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Lead decreases cell survival, proliferation, and neuronal differentiation of primary cultured adult neural precursor cells through activation of the JNK and p38 MAP kinases

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ABSTRACT

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

1. Introduction

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

The heavy metal lead is a ubiquitous environmental contaminant and a major public health concern. The combustion of leaded gas in the U.S. in the 20th century released approximately 4 million metric tons of lead into the environment (Toscano and Guilarte, 2005b). The phasing out of leaded paint and gasoline has contributed to a significant decline in ambient lead levels as well as mean blood lead levels (12.8–1.6 μg/dL from 1976 to 2002) in the U.S. population (Toscano and Guilarte, 2005b; ATSDR, 2007). However, lead can persist in the soil for decades and no level of lead is considered safe (White et al., 2007). In addition to its well-characterized developmental neurotoxicity, cumulative lead exposure can also cause neurological impairment in adults (van Wijngaarden et al., 2009). Monkeys and rats exposed to low concentrations of lead have increased cognitive decline and

Abbreviations: aNPC, adult neural precursor cells; SGZ, subgranular zone; DG, dentate gyrus; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen activated protein; DMSO, dimethyl sulfoxide; BrdU, 5-bromo-2′-deoxyuridine; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; PFA, paraformaldehyde; PBST, phosphate-buffered saline with 0.1% Triton-X 100.

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

Lead may facilitate and accelerate cognitive decline through impaired adult hippocampal neurogenesis. Several studies have examined the effect of early life lead exposure on adult hippocampal neurogenesis and found that developmental lead exposure is associated with altered proliferation, survival, and dendritic morphology of adult-born neurons in the hippocampus and altered hippocampus learning and memory in rats (Verina et al., 2007; Jaako-Movits et al., 2005). However, results have not been entirely consistent among various studies. Gilbert et al. (2005) found that lead-treated rats have reduced adult-born cell ($BrdU^+$) survival but no change in cell proliferation, while a similar study reported that lead decreases adult-born cell survival as well as proliferation (Verina et al., 2007). Furthermore, Jaako-Movits et al. (2005) found that lead impaired adult-born cell proliferation and neuronal maturation, while Verina et al. found no effect of lead on neuronal differentiation (Verina et al., 2007). Although these studies are very interesting, the inconsistent results warrant further investigation. Moreover, the early life exposure paradigms used in these *in vivo* studies may have introduced potential confounding factors due to the adverse effects of lead during development (Gilbert et al., 2005; Jaako-Movits et al., 2005; Verina et al., 2007). Only one study to date has assessed the effect of postnatal lead exposure alone on adult neurogenesis (Schneider et al., 2005). Schneider et al. (2005) exposed male rats to 1500 ppm lead acetate for 30–35 days starting at postnatal day 25 and found that lead impaired adult-born cell proliferation in the SGZ. However, they did not assess the effect of lead on other stages of adult neurogenesis or cognitive behavior. Thus, additional research is needed to determine whether adult-only lead exposure is sufficient to impair adult hippocampal neurogenesis and to characterize the signaling mechanisms underlying lead-induced impairment in adult neurogenesis. In this study, we used primary cultured aNPCs isolated from the hippocampus (SGZ-aNPCs) of adult mice as an *in vitro* model system to test the hypothesis that lead exposure impairs adult hippocampal neurogenesis and to elucidate the underlying signaling mechanisms.

2. Materials and methods

2.1. Reagents

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

2.2. Cell culture

The University of Washington Institutional Animal Care and Use Committee approved all experimental procedures. The primary aNPCs were prepared as previously described (Guo et al., 2012; Pan et al., 2013) from the SGZ of the DG from 6–7 week-old male C57BL/6J mice (Taconic, Hudson, NY). The solutions and media used during the aNPC isolation were filter sterilized. Briefly, the whole brain from four adult male mice was harvested and placed in HBSS (Invitrogen). Each brain was then sliced into 1 mm sections using an adult mouse brain matrix (Kent Scientific, Torrington, CT), and then the SGZ was isolated from these sections via microdissection under a dissection microscope. The SGZ tissue was placed in Solution A (30 mM Glucose, 26 mM $NaCO_3$, 2 mM HEPES pH 7.4 (Invitrogen) in HBSS (Invitrogen)) and spun down for 10 min at 1000 rpm. The pelleted tissue was then resuspended and a combination of mechanical and enzymatic digestion (MACS Neural Tissue Dissociation Kit, Miltenyi Biotec, San Diego, CA) was used to dissociate the tissue. To stop the digestion, DMEM/F-12 medium (Invitrogen) with 10% Fetal Bovine Serum (FBS, Invitrogen) was added and the SGZ tissue was then filtered through a cell strainer (70 μm cell strainer, Fisher Scientific, Waltham, MA) and spun down for 3 min at 1000 rpm. The pellet was washed once with DMEM/F-12 medium with 10% FBS and once with DMEM/F-12 medium with 10% FBS plus Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) solution (1:10 Percoll in PBS) followed by spins at 1000 rpm for 3 and 15 min, respectively. The pellet was washed once with Solution A and once with initial proliferation medium (Neurobasal medium (Invitrogen); 1X B27 supplement without Vitamin A (Invitrogen); 2 mM L-Glutamine (Invitrogen); 100 U/ml penicillin/streptomycin (Invitrogen), 20 ng/ml of epidermal growth factor (EGF; EMD Chemicals) and 10 ng/ml basic fibroblast growth factor (bFGF; EMD Chemicals) followed by 5 min spins at 1500 rpm. The cells were then plated 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 3–4 d unless noted otherwise. Primary neurospheres formed after 7–14 d, at which time, the neurospheres were collected, enzymatically and mechanically dissociated, and then resuspended in growth media (Advanced DMEM/F-12, 1X N2 Supplement (Invitrogen), 1X B27 Supplement, 100 U/ml Penicillin/streptomycin, 2 mM L-Glutamine, 2 μg/ml Heparin sodium salt (Sigma), 20 ng/ml EGF, and 10 ng/ml bFGF). The neurospheres were maintained in petri dishes in the growth media and passaged ⩽10 times.

2.2.1. Drug treatment

For experiments, the neurospheres were dissociated (0.125% trypsin–EDTA (Invitrogen) for 5 min; 0.014% soybean trypsin inhibitor (Sigma) for 5 min) and seeded as a monolayer culture on

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