (Goering et al., 1993; Torra et al., 1995), lung (Shukla et al., 2000), testis (Mao et al., 2012), and bone (Wallin et al., 2013). Recent studies have suggested that Cd is also a potential neurotoxicant. *In vitro* studies have shown that Cd exposure induce cell death in various neural cells (Chen et al., 2008; Lopez et al., 2003; Wang et al., 2017; Yuan et al., 2013). Several animal studies also reported that Cd exposure induces impairments in the cerebral cortex and cerebellum, and also causes deficits in passive avoidance, schedule controlled responding, and conditioned suppression (Ali et al., 1986; Gabbiani et al., 1967; Goncalves et al., 2010; Goncalves et al., 2012; Maodaa et al., 2016; Nation et al., 1984; Nation et al., 1983; Wong and Klaassen, 1982). However, the biochemical basis of neurotoxicity of Cd is still not fully understood.

A few epidemiological researches have reported association between Cd exposure and deficits in cognitive and olfactory functions in humans (Ciesielski et al., 2013; Ciesielski et al., 2012; Mascagni et al., 2003). Our recent *in vivo* findings in mice provide direct evidence that low-level Cd exposure through drinking water causes

impairments of cognition and olfactory memory in mice (Wang et al., 2018)，but the potential mechanisms are still unknown.

Adult neurogenesis is a process that generates new functional neurons from adult neural progenitor/stem cells (aNPCs). This process is restricted to two specific regions in adult brains under normal physiological conditions: the subgranular zone (SGZ) in the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Ming and Song, 2011; Ming and Song, 2005). Researchers have discovered that adult-born neurons from SGZ-aNPCs and SVZ-aNPCs are important for the hippocampus-dependent learning and memory, as well as olfaction, respectively (Bond et al., 2015; Pan et al., 2012a; Pan et al., 2012b; Pan et al., 2012c; Zou et al., 2012b). Our previous study has found that Cd exposure impairs adult neurogenesis, both *in vitro* and *in vivo.* Since we have found that Cd exposure induces deficits in hippocampus-dependent learning and memory, and olfactory memory in mice (Wang et al., 2018), we hypothesize that Cd may induce these toxic effects through affecting adult neurogenesis.

MEK5 is a highly specific activator of ERK5. The effects of MEK5 are attributed to its ability to activate ERK5 (Diaz-Meco and Moscat, 2001; Drew et al., 2012). The MEK5/ERK5 signaling pathway has been implicated in cell survival, angiogenesis, cell motility, differentiation, and proliferation (Drew et al., 2012; English et al., 1999; Nishimoto et al., 2005). By using a gain-of-function knock-in mouse model that allows inducible and conditional expression of active MEK5 (caMEK5), we have found that activation of ERK5 through MEK5 increases adult neurogenesis both in the SGZ and SVZ, and further improves the cognitive and olfactory functions (Wang et al., 2015; Wang et al., 2014). In this study, by using this transgenic mouse model, we determined

if enhancing adult neurogenesis could rescue mice from Cd-induced impairments of hippocampus-dependent learning and memory, and olfactory memory.

6.2 Materials and Methods

Animals

The ROSAcaMEK5 knock-in mouse line was generated in our laboratory as previously described (Wang et al., 2014). The Nestin-CreER transgenic mouse line has been described previously (Kuo et al., 2006). The Nestin-CreER^{TM}:caMEK5 mouse line was generated by crossing the ROSAcaMEK5 mice with Nestin-CreER^{TM} mice. All animals were housed (4-5 animals per cage) under standard conditions (12 h light/dark cycle) with food and water provided *ad libitum*. Both male and female mice were used in this study. All animal care and treatments were approved by the University of Washington Institutional Animal Care and Use Committee.

Animal treatment

Male and female caMEK5 mice received normal drinking water (control) or drinking water with 0.6 mg/L Cd (in the form of $CdCl_2$) starting at 8 weeks of age. Animal drinking water with $CdCl_2$ (Cat. 202908, Sigma-Aldrich, St. Louis, MO) was prepared from a stock solution and replaced every 1-2 week. Water consumption was monitored every 1-2 week during the whole exposure period. The Cd-treated group of mice were exposed to 0.6 mg/L Cd in drinking water for 38 weeks, then all animals were switched to normal drinking water for the remainder of the study. Mouse body weight was

recorded every 1-2 weeks throughout the whole exposure period. The experiment cohorts had n = 8-10 animals per treatment / sex.

To induce Nestin-CreER^{TM} mediated recombination of caMEK5 in neural progenitor/stem cells, mice were given freshly made tamoxifen (200 mg/kg; Sigma) by oral gavage once a day for 4 d in each cycle for 3 cycles with 2 weeks intervals.

The preparation use and disposal of hazardous reagents were conducted according to the guidelines set forth by Environmental Health and Safety Office at the University of Washington. All animal care and treatments were approved by the University of Washington Institutional Animal Care and Use Committee.

Open field test

The open field test was used to assess the effects of Cd on anxiety and locomotor activity. Mice were placed into a 10 inches (width) × 10 inches (depth) × 16 inches (height) TruScan Photo Beam Tracking arena (Coulbourn Instruments, Holliston, MA) with clean Plexiglas sidewalls and their movement was monitored by two sets of infrared breams spaced 0.6 inch apart, providing a spatial resolution of 0.3 inch. The animal was allowed to freely explore in the arena without prehabituation for 20 min and the data were collected by TruScan 2.0 software (Coulbourn Instruments, Holliston, MA). The total move distance, total move time, and average speed are used to examine the effects of Cd on locomotor activity. The time and distance spend in margin and center, as well as center entries are used to assess anxiety.

Elevated plus maze test

The elevated plus maze test was used to examine the effect of Cd on anxiety. The elevated plus maze apparatus (San Diego Instruments, San Diego, CA) was used in this test. The maze (26 inches × 26 inches × 15.25 inches) consists of two open arms, two closed arms, and a center area. Each closed arm has a 7-inch wall on two sides, and the center area measures 2 inches × 2 inches. During the test, the maze was placed in the center of the behavior room, and animals were placed into the center of the apparatus facing toward an open arm and allowed to freely explore for 5 min. The open and closed arm ends were defined as the distal 1/3 of the arms. A video camera and ANYmaze software (San Diego Instruments, San Diego, CA) were used to track and analyze the movement of mice during the test.

Novel object location test

The novel object location test was used to examine the effects of Cd on hippocampus-dependent spatial working memory. This assay was performed as previously described. Briefly, each animal was placed into an open field arena (Coulbourn Instruments, Holliston, MA) with two identical objects placed in 2 different corners. During the training session, the mouse was allowed to freely explore the two objects for 5 min and then returned to its homecage. To exlude preference of specific location, alternating coners were used for object presentation. Testing was performed 1 h after training, the animal was returned to the arena with the same two objects; one object remained in its original location and the other had been moved to a new location. The time the animal spent actively investigating each object during the training and

testing was recorded by camera and quantified after the test. Each training and testing session was scored and analyzed by an experimenter blinded to the animal's treatment.

Cued and contextual fear conditioning tests

Contexutal fear memory is another form of hippocampus-dependent learning and memory. In this study, a modified cued and contextual fear conditioing test using weak foot shock conditioning paradigm (3 × 0.3 mA, 2 s shocks with 2 min inter-trial intervals) was used as previously described (Engstrom et al., 2017; Pan et al., 2012a). For the conditioning session, the mouse was placed into the foot shock context (10 inches × 10 inches × 16 inches arena with grid shock floor (Coulbourn Instruments, Holliston, MA)) 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, a 0.3 mA foot-shock (unconditioned stimulus, US) was delivered. This cycle was repeated two more times before the mouse was returned to its home cage. The CS and US were automatically delivered by the TruScan software (Coulbourn Instruments, Holliston, MA). The contextual fear memory test was then conducted 24 h after conditioning session. The mouse was placed back into the foot shock context for 2 min in the absence of tone or foot-shock. For the cued test, which was performed 2 h after context test, the animal was placed into a novel context (new room; hexagonal Plexiglas arena) and allowed to freely explore in the new context for 2 min. The CS (tone) was then presented for 2 min. For the novel context test, which was performed 2 h after the cued test, the mouse was placed into another novel context (new room; rat cage) and allowed to freely explore for 2 min with no presentation of either tone or foot shock. In all three tests, persistent

freezing behavior (four paws on the ground, no head or body movement besides breathing) was recorded by video camera and manually quantified during the 2 min scoring period by an experimenter blinded to animal treatment.

Olfactory habituation/dishabituation test

This test was conducted as previously described (Pan et al., 2012b; Wang et al., 2015; Zou et al., 2012a). Briefly, mice were trained with mineral oil-laced cotton swabs for four of 60 s presentations with 2 min intervals between each presentation. This pretaining was to ensure that subsequent exposure to odorant laced cotton swabs did not induce a response due to the novelty of the cotton swab. Then, the olfactory habituation/dishabituation test was conducted by presenting citralva , isoamyl acetate, and vanillin sequentially with four 60 s presentations (with 2 min interval) for each odorant. During the test, the duration of the mouse's investigation (sniffing) of the cotton swab was recorded by video camera and scored by an experimenter blinded to animal treatment. The investigation of the swab was defined as the animal's nose approaching to and within 1 cm to the swab. A significant decrease in investigating time during subsequent presentation of the same odorant indicates odor habituation, and an increase in the time spent in investigating the swab with a new odorant indicates dishabituation.

Short-term olfactory memory

Short-term olfactory memory was assessed as previously described (Pan et al., 2012b; Wang et al., 2015). The animals were presented with a cotton swab laced with

the same odorant during two 5 min sessions separated by different intervals (60-240 min). A different odorant was used for each interval time point. To avoid cross-interference of olfactory detection and memory, only one time interval was tested on each day. The investigating time on the swab was recorded by video camera and scored by an experimenter blinded to animal treatment after the test. A significant decrease in investigation of the swab during the 2nd presentation of the same odorant indicates olfactory memory for that odorant.

Sand-digging based odor-cued associative olfactory learning test

This behavior test was conducted following the protocol as previously described (Wang et al., 2018). Before the test, mice were food restricted to maintain 85-90% of normal body weight for 6 days before the beginning and throughout the entire test. At the beginning, mice were pre-trained to learn to retrieve the food reward buried in sand. The pretraining session consisted of three consecutive days during which the food pellet was put on the top of sand (day 1), partially buried (day 2), and deeply buried in sand (day 3) with 4 trials per block (1 min interval between each trial), 2 blocks each day and a 4 h interval between the 2 blocks. By the end of day 3, mice had learned to retrieve the food pellet within 50 s. The day after pre-training session, mice were subjected to the olfactory discrimination test for 4 trials per block, 2 blocks per day with a 4 h interval for 10 days. During the test, both dishes were filled with sand with one scented with (+)-carvone and another with (-)-carvone. A food pellet was buried deeply in the sand with (+)-carvone. The two dishes were placed on each side twice per test block but no more than three consecutive times each day to avoid spatial cue. Scoring for correct or

incorrect choice was based on the mouse's first dig. If the mouse's first choice was correct, it was allowed to finish the digging and to retrieve the food reward. If the mouse made a wrong first choice, it was allowed to finish the digging on that side but not allowed to self-correct. In this training, mice were trained to learn to associate the (+)-carvone with the food reward. During the test, the experimenter was blinded to the animal treatment.

Blood Cd analysis

The Environmental Health Laboratory at the University of Washington measured blood Cd levels using inductively coupled plasma mass spectrometry. The experimenter who measured the blood Cd concentration was blinded to the treatment of animals. The final blood Cd analysis was conducted at the end of the behavior tests by using equipment Agilent 7900 (Agilent Technologies, Santa Clara, CA) which is more sensitive and has a detection limit of < 0.08 μg /L.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) and Excel (Microsoft, Redmond, WA). For behavioral tests, one-way ANOVA was used for the sand digging based test in each group separately. Two-way analysis of variance (ANOVA) with Holm-Sidak post-test ($\alpha = 0.05$) was used to analyze the open field, elevated plus maze, contextual fear after tamoxifen treatment in order to account for the treatment of tamoxifen (tamoxifen vs vehicle) and Cd exposure (control vs Cd). Student's two-tailed t test was used to analyze the other

behavioral results. All data were expressed as mean ± SEM. n.s. not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

6.3 Results

Both male and female mice do not exhibit weight loss, locomotor deficits, or anxiety following Cd exposure

In this study, we exposed 8-week old female and male caMEK5 mice to 0.6 mg/L Cd through drinking water for 38 weeks to model low-level Cd exposure via ingestion (Fig. 6.1 A). We recorded body weight and water consumption every 1-2 weeks during the whole exposure period and did not observe any significant difference in body weight (Fig. 6.1 B) or water consumption (Fig. 6.1 C) between control and Cd-treated mice

In order to examine the effects of Cd on locomotor activity and anxiety, we conducted the open field test before and after Cd exposure. We did not find any significant difference in activity between control and Cd-treated group before exposure (Fig. 6.2 A), suggesting no intrinsic differences exist between the control and Cd treated mice. After Cd exposure (Fig. 6.2 B), both the control and Cd-treated mice traveled the same total distance with similar moving time and speed in the open field test, suggesting that Cd exposure does not affect locomotor activity. Furthermore, both groups spent similar amounts of time along the margins or center traveled similar distance along the margin and to the center area and had similar center entries suggesting that Cd exposure does not cause overt anxiety in female or male mice.

The elevated plus maze test was also conducted after the cessation of the Cd exposure to assess the effect of Cd on anxiety (Fig. 6.3). There was no significant

difference between control and treated mice on the percent of total time spent or total distance traveled in the open arms, the percent of open arm entries, or total distance travelled in the elevated plus maze. All the data from the open field and elevated plus maze test suggest that Cd exposure did not induce overt anxiety in both genders.

Cd exposure causes impairment of hippocampus-dependent spatial memory in the novel object location (NOL) test

In order to determine if this Cd exposure level induce impairments of hippocampus-dependent spatial working memory, we conducted a 1 h NOL test before, during Cd exposure in control and Cd-treated mice. At each time point tested, there was no difference in the exploration time of each object or each location (location A vs B) in the training session (data not shown), suggesting that none of the mice exhibited a preference for either location or object.

Before Cd exposure, all mice spent significantly more time exploring the object in the novel location (C) vs. familiar location (A) in the test session (Fig. 6.4 A and B), indicating that they had the memory of the original object location during training sessions. Five weeks into the Cd exposure, the Cd-treated female mice spent almost equal amounts of time in exploring the two objects (Fig. 6.4 C), indicating that the Cd-treated mice did not discriminate between the old and new object locations, thus had no short-term spatial memory. After 11 weeks of Cd exposure, the male caMEK5 Cd-treated mice started to exhibit this impairment, which is 6 weeks later then the female cohort (Fig. 6.4 D). Furthermore, the Cd-treated mice continued to exhibit a deficit in this

form of spatial memory 17 (female) and 15 (male) weeks into the Cd exposure (Fig. 6.4 E and F).

caMEK5 activation rescues caMEK5 mice from Cd-induced impairments of hippocampus-dependent spatial working memory in NOL test

To determine the rescue effect of caMEK5 activation on the Cd-induced impairments of hippocampus-dependent spatial memory, the control and Cd-treated mice were subjected to the NOL test again after 3 cycles of tamoxifen treatment. Before tamoxifen treatment, all of female and male control mice exhibited spatial working memory while the Cd-treated mice did not (Fig. 6.5 A and B). In the female cohort, six weeks after tamoxifen treatment, Cd-tamoxifen treated mice spent significant more time in exploring the object in the novel location (C),while the Cd-vehicle mice did not (Fig. 6.5 C). This result suggest that tamoxifen treatment rescued mice from Cd-induced hippocampus-dependent spatial working memory deficits. Furthermore, 40 weeks after tamoxifen treatment (27 weeks after Cd exposure), the Cd-tamoxifen group still had the memory while the Cd-vehicle group did not, which suggests that the rescue effects persists (Fig. 6.5 E). In male cohort, the Cd-tamoxifen treated mice started to exhibit spatial working memory at 14 weeks after tamoxifen treatment, while the Cd-vehicle group did not (Fig. 6.5 D). Then, 42 weeks after tamoxifen treatment (27 weeks after Cd exposure), all male groups exhibited spatial working memory (Fig. 6.5 F), suggesting that the Cd-induced impairments of spatial working memory in male group does not persist.

caMEK5 activation rescues caMEK5 mice from Cd-induced impairments of short-term olfactory memory.

We conducted a cotton-tip based test to assess short-term olfactory memory after Cd and tamoxifen treatment. In female cohort (Fig. 6.6 A-C), mice were presented with the same odorant twice with a time interval between the two presentations (60, 120 and 180 min) in three separate days. Both control (vehicle and tamoxifen) and Cd-treated (vehicle and tamoxifen) mice spent significantly less time sniffing the odor-laced cotton tips during the second presentation compared to the first presentation at the 60 min interval (Fig. 6.6 A), suggesting that they had memory for the odorant presented 60 min earlier. At the 120 min time interval (Fig. 6.6 B), while the control-vehicle, control-tamoxifen, and Cd-tamoxifen treated mice still exhibited olfactory memory, the Cd-vehicle treated mice did not. At the 180 min time point (Fig. 6.6 C), both Cd-vehicle and Cd-tamoxifen group had lost their memory for the odorant while the control-vehicle and control-tamoxifen still had the memory.

In the male cohort (Fig. 6.6 D-F), the test was conducted at 120, 180, and 240 min time interval on three separate days. At 120 min interval (Fig. 6.6 D), all mice exhibited olfactory memory for the odorant. At 180 min (Fig. 6.6 E), the Cd-vehicle treated mice lost their memory while the other group mice still retained memory of the odorant. At 240 min time interval (Fig. 6.6 F), all mice did not remember the odorant prensented before.

These data suggest that Cd exposure in mice shortened their capacity to remember a specific odorant, and tamoxifen treatment improved but did not fully recover the impaired short-term olfactory memory.

caMEK5 activation rescues mice from Cd-induced impairments of odor-cued associative learning and memory

Next, we subjected control and Cd-treated mice to a sand-digging odor-cued olfactory learning and memory test to assess the odorant-cued associative olfactory learning and memory. Mice were pre-trained for 3 days to associate food reward with sand digging, and both cohorts (female and male) of mice learned the task by the end of the pre-training (Fig. 6.7 A). In the female cohort, over the course of 13 days training, the control-vehicle, control-tamoxifen, and Cd-tamoxifen treated mice gradually learned the task and retrieved the food reward correctly by the end of the 13-day training, while the Cd-vehicle group did not learn to associate the food reward with right odorant and only found the reward by the 50% random chance (Fig. 6.7 B).

The male cohort, the control-vehicle, Cd-vehicle, and Cd-tamoxifen group learned the task by the end of the 10-day training, while the Control-tamoxifen group did not. Furthermore, the Cd-vehicle group learned the task two days later than the control-vehicle group, and 1 day later than the Cd-tamoxifen group (Fig. 6.7 C).

These data indicate that Cd exposure impairs odor-cued associative olfactory learning and memory, and tamoxifen treatment rescued these deficits in female caMEK5 mice. However, Cd exposure did not cause significant impairment in the male group.

caMEK5 activation rescues female caMEK5 mice from Cd-induced impairments of contextual fear memory.

We conducted fear conditioning tests to investigate the effect of caMEK5 on the Cd-induced impairments of contextual fear memory, another form of hippocampus-dependent learning and memory, and on cued fear memory which is hippocampus-independent. In this study, we used a challenging training paradigm in which animals received 3 x 0.3 mA (2 s) foot shocks during the fear conditioning session (Fig. 6.8 A) as previouly reported (Engstrom et al., 2017; Pan et al., 2012a). Mice were assessed for the amount of time they froze in the testing arena 24 h after the footshock training as a measurement of their fear memory. In the female cohort, Cd-vehicle treated mice exhibited significantly reduced contextual fear memory compared to control-vehicle, control-tamoxifen, and Cd-tamoxifen treated mice (Fig. 6.8 B). However, we did not find this significant difference in the male group (Fig. 6.8 C). Furthermore, in both male and female cohorts, Cd and tamoxifen-treatment did not cause any significant change in any animal in auditory-cued fear memory- a form of amygdala -dependent, but hippocampus-independent memory, or in general freezing behavior in a novel context (Fig. 6.8 B and C). Therefore, Cd treatment does not affect the hippocampus-independent, auditory-cued memory, or lead to generalized freezing behavior in caMEK5 mice. Collectively, these data suggest that Cd exposure impairs the formation and/or retrieval of contextual fear memory in caMEK5 female mice, and caMEK5 activation can rescue mice from this impairment.

Blood Cd concentration at the end of the behavior tests

At the end of the study, we collected blood from all mice at 68 and 71 weeks to measure the final blood Cd levels (Fig. 6.9). The final Cd concentrations of Cd-treated

mice in female cohort was 0.25 ± 0.034 μg/L, while the blood Cd concentrations of control mice was 0.17 ± 0.028 μg/L. In the male group, the final blood Cd concentrations of Cd-treated mice was 0.134 ± 0.014 μg/L. In the control group, most of blood Cd level was below the detection limit (< 0.08 μg/L).

6.4 Discussion

Our previous studies have demonstrated that Cd exposure impairs hippocampus-dependent memory, olfactory memory, and adult neurogenesis in the SGZ and SVZ. However, the potential mechanisms of Cd neurotoxicity in cognitive and olfactory functions are still unknown. Since adult neurogenesis is involved in functions of cognition and olfaction, it is very likely that Cd may impairs hippocampus-dependent learning and memory, as well as olfactory memory through affecting adult neurogenesis. By using caMEK5 mice, a gain-of-function knock-in mouse that allows inducible and conditional activation of adult neurogenesis, the goal of this study was to determine whether enhancing adult neurogenesis by genetic manipulation could rescue the toxic effects of Cd on cognitive and olfactory functions in mice.

In this study, we used a line of knock-in mice in which endogenous ERK5 in adult neural progenitor/stem cells can be conditionally activated by tamoxifen-induced expression of active MEK5. Our recent studies have demonstrated that expression of caMEK5 increases the number of adult-born neurons in the SGZ and adult-born interneurons in the olfactory bulb by promoting cell survival, neuronal differentiation and maturation of adult-born stem cells without affecting cell proliferation, migration, or the stem cell pool, and thereby improving hippocampus-dependent learning and memory,

as well as olfactory functions in mice (Wang et al., 2015; Wang et al., 2014). The caMEK5 transgenic mouse line is a unique and powerful tool for us to examine if adult neurogenesis is involved in Cd-induced impairments of hippocampus-dependent learning and memory, as well as olfactory functions.

Animals were exposed to 0.6 mg/L Cd through drinking water for 38 weeks for behavior studies. Compared with our previous study, the Cd exposure level (0.6 mg/L) used in the study was 5 times lower, and the exposure time (38 weeks) was 18 weeks longer, which makes our current exposure paradigm more relevant to general population exposure levels. We assessed hippocampus-dependent spatial working memory in a novel object location test at multiple time points before, during, and after Cd exposure to “probe” the onset and sustainability of Cd-induced spatial memory impairment. In the female cohort, we discovered a loss of spatial working memory at 5 weeks into Cd exposure, and confirmed that the memory for novel object location was still impaired at 27 weeks after the Cd exposure was stopped. In male cohort, the spatial working memory loss started at 11 weeks into Cd exposure. We were able to detect spatial memory loss at 11 weeks after Cd exposure stopped but at 27 weeks after Cd exposure cessation, we found that the spatial working memory impairment had recovered in all Cd-treated mice.

We also conducted other behavioral tests after Cd exposure in both female and male cohorts. In the female cohort, a test of short-term olfactory memory was conducted at 7-8 weeks after Cd exposure, the sand-digging test was conducted at 15-16 weeks after Cd exposure, and contextual fear memory test was conducted at 23 weeks after Cd exposure. In the male cohort, the short-term olfactory memory was conducted at 13-

14 weeks after Cd exposure, the sand-digging test was conducted at 18-19 weeks after Cd exposure, and contextual fear memory test was at 22 weeks after Cd exposure. In the female cohort, we found Cd-induced impairments in all of the behavioral tests conducted after Cd exposure. The results of the female cohort behavior tests suggest that Cd exposure impairs hippocampus-dependent learning and memory, as well as olfactory memory, and the impairments persists at 27 weeks after cessation of Cd exposure. In the male cohort, we found significant Cd-induced short-term olfactory memory loss in the short-term olfactory memory test. However, we only found slight deficits in sand-digging test and no deficits in contextual fear memory test. All of the behavioral test (including the NOL test) results indicate that Cd exposure induces impairments of hippocampus-dependent memory and olfactory memory in male mice, but the impairments are less pronounced than those seen in female mice.

After we confirmed that Cd induced hippocampus-dependent memory loss in the novel object location test in female and male cohorts, all mice received 3 cycles of tamoxifen/vehicle treatment to examine whether enhancing adult neurogenesis can rescue caMEK5 mice from Cd induced memory loss. In the female cohort, we found that caMEK5 activation have significant effects on hippocampus-dependent learning and memory, as well as olfactory memory. The Cd-tamoxifen treated mice showed enhanced spatial working memory, contextual fear memory, short-term olfactory memory, and odor-associative learning and memory when compared with Cd-vehicle treated mice. In the male cohort, caMEK5 activation also had significant effects on spatial working memory and short-term olfactory memory. In the sand-digging test, although both Cd-vehicle and Cd-tamoxifen treated mice learned to associate specific

odorant with food award, the Cd-tamoxifen group learned the test 1 day earlier than the Cd-vehicle group. In the contextual fear memory test, since Cd-vehicle treated mice recovered from previous Cd-induced impairment, we could not tell if caMEK5 activation could rescue mice from impaired contextual fear memory. Together with female and male cohorts, these results suggest that enhancing adult neurogenesis through caMEK5 activation could rescue mice from Cd-induced impairments of hippocampus-dependent learning and memory, as well as olfactory memory.

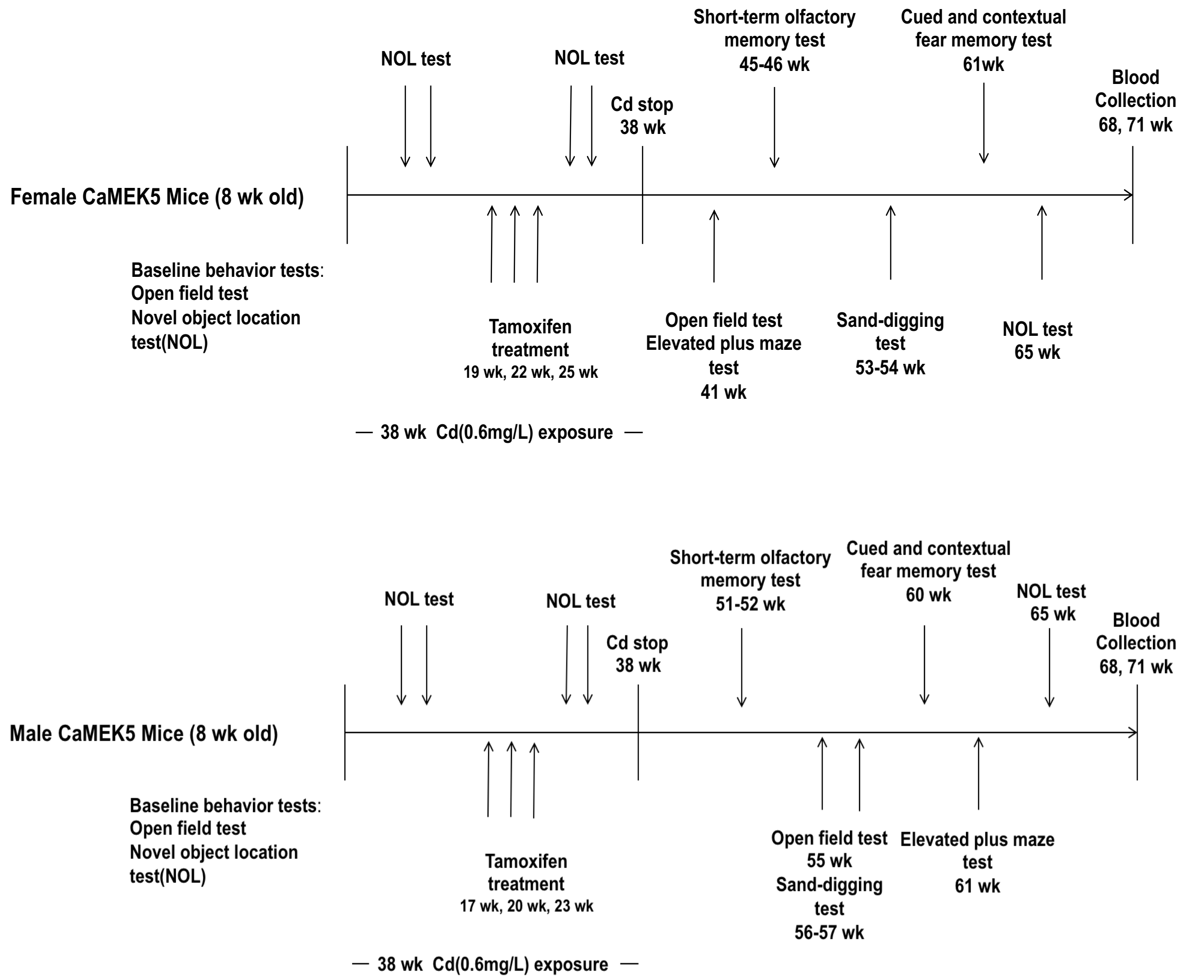
When compared the results of female and male cohorts, we noticed that female caMEK5 mice are more vulnerable to the toxic effects of Cd on cognition and olfactory memory. Cd exposure induced impaired spatial working memory loss much earlier in female cohort (5 weeks vs 11 weeks). Meanwhile, the Cd-induced impairments of cognition and olfaction in female mice persists longer than male cohort. This result may be due to the difference in blood Cd concentration between female and male mice. Our study found that the final blood Cd concentration in Cd-treated female mice is 0.25 ± 0.034 μg/L, while the concentration in Cd treated male mice is 0.134 ± 0.014 μg/L. The Cd blood concentration in female mice is almost twice the concentration in male mice, so the Cd-induced impairments in female mice may be more severe. Furthermore, in female caMEK5 mice, rescue effects in spatial working memory started at 6 weeks after tamoxifen treatment, which is 5 weeks earlier than male mice. This result suggests that the effects of tamoxifen treatment in adult neurogenesis may be more effective in female mice. Based on these results, further study about the effects of Cd and tamoxifen treatment on adult neurogenesis in female and male caMEK5 mice is needed

to investigate the potential mechanisms of the different effects of Cd in female and male mice.

6.5 Conclusion

In summary, our data demonstrate that caMEK5 activation, which has been proved to enhance adult neurogenesis, rescues mice from Cd-induced impairments of hippocampus-dependent learning and memory, as well as olfactory memory, and the rescue effect is more effective in female mice. These findings further support our hypothesis that Cd may impair cognitive and olfactory functions by affecting adult neurogenesis.

6.6 Figures and Tables



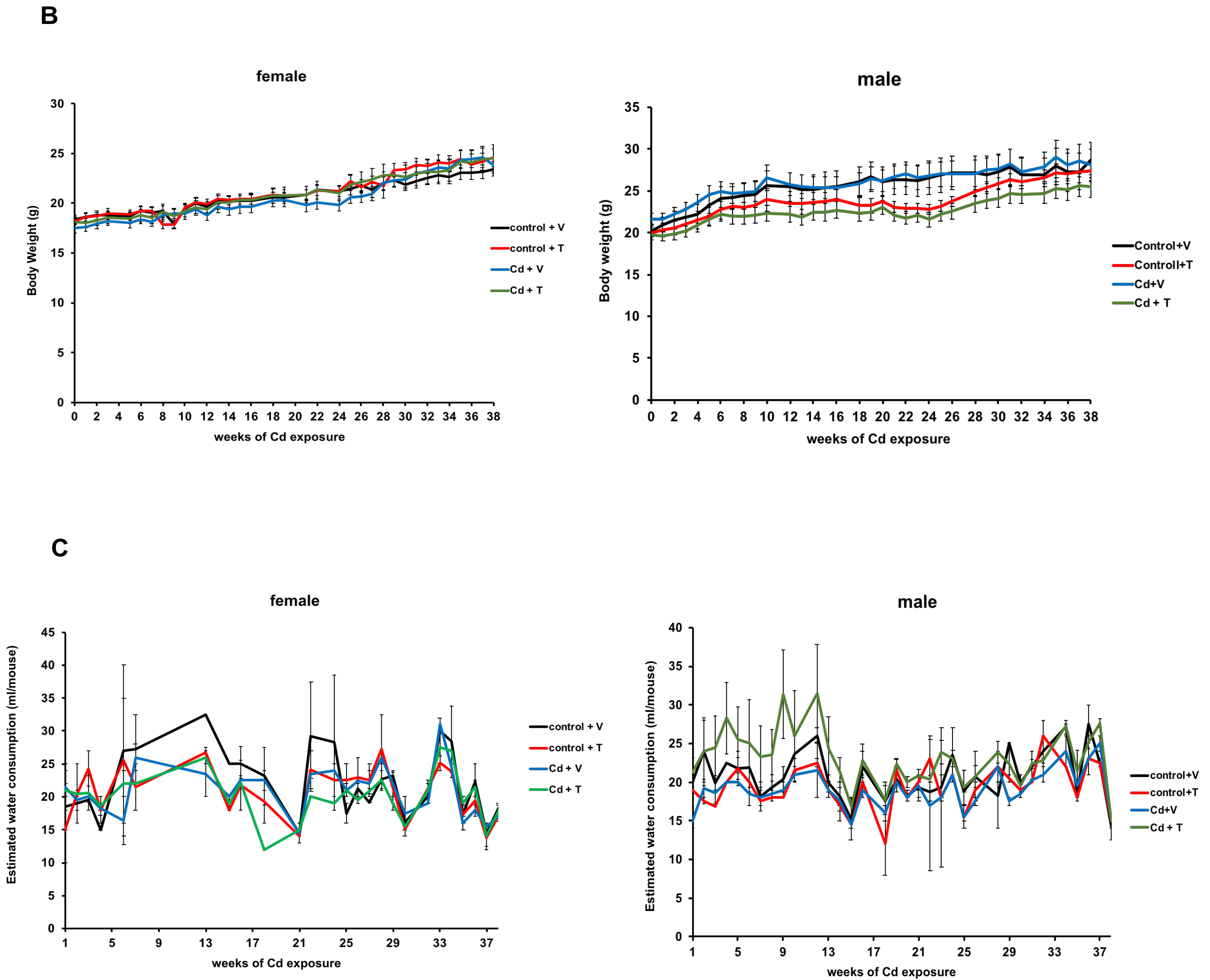
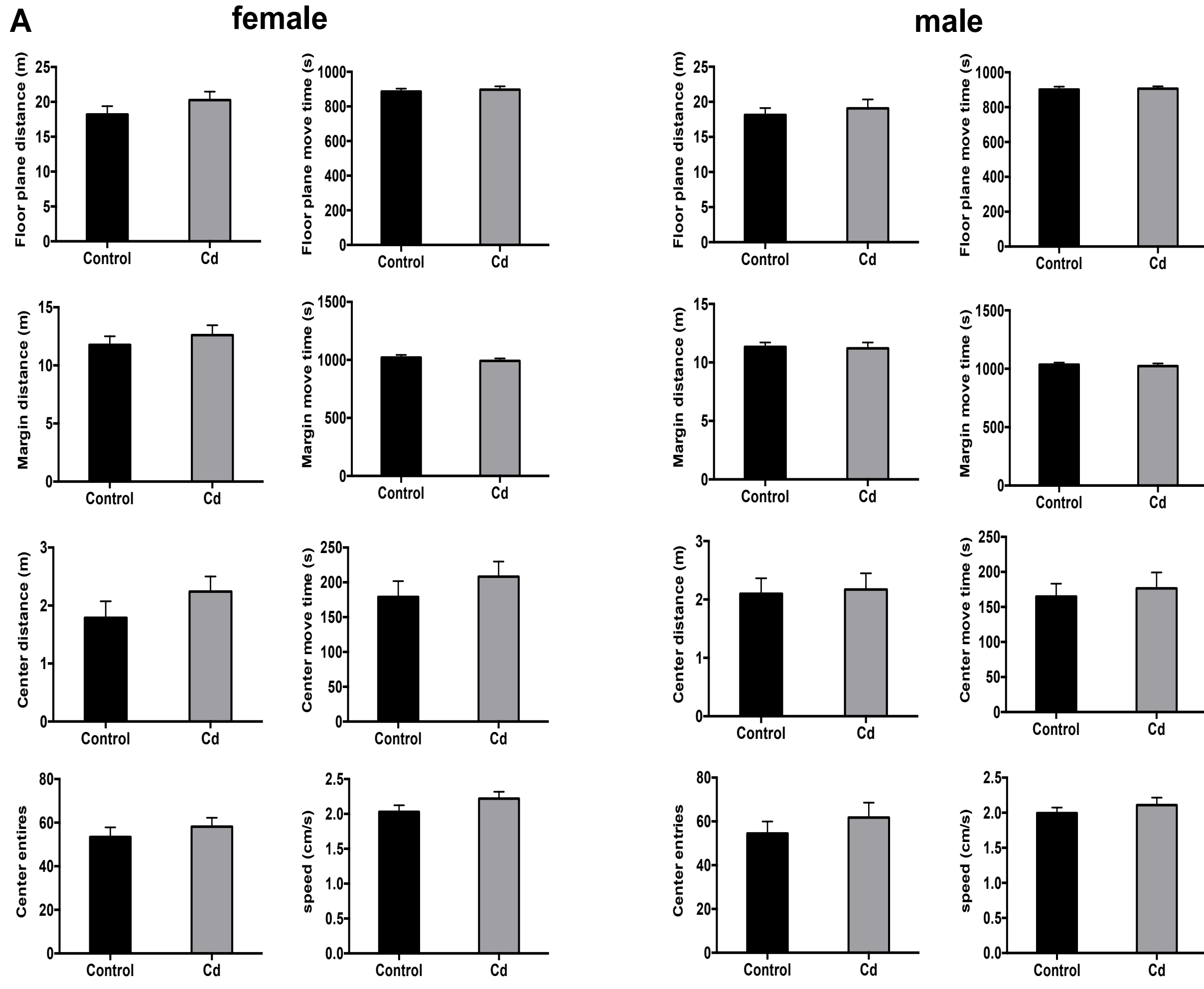


Figure 6.1. Cd exposure does not affect body weight and water consumption in female and male caMEK5 mice. A, Experiment design and timeline for behavior test. B, The Cd-treated mice did not exhibit a significant difference in body weight during the whole exposure period when compared with control mice. C, The Cd-treated mice did not exhibit a significant difference in water consumption during the whole exposure period when compared with control mice. Data are presented as mean ± SEM with n = 9-10 in each group.


A
female
male
Floor plane distance (m)
Floor plane move time (s)
Margin distance (m)
Margin move time (s)
Center distance (m)
Center move time (s)
Center entries
speed (cm/s)
Control
Cd

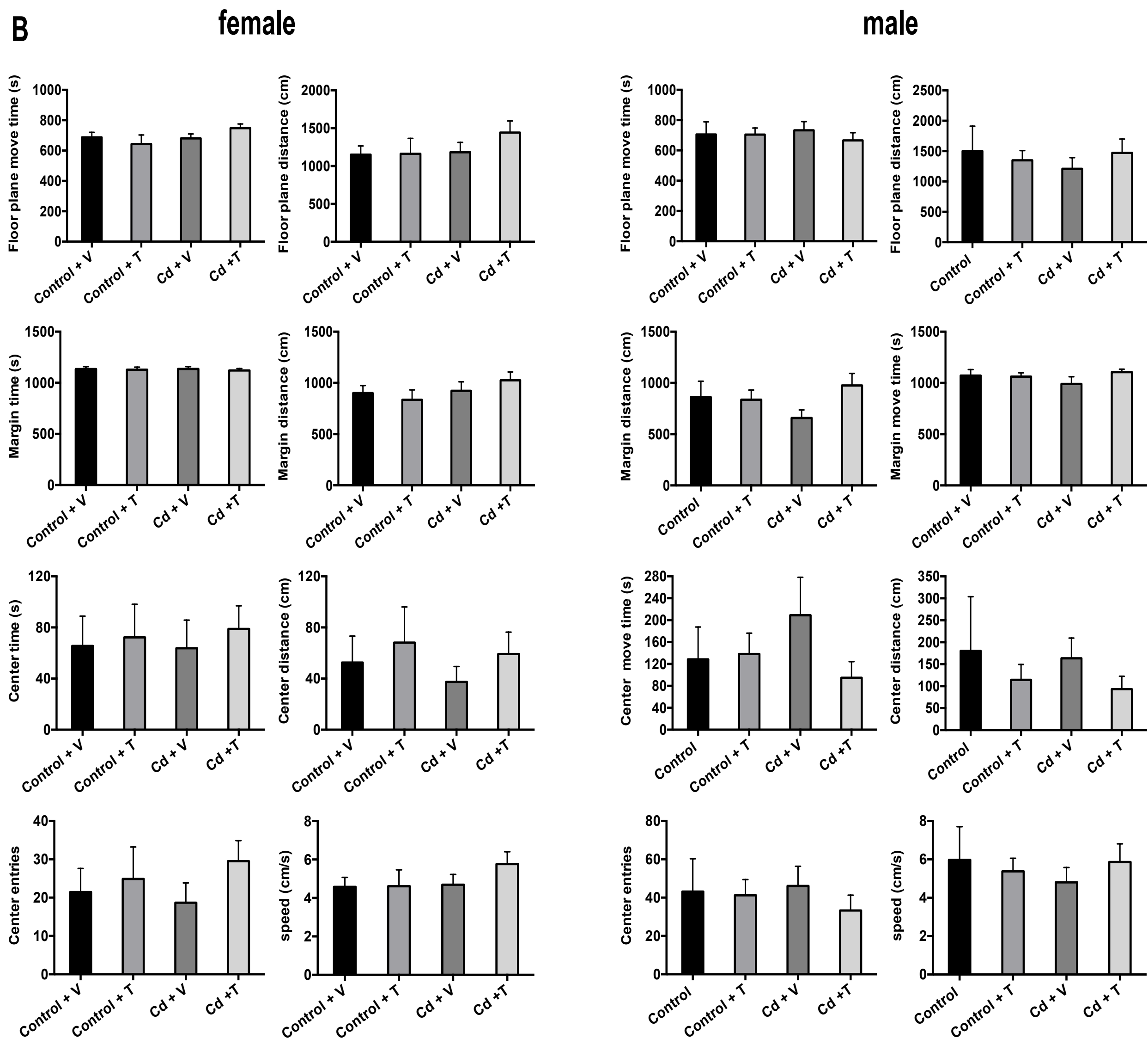


Figure 6.2. Cd exposure does not affect the locomotor activity or anxiety in open field test. A. Before Cd exposure. B, After Cd exposure and tamoxifen treatment. Data are presented as mean ± SEM with n= 19-20 in each group in (A) and n = 9-10 in each group in (B).

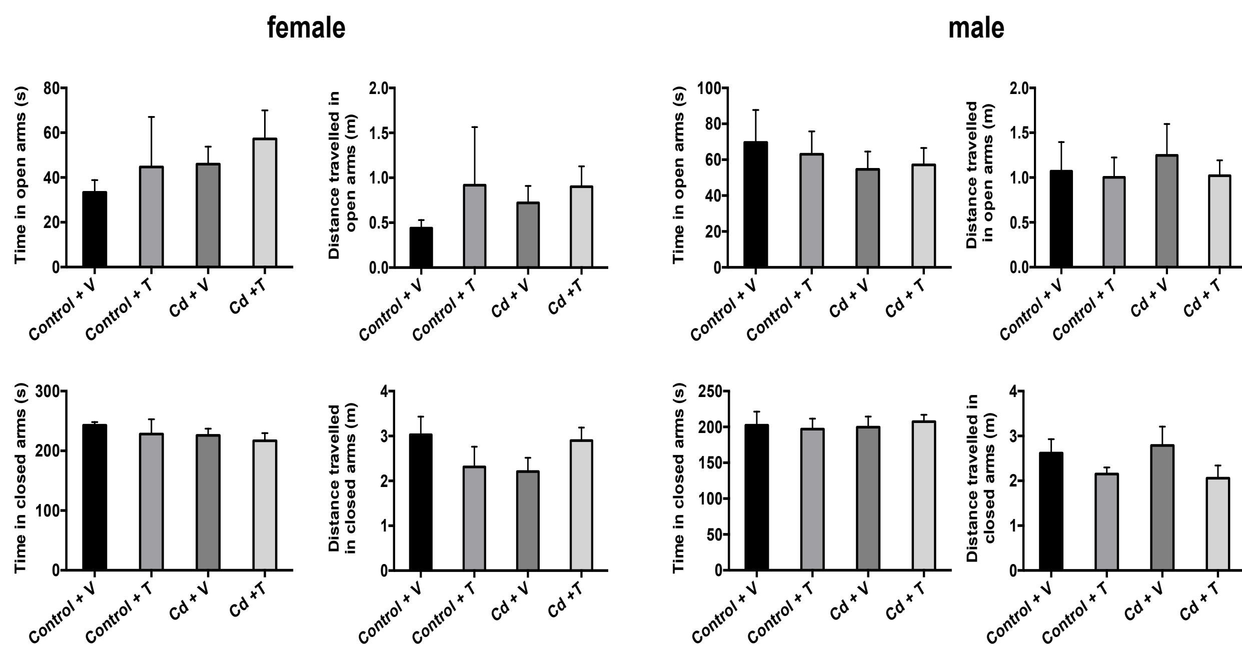


Figure 6.3. Cd exposure and tamoxifen treatment do not cause anxiety in elevated plus maze test. Data are presented as mean ± SEM with n = 9-10 in each group.

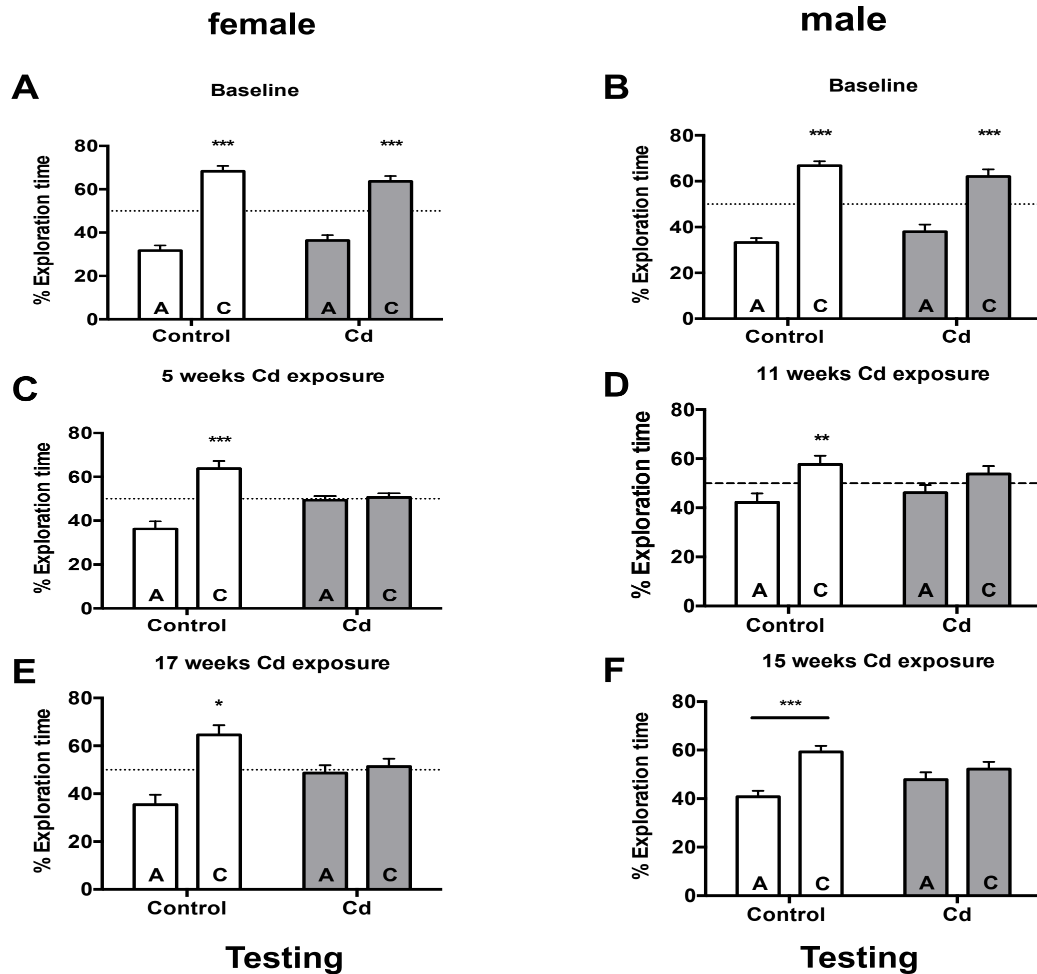


Figure 6.4. Cd exposure impairs hippocampus-dependent spatial working memory in the novel object location (NOL) test. (A, B) All mice in female and male cohorts had intact spatial working memory prior to Cd exposure. (C) At 5 weeks into Cd exposure, the Cd-treated female mice started to show spatial working memory deficits in the NOL test. (D) At 11 weeks into Cd exposure, the Cd-treated male mice started to exhibit spatial working memory deficits in the NOL test. (E) At 17 weeks into Cd exposure, the Cd-treated female mice continued to exhibit a deficit in spatial working memory. (F) At 15 weeks into Cd exposure, the Cd-treated male mice continued to exhibit a deficit in spatial working memory. Data are presented as mean ± SEM with n = 9-10 in each group. *p<0.05; **p<0.01; ***p<0.001.

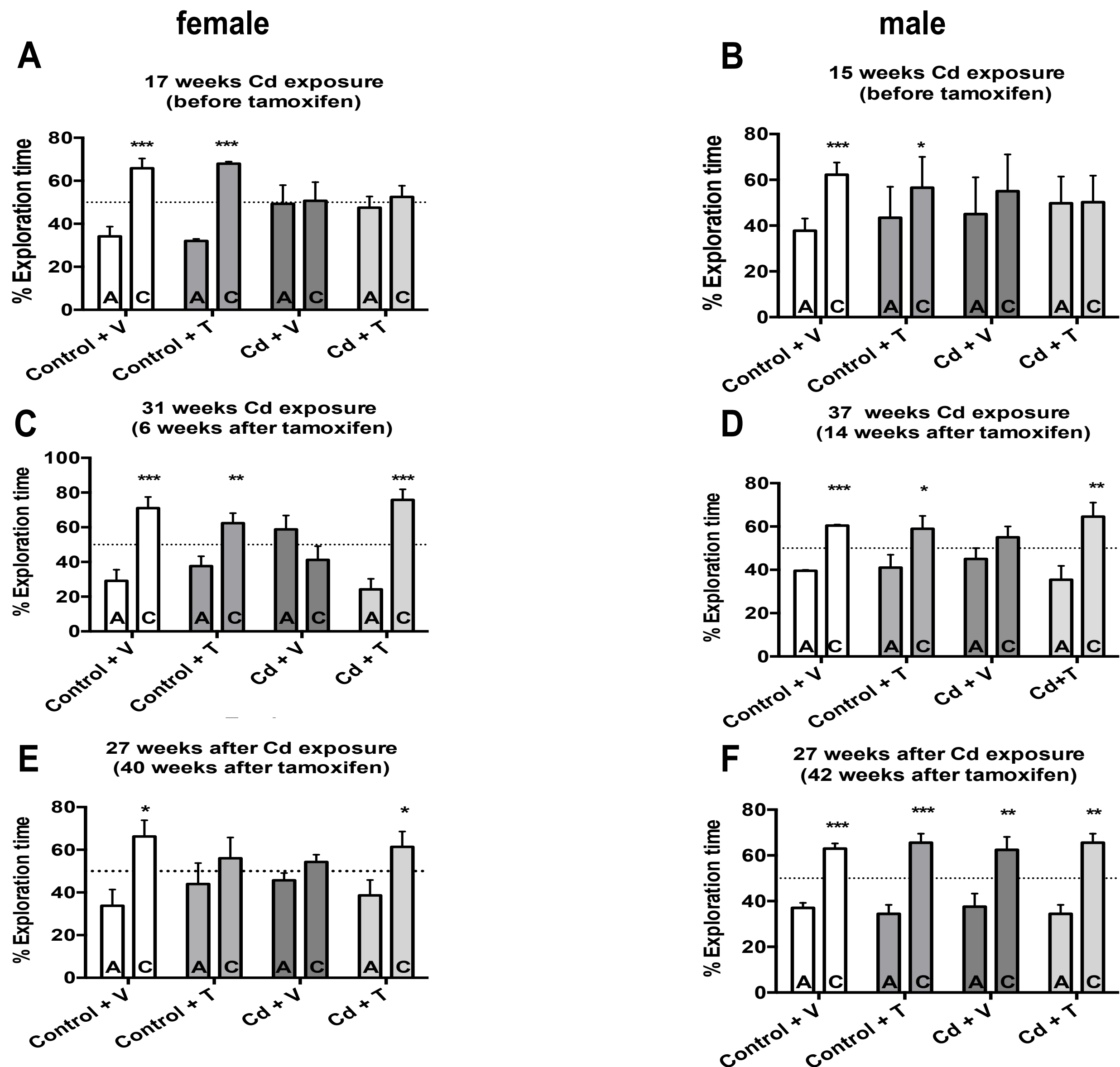


Figure 6.5. caMEK5 activation rescues mice from Cd-induced impairments of spatial working memory in NOL test. (A, B) Before tamoxifen treatment, all Cd-treated mice exhibited deficit in spatial working memory while control mice had intact spatial working memory. (C) In female cohort, 6 weeks after tamoxifen treatment, Cd-tamoxifen group started to exhibit spatial working memory in the NOL test, while Cd-vehicle group did not. (D) In male cohort, 14 weeks after tamoxifen treatment, Cd-tamoxifen group started to exhibit spatial

working memory in the NOL test, while Cd-vehicle group did not. (E) In female cohort, at 27 weeks after Cd exposure (40 weeks after tamoxifen treatment), Cd-tamoxifen group continued to exhibit spatial working memory, but Cd-vehicle group still had impaired spatial memory. (F) In male cohort, at 27 weeks after Cd exposure (42 weeks after tamoxifen treatment), all mice exhibited spatial working memory in the NOL test. Data are presented as mean ± SEM with n = 9-10 in each group. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

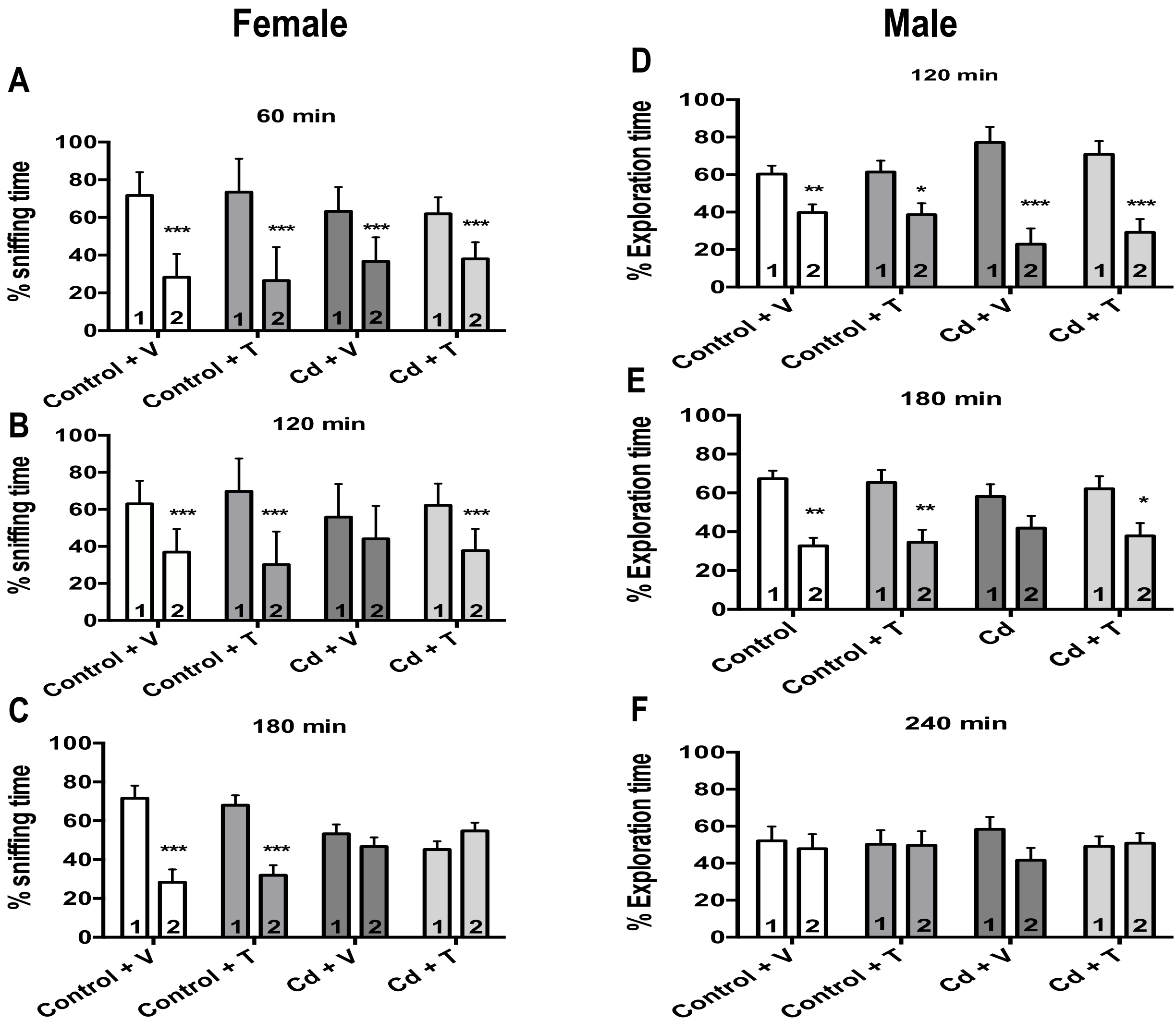
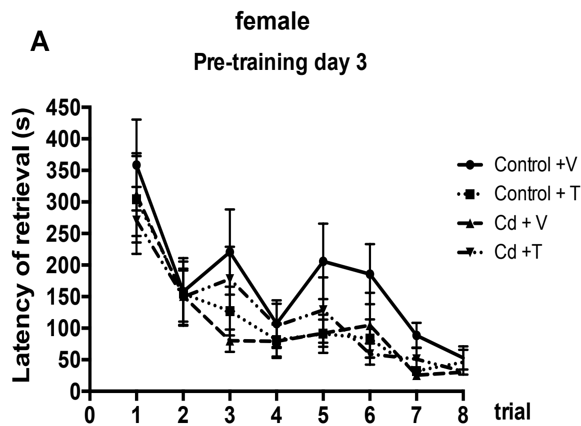
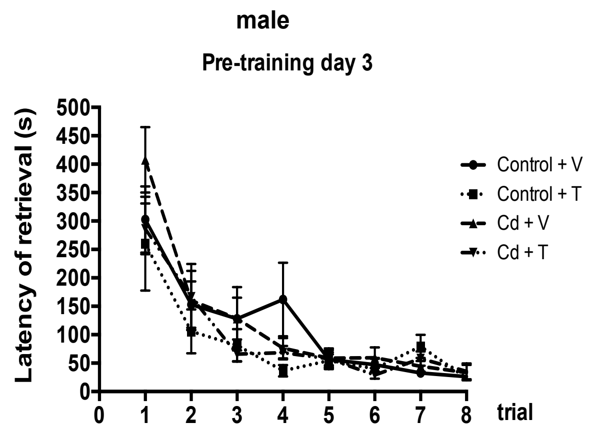


Figure 6.6. caMEK5 activation rescues mice from Cd-induced short-term olfactory memory deficits. Mice were presented with cotton swbs laced with the same odorant twice with time intervals as indicated. A decrease investigation during the second presentation of the same odorant suggests olfactory memory of the specific odorant. (A-C) Short-term olfactory tests with different time intervals in female cohort. (D-F) Short-term olfactory tests with different time intervals in male cohort. Data are presented as mean ± SEM with n = 9-10 in each group. *p<0.05; **p<0.01; ***p<0.001

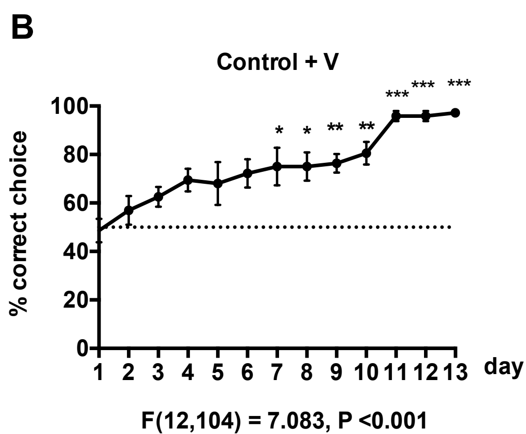
A

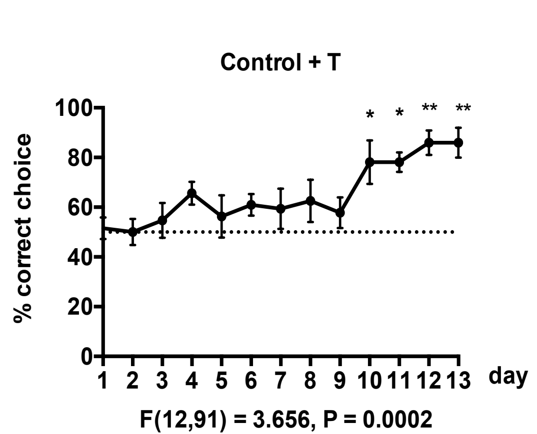


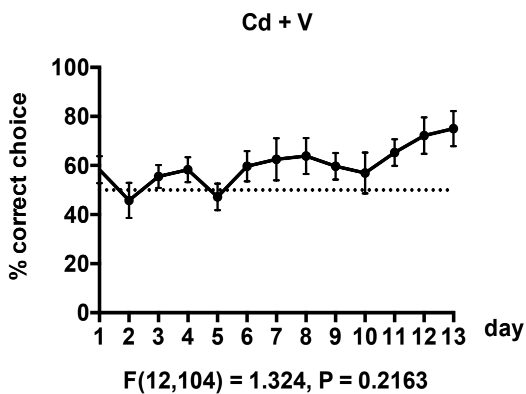


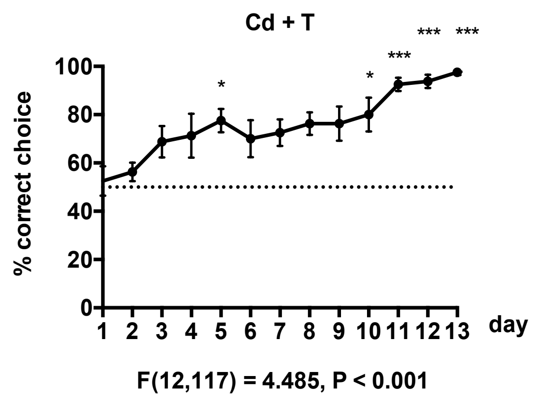
B

female









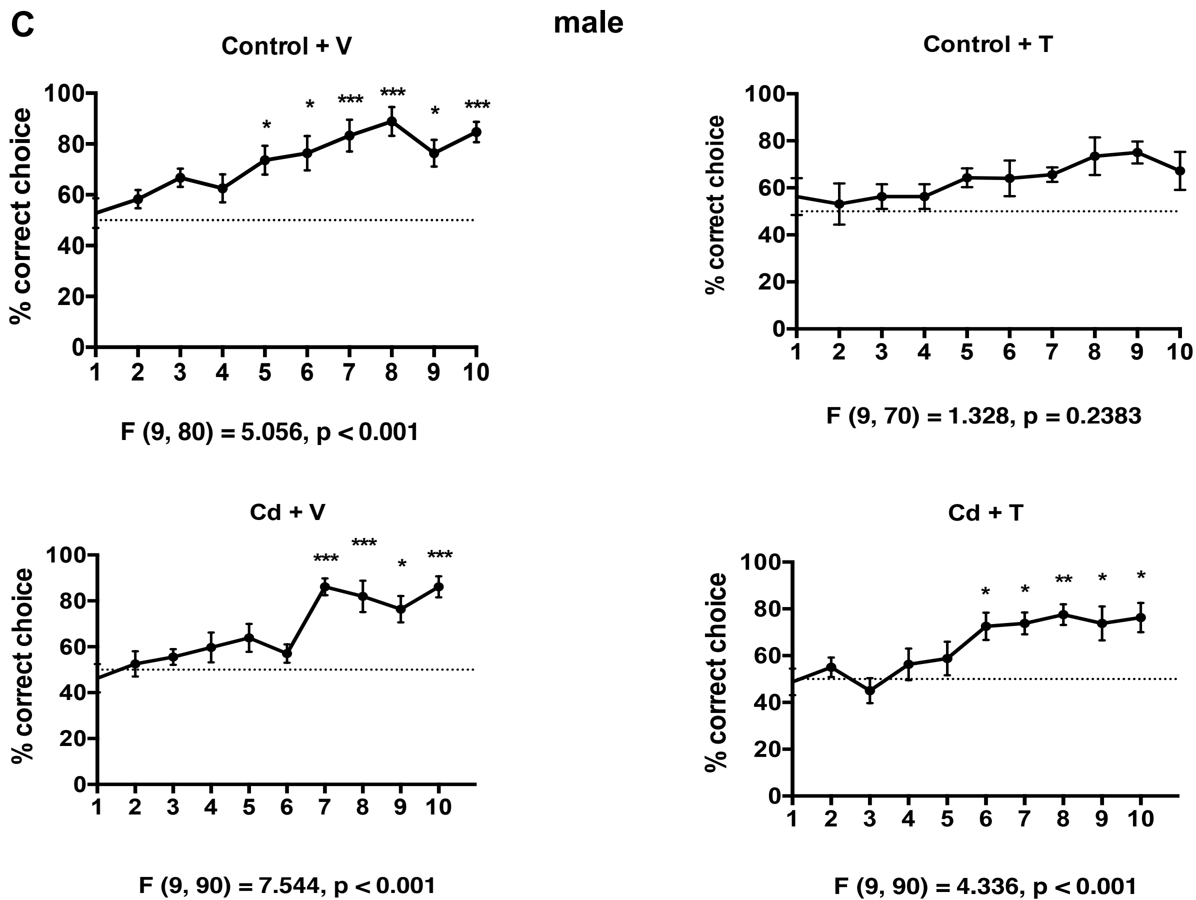


Figure 6.7. caMEK5 activation rescues mice from Cd-induced impairments in odor-cued associative learning and memory. A, On the third day of pretraining, all mice learned to retrieve food reward deeply buried in the sand. B, In female cohort, all mice learned to associate specific odorant with food reward over 13 days training except Cd-vehicle group. C, In male cohort, all mice learned to associate specific odorant with food reward over 10 days training except the control-tamoxifen group. Data are presented as mean ± SEM with n = 9-10 in each group. *p<0.05; **p<0.01; ***p<0.001.

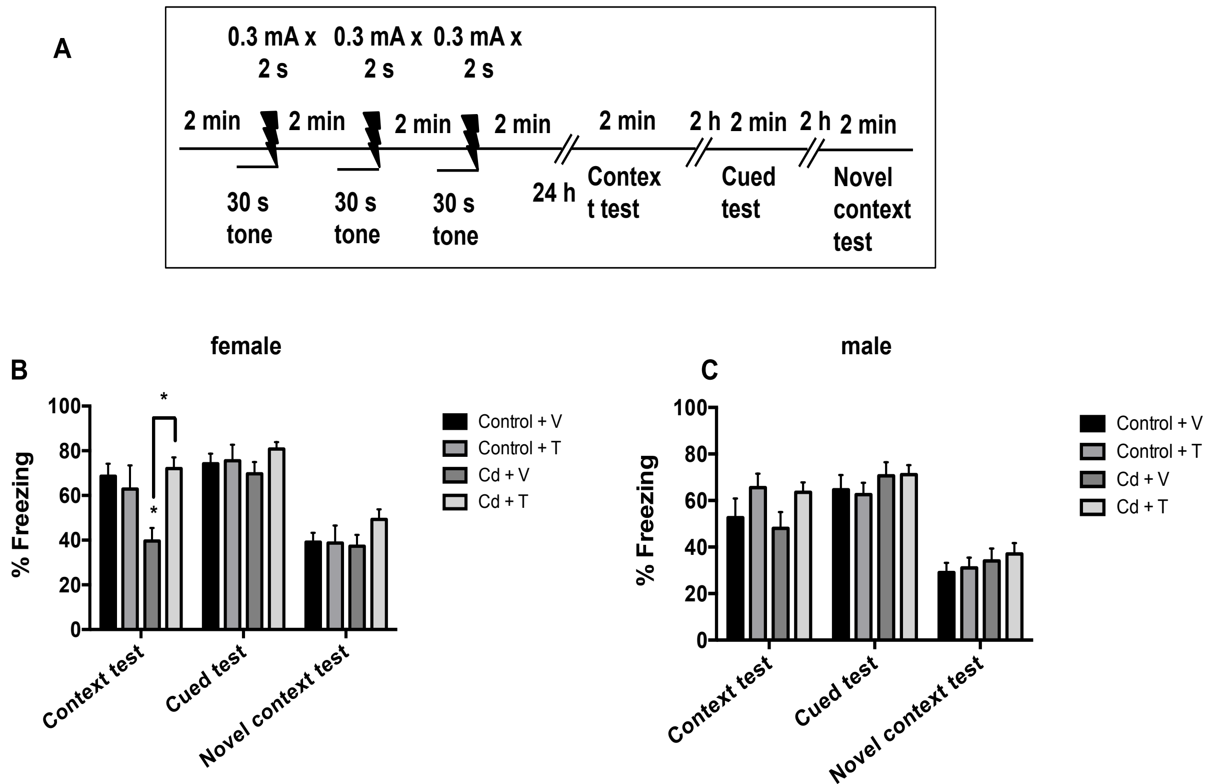


Figure 6.8. caMEK5 activation rescues female mice from Cd-induced contextual fear memory. A, Schematic of cued and contextual fear conditioning test. B, In the female cohort, the Cd-vehicle treated group exhibited significant lower freezing behavior in context test than the other groups, but auditory-cued fear memory (cued test) and general freezing behavior (novel context test) were not affected. C, In the male group, all mice exhibited similar freezing behavior in context test, cued test and novel context test. Data are presented as mean ± SEM with n = 9-10 in each group. *$p<0.05$.

Female	*In vivo* Cd exposure	
	Control	Cd (0.6 mg/L)
Cd in blood (ug/L)	< 0.08(detection limit) (n=3) 0.17 ± 0.028 (n=12)	< 0.08(detection limit) (n=3) 0.25 ± 0.034 (n=14)

Male	*In vivo* Cd exposure	
	Control	Cd (0.6 mg/L)
Cd in blood (ug/L)	< 0.08(detection limit) (n=17) 0.09 (n=1)	< 0.08(detection limit) (n=7) 0.134 ± 0.014 (n=12)

Figure 6.9. Blood Cd concentration of female and male cohorts. At the end of the study, the blood of mice was collected to measure the final blood Cd concentration. Data are presented as mean ± SEM with n = 15-19 in each group.

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Chapter 7: Conclusion and future directions

Cadmium (Cd) is a ubiquitous heavy metal pollutant that can cause toxic effects to many organs, including kidney, liver, lung, and bone (Chen et al., 2009; Dudley et al., 1982; Goering et al., 1993; Satarug et al., 2010; Torra et al., 1995). Cd is also a potential neurotoxicant that its exposure is suggested to be associated with cognition and olfaction deficits in humans (Ciesielski et al., 2013; Ciesielski et al., 2012; Mascagni *et al.*, 2003). However, there was no direct evidence show that environmental relevant Cd exposure impairs cognition and olfaction in animal models. In addition, little is known regarding the underlying mechanisms of Cd neurotoxicity.

My contribution to the field of Cd neurotoxicity stemmed from the hypothesis that Cd exposure impairs cognitive and olfactory functions through affecting adult neurogenesis. In my dissertation research, I studied the toxic effects of Cd exposure on hippocampus-dependent memory and olfactory functions with a focus on its effects on adult neurogenesis. I found that environmentally relevant Cd exposure impairs hippocampus-dependent learning and memory (spatial working memory and contextual fear memory), as well as olfactory memory (short-term olfactory memory and odor-cued associative memory) in mice. In addition, I confirmed that Cd affects the process of SGZ and SVZ adult neurogenesis, both *in vitro* and *in vivo.* I also found that the toxic effects of Cd on adult neuronal stem/progenitor cells may be mediated by the activation of the JNK and p38 MAP Kinases signaling pathways. These data provide direct evidence that Cd exposure impairs cognition and olfactory function, as well as adult neurogenesis.

Furthermore, I demonstrated that inducible and conditional enhancement of adult neurogenesis by genetic manipulation can rescue mice from Cd-induced impairments of

hippocampus-dependent learning and memory (spatial working memory, contextual fear memory), as well as olfactory memory (short-term olfactory memory, odor-cued associative learning and memory). This exciting and novel finding further suggest that Cd may induce impairments of cognition and olfaction through affecting the process of adult neurogenesis, both in SGZ and SVZ/OB.

In the future, additional characterization of the effects of Cd on adult neurogenesis, such as dendritic complexity of immature adult-born neurons, is needed to get a full spectrum of Cd neurotoxicity. Meanwhile, in my current study, I have not examined the status of adult neurogenesis in caMEK5 mice before and after genetic manipulation, so more characterization of adult neurogenesis in caMEK5 mice after Cd and tamoxifen treatment is required to confirm that the rescue effects we found in behavior tests are due to the increase of adult neurogenesis.

Besides affected adult neurogenesis, I am also wondering if there is any other potential mechanism of neurotoxicity of Cd. Hippocampus-dependent memory is initiated by Ca^{2+} signals. (Danysz et al., 1988; Morris et al., 1986; Tsien et al., 1996). Studies have found that Ca2+/cAMP/ERK1/2 MAP kinases /MSK1/CREB signaling pathway is involved in hippocampus-dependent learning and memory (Athos et al., 2002; Atkins et al., 1998; Bourtchuladze et al., 1994; Impey et al., 1998a; Impey et al., 1998b; Pittenger et al., 2002). Cd is a potent competitive inhibitor of several Cd channels (Carageorgiou and Katramadou, 2012; Thevenod and Jones, 1992). In addition, Cd can inhibit the Ca^{2+}-stimulated adenylyl cyclases in neurons (Chan et al., 2005). These findings suggest that Cd may induce neurotoxicity by affecting the Ca^{2+} signal transduction pathway in hippocampal neurons.

Studies have found that the hippocampus is vulnerable to damage in Alzheimer's disease patients. ApoE, a protein that plays an important role in Alzheimer's disease, may regulate adult neurogenesis in the hippocampus (Li et al., 2009; Yang et al., 2011). Research has proved that the ApoE4 variant is a genetic risk factor for onset of sporadic Alzheimer's disease (Roses, 1996). Our lab has found that there is a gene and environment interaction between ApoE4 and lead exposure on learning and memory (Engstrom et al., 2017). Based on this information, it would be very interesting to study if there is any gene and environment interaction between Cd exposure and specific genes, such as ApoE4.

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