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Cadmium impairs the survival and proliferation of cultured adult subventricular neural stem cells through activation of the JNK and p38 MAP kinases

Hao Wang, Anna K Engstrom, Ph.D, and Zhengui Xia, Ph.D*

Abstract

Cadmium (Cd) is a heavy metal with a long biological half-life in humans and is recognized as a toxic pollutant. Cd is also a potential neurotoxicant and its exposure is associated with olfactory impairment in humans. However, the molecular and cellular mechanisms of Cd neurotoxicity are not well defined. Adult neurogenesis is a process that generates functional neurons from adult neural stem/progenitor cells (aNPCs). It occurs in specific regions of the adult brain including the subventricular zone (SVZ) along the lateral ventricles in mammals, a process that is critical for olfaction. Various external stimuli can modulate adult neurogenesis and the effect of neurotoxicants on adult neurogenesis is just beginning to be elucidated. Since Cd exposure can impair olfaction in humans, the goal of this study is to investigate the effects of Cd on SVZ adult neurogenesis and underlying mechanisms using primary cultured SVZ-aNPCs. In this study, we report that low-level Cd exposure decreases cell number, induces apoptosis, and inhibits cell proliferation in SVZ-aNPCs. Furthermore, Cd exposure significantly increases phosphorylation of c-Jun NH2-terminal kinase (JNK), and p38 MAP kinase in these cells, indicative of JNK and p38 activation. Pharmacological inhibition of JNK or p38 MAPK kinases attenuated Cd-induced cell loss and apoptosis. Cd treatment did not cause cell loss or apoptosis in SVZ-aNPCs prepared from transgenic mice null for the neural-specific JNK3 isoform. These data suggest a critical role for p38 and JNK3 MAP kinases in Cd neurotoxicity. These results are, to our knowledge, the first demonstration that Cd impairs SVZ adult neurogenesis *in vitro*, which may contribute to its neurotoxicity in olfaction.

Keywords

Cadmium; Adult neurogenesis; Neurotoxicity; Apoptosis; Proliferation; MAPK signaling pathway

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

*Corresponding Author: Institution: Toxicology Program in the Department of Environmental and Occupational Health Science, University of Washington.

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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 food 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). Human epidemiology studies found 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 of 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: 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, 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; Kikuchi 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.

2. Materials and methods

2.1. 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 (H₂O) to make a 1mM [Cd²⁺] 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.

2.2. 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 ventricle 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) with 30 mM Glucose, 2mM pH 7.4 HEPES (Invitrogen), and 26 mM NaCO₃) 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) with 10% FBS (Invitrogen) 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, Pittsburgh, PA) 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% CO₂. 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 (Sigma), 20 ng/ml EGF, and 10 ng/ml bFGF). The neurospheres were maintained in petri dishes in the growth media and passaged 11 times.

2.3. 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 (Sigma) 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 (CdCl₂) was dissolved in H₂O while the JNK and p38 inhibitors were dissolved in DMSO, so either an equal volume of H₂O 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.

2.4. 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 H₂O 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.

2.5. 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.

2.6. Western blot analysis

The cells were seeded at 5×10^5 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).

2.7. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software (version 6.0c for Mac, GraphPad Software Inc., San Diego, CA) or Excel (Microsoft, Redmond, WA). All of the immunocytochemical data are from at least three independent experiments with triplicates for each data point ($n = 9$ 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 pairwise 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 $\pm$ SEM., n.s. not significant, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results

3.1. 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. 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.

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

To determine whether low-level Cd exposure can induce toxic effects on SVZ-aNPCs, cells were treated with 0 to 0.45 μM CdCl₂ (50 ppb Cd²⁺) for 96 h *in vitro*. Cd significantly decreased the total cell number, starting at 0.09 μM CdCl₂ (10ppb Cd²⁺), in a dose-dependent manner (Fig. 2A–D). In addition, Cd treatment induced apoptosis, quantified by nuclear condensation and fragmentation, beginning at 0.09 μM CdCl₂, (Fig. 2E).

Furthermore, Cd significantly increased the number of cells positively immunostained for cleaved caspase-3, indicating caspase-3 activation (Fig. 2F, G). These data suggest that Cd causes cell loss and induces apoptosis in SVZ-aNPCs.

3.3. 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 CdCl₂, we found a significant decrease in the percentage of BrdU⁺ cells in a dose-dependent manner after a 24 h-treatment (Fig. 3A–D). These data indicate that Cd inhibits proliferation of SVZ-aNPCs *in vitro*. Combined with data in Fig. 2, these results suggest that Cd decreases the total cell number through apoptosis and inhibition of proliferation.

3.4. 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 the JNK signaling is activated by Cd. SVZ-aNPCs were treated with 0.45 μM CdCl₂ 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. 4A, B). Pre-incubation with a pan-JNK inhibitor SP600125 (0.5 μM) for 1 h significantly reversed the cell number loss and apoptosis induced by Cd treatment (0.45 μM CdCl₂ for 96 h) (Fig. 4C, D). These data suggest that activation of the JNK signaling pathway plays an important role in Cd-induced toxic effects on SVZ-aNPCs.

3.5. 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 CdCl₂ (20ppb Cd²⁺) 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. 5A, 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.

3.6. 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. 6A, B). Furthermore, pre-treatment with 0.5 μ M p38 inhibitor (SP202190) for 1h almost completely blocked the toxic effects of Cd on apoptosis and total cell number (Fig. 6C, D). These results suggest that the activation of p38 also contributes to Cd-induced toxic effects on SVZ-aNPCs.

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 proliferation.

When cells were treated with Cd for up to 96h, we found that p-JNK and p-p38 was elevated for the first 2 hr (Fig. 4) but not later in the treatment (data not shown). Meanwhile, Cd-induced cell death occurred at 96 hr (Fig. 2) but not at 24 hr or 48 hr after treatment (data not shown). However, pharmacological inhibition of JNK or p38 prevented cell loss and apoptosis induced by Cd treatment. These results suggest that JNK and p38 activation, although transient and occurred much earlier than cell death, is critical for Cd-induced cytotoxicity in SVZ-aNPCs. These results are consistent with other reports that Cd induce 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 epidemiology studies have found that Cd exposure at levels that had previously been considered to be without adverse effect and common in the U.S. 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 10ppb Cd^{2+}). This concentration is close to the US EPA's maximum allowable contaminant level of Cd in drinking water (5ppb). Thus, our findings suggest that Cd may exert adverse effects on SVZ adult neurogenesis under environmentally relevant exposure levels.

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.

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Fig. 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 .

Fig. 2. Cd decreases the total cell number and increases apoptosis in SVZ-aNPCs
 (A–C) Representative Hoechst nuclei staining from (A) Control, (B) 0.09μM, and (C) 0.45μM CdCl₂-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 CdCl₂. Arrows point to apoptotic cells with condensed and/or fragmented nuclei (enlarged images in upper left corner) in B and C. (F, G) Cd activates caspase-3 in SVZ-aNPCs. (F) Representative Hoechst nuclei (blue) and active caspase-3 (green) co-staining from Control and 0.45μM CdCl₂-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. At least 500 cells per coverslip were quantified in each treatment. Scale bar, 50 μm. Data represent mean ± SEM., n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 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 CdCl₂-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. At least 500 cells per coverslip were quantified in each treatment. Scale bar, 50 μm. Data represent mean ± SEM., n.s., not significant; *p < 0.05; ***p < 0.001.

Fig. 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 μ M CdCl₂ 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 μ M of the pan-JNK inhibitor (SP600125) for 1h and then treated with 0.45 μ M CdCl₂ for an additional 96h. Quantification of (C) the percent apoptotic cells and (D) the total cell number. n=3 independent experiments for a total of 9 coverslips per data point. At least 250 cells per coverslip were quantified in each treatment. Data represent mean $\pm$ SEM., n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 5. Deletion of the neural specific JNK3 isoform attenuates Cd-induced apoptosis and cell loss of SVZ-aNPCs

SVZ-aNPCs isolated from WT and JNK3 KO littermate mice were treated for 96h with several concentrations of Cd²⁺ as indicated. Quantification of (A) the percent apoptotic cells and (B) the total cell number. n=3 independent experiments for a total of 9 coverslips per data point. At least 250 cells per coverslip were quantified in each treatment. Data represent mean ± SEM., n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 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. n=3 independent experiments for a total of 9 coverslips per data point. At least 250 cells per coverslip were quantified in each treatment. Data represent mean $\pm$ SEM, n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001.