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RESEARCH ARTICLES

Gestational marginal zinc deficiency impaired fetal neural progenitor cell proliferation by disrupting the ERK1/2 signaling pathway

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Abstract

This study investigated if a marginal zinc deficiency during gestation in rats could affect fetal neural progenitor cell (NPC) proliferation through a down-regulation of the extracellular signal-regulated kinase (ERK1/2) signaling pathway. Rats were fed a marginally zinc-deficient or adequate diet from the beginning of gestation until embryonic day (E)19. The proportion of proliferating cells in the E19 fetal ventricular zone was decreased by marginal zinc deficiency. Immunostaining for phosphorylated ERK1/2 in the cerebral cortex was decreased in the marginal zinc fetuses, and this effect was strongest in the ventricular zone. Furthermore, phosphorylation of the upstream mitogen-activated ERK kinases (MEK1/2) was not affected, suggesting that marginal zinc deficiency could have increased ERK-directed phosphatase activity. Similar findings were observed in cultured rat embryonic cortical neurons and in IMR-32 neuroblastoma cells, in which zinc-deficiency decreased ERK1/2 phosphorylation without affecting MEK1/2 phosphorylation. Indeed, zinc deficiency increased the activity of the ERK-directed phosphatase protein phosphatase 2A (PP2A) in the fetal cortex and IMR-32 cells. Inhibition of PP2A with okadaic acid prevented the decrease in ERK phosphorylation and proliferation of zinc-deficient IMR-32 cells. Together these results demonstrated that decreased zinc availability reduces ERK1/2 signaling and decreased NPC proliferation as a consequence of PP2A activation. Disruption of fetal neurogenesis could underlie irreversible neurobehavioral impairments observed after even marginal zinc nutrition during a critical period of early brain development.
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Keywords; Trace element; Mineral; Nutrition; Fetal brain development; Cell signaling

1. Introduction

Zinc deficiency represents a major risk for pregnant women given that it can compromise fetal development leading to irreversible consequences for neuronal physiology and cognitive function. It was recently estimated that 15%–20% of the global population is at risk of inadequate dietary zinc intake [1]. Global incidence of stunting is associated with dietary zinc intake [1] and zinc supplementation during pregnancy can prevent congenital malformations including neural tube defects resulting from inadequate intake [2,3], or impaired absorption in the case of acrodermititis enteropathica [4]. Subtler effects of prenatal zinc supplementation include improved function of the autonomic nervous system later in life [5]. Severe zinc deficiency is clearly a major public health concern in populations that rely heavily on grain-based diets with a high phytate/zinc ratio. However, the prevalence of marginal zinc deficiency, leading to less severe effects including impaired neuronal and cognitive development, is likely to be much greater [6].

Zinc deficiency during critical periods of brain development can disrupt neurogenesis. Severe dietary zinc deficiency from the beginning of pregnancy leads to gross brain malformations associated with decreased proliferation in the ventricular zone of the fetal rat brain at embryonic day (E)12 [7]. Similarly, severe zinc deficiency during postnatal brain development decreases the number of neurons in the internal granule cell layer of the cerebellum [8] and decreases the number of neural progenitor cells (NPCs) expressing the proliferative cell marker Ki67 in the subgranule zone and granule cell layer of the dentate gyrus [9]. These findings suggest that zinc deficiency can impair neurogenesis by decreasing NPC proliferation. However, it remains unclear if marginal zinc deficiency during pregnancy can affect neurogenesis.

Zinc plays a key role in the regulation of the extracellular signal-regulated kinase 1/2 (ERK1/2; EC 2.7.11.24) mitogen-activated protein kinase cascade in neuronal cells (reviewed in Ref. [10]). ERK1/2 signaling is a critical cell signal mediating the response to neurotrophic stimuli, such as nerve growth factor and brain-derived neurotrophic factor (BDNF), to support NPC proliferation, neuronal differentiation and survival. Mutations that disrupt ERK1/2 signaling

Abbreviations: ERK1/2, extracellular signal-regulated kinases 1/2; NPC, neural progenitor cell; E, embryonic day; MEK1/2, mitogen-activated ERK1/2 kinases 1/2; PP2A, protein phosphatase 2A; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; RCN, rat cortical neural progenitor cells; CREB, cyclic AMP response element-binding protein; IPCs, intermediate progenitor cells.

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have been associated with abnormal brain development leading to cognitive dysfunction [11]. Impaired NPC proliferation may contribute to these effects because incorporation of bromodeoxyuridine (BrdU) is decreased in the brains of mice with a conditional deletion of ERK2 in NPCs [12]. In addition, BrdU incorporation is increased in the brains of mice with a mutation that activates ERK1/2 signaling [13].

Disruption of ERK1/2 signaling may contribute to impairments in neurogenesis resulting from zinc deficiency. Marginal zinc deficiency decreases the levels of phosphorylated ERK1/2 in the fetal rat brain at E19 [14]. In cell culture, zinc deficiency decreases ERK1/2 phosphorylation in human IMR-32 neuroblastoma cells and in rat embryonic cortical neuron (RCN) cultures [15,16]. Moreover, this phenomenon is associated with an arrest of the cell cycle in G0/G1 phase in IMR-32 cells [15].

This study investigated the impact of maternal marginal zinc nutrition in rats on fetal neurogenesis. Human and rat embryonic cell culture models were also used to investigate the mechanisms underlying impaired NPC proliferation and the associated disruption of the ERK1/2 signaling pathway resulting from zinc deficiency.

2. Materials and methods

2.1. Materials

IMR-32 cells were from the American Type Culture Collection (Rockville, MA, USA). Cell culture media and reagents were from Thermo Scientific (Logan, UT, USA). Primary antibodies for total ERK1/2, phosphorylated ERK1/2 (T202/Y204), total mitogen-activated ERK1/2 kinase 1/2 (MEK1/2; EC 2.7.12.2) and phosphorylated MEK1/2 (S217/S221) were from Cell Signaling Technologies (Danvers, MA, USA). Primary antibodies for Ki67 were from BD Biosciences (San Jose, CA, USA). Primary antibodies for protein phosphatase 2A (PP2A; EC 3.1.3.16) C subunit and activity assay kit were from Millipore (Billerica, MA, USA). PVDF membranes were from BIO-RAD (Hercules, CA, USA). The ECL plus immunoblotting system was from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). All other reagents were from the highest quality available and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Animals and animal care

All procedures were in agreement with standards for the care of laboratory animals as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Animal Resource Services of the University of California at Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Experimental protocols were approved before implementation by the University of California at Davis Animal Use and Care Administrative Advisory Committee and were administered through the Office of the Campus Veterinarian.

Adult Sprague–Dawley rats (Charles River, Wilmington, MA, USA) (200–225 g) were housed individually in stainless steel cages in a temperature (22–23°C) and photoperiod (12-h light/dark) controlled room. An egg-white protein-based diet with adequate zinc (25 μg zinc/g) was the standard control diet [17]. Animals were fed the control diet for 1 week before breeding. Males and females were caged together overnight and the following morning, E0, after the presence of a sperm plug confirmed a successful breeding; female rats (five animals/group) were divided into two groups and fed *ad libitum* a control diet (25 μg zinc/g diet) or a diet containing a marginal concentration of zinc (10 μg zinc/g diet; MZD). Food intake was recorded daily, and body weight was measured at 3-day intervals. On E19, the dams were anesthetized with isoflurane (2 mg/kg body weight), and laparotomies were performed. The gravid uterus was removed, and fetuses were weighed. Brains were weighed, and immediately fixed in 4% (wt/vol) paraformaldehyde for immunohistochemistry or microdissected on ice, frozen in liquid nitrogen and stored at −80°C. The concentration of zinc in the diets and in maternal plasma was measured by inductively coupled plasma atomic emission spectroscopy, as described by Clegg et al. [18].

2.3. Cell cultures and incubation

Zinc-deficient fetal bovine serum (FBS) was prepared by chelation with diethylenetriamine pentaacetic acid, as previously described [19,20]. The chelated FBS was subsequently diluted with Dulbecco's modified Eagle's medium (DMEM) high glucose to a final concentration of 3 mg protein/ml to match the protein concentration of the control nonchelated medium [10% (vol/vol) FBS]. Aliquots of the zinc-deficient medium were supplemented with $ZnSO_4$ to a final nominal zinc concentration of 1.5, 5, 15 or 50 μM.

RCNs were isolated from the fetal rat brain at E19 as previously described [15]. Isolated cells were suspended in neurobasal culture medium supplemented with 2% (vol/vol) serum-free additive B27. Cell cultures were incubated at 37°C in an atmosphere of 95% (vol/vol) air, 5% (vol/vol) CO_2 with 90%–95% (vol/vol) humidity. After 48 h in culture, medium was replaced by chelated medium containing 1.5 (zinc deficient; ZD) or 15 μM zinc (control; C).

IMR-32 cells were cultured at 37°C in high-glucose DMEM supplemented with 10% (vol/vol) FBS and 50 U/ml penicillin, 50 μg/ml streptomycin. Cells were grown in medium containing 10% (vol/vol) nonchelated FBS until 70% confluency. Medium was subsequently changed to chelated medium containing 5 (ZD) or 50 μM zinc (C). For inhibition of PP2A, 5 nM okadaic acid or vehicle was added at the time that the medium was changed to chelated medium.

2.4. Immunoblots

Extraction of total cellular protein from fetal brains was done as previously described [14]. For extraction of total protein from cell cultures, cells in 30-mm dishes were washed in cold phosphate-buffered saline (PBS) and scraped in 50 μl lysis buffer, and total cell extracts were prepared as previously described [15].

Protein concentration was measured by the Bradford assay [21], and aliquots containing 25–50 μg of protein were separated by reducing 10% (wt/vol) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Colored molecular weight standards were run simultaneously. Membranes were blocked for 1 h in 5% (wt/vol) bovine serum albumin (BSA) and then incubated overnight in blocking buffer containing a 1:1000 dilution of the corresponding primary antibody. After incubation with a horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:10,000 dilution) for 90 min at room temperature, the conjugates were visualized with chemifluorescent detection in a Storm 840 Phosphoimager from Amersham Pharmacia Biotech. Image Quant software from Molecular Dynamics (Sunnyvale, CA, USA) was used to quantify band intensity.

2.5. Immunohistochemistry

Fetal rat brains fixed in 4% (wt/vol) paraformaldehyde were dehydrated through an ethanol gradient, embedded in paraffin, cut into 10-μm sections and mounted onto microscope slides. Slides were deparaffinized in xylene and rehydrated through a series of alcohol washes. For antigen retrieval, slides were incubated in citrate buffer [10 mM citric acid, 0.05% (vol/vol) Tween 20, pH 6.0] and heated in a microwave oven for 15 min. To quench endogenous peroxidase activity, slides were incubated with 0.6% H_2O_2 in PBS for 30 min. To increase permeability of the tissue for subsequent steps, slides were treated with 0.005 % (wt/vol) trypsin in PBS at 37°C for 5 min. Sections were blocked with subsequent treatments of avidin, biotin (Vector Laboratories, Burlingame, CA, USA) and 5% (wt/vol) BSA, 0.05% (vol/vol) Tween-20 in PBS for 1 h. Primary antibodies for phosphorylated ERK1/2 were diluted (1:500 vol/vol) with 5% (wt/vol) BSA blocking buffer and applied to the sections overnight at 4°C. Negative controls were incubated with blocking buffer without a primary antibody. Sections were incubated for 2 h at room temperature with a biotinylated secondary antibody diluted in blocking buffer (Vector Laboratories). To visualize the antigen–antibody complex, the secondary antibodies were labeled with avidin–horseradish peroxidase (Vector Laboratories) for 2 h at room temperature followed by exposure to 3,3-diaminobenzene (DAB; Vector Laboratories) for 3 min. Slides were washed and dehydrated, and coverslips were attached with Permount (Fischer Sci, Hampton, NH, USA). Two sections per animal (one from each litter, $n=5$/group) were imaged on an Olympus BX51 microscope (Tokyo, Japan).

2.6. Immunofluorescence

Fetal rat brains were dissected out and fixed in 4% (wt/vol) solution of paraformaldehyde in PBS overnight. Cryoprotection was then performed in 15% (wt/vol) sucrose in PBS for 24 h and 30% (wt/vol) sucrose for 3 days, after which brains were submerged in Cryoplast (Biopack, Buenos Aires, Argentina), frozen, cut into 18-μm sections on a Leica CM 1850 cryotome (Leica Microsystems, Nussloch, Germany) and mounted on positively charged microscope slides. Sections were blocked in 5% (wt/vol) BSA, 0.05% (wt/vol) Tween-20 in PBS for 1 h and then incubated overnight at 4°C with mouse anti-Ki67 (1:100) monoclonal primary antibody. Sections were washed in PBS and incubated for 2 h at room temperature with Cy3-conjugated donkey anti-mouse IgG (1:200) (Jackson Immuno Research Co. Laboratories, West Grove, PA, USA). After immunostaining, cell nuclei were stained with Hoechst 33342 [22] and sections were imaged using an Olympus FV 300 laser scanning confocal microscope or Leica DMI 3000B epifluorescent microscope. Image pro software (Rockville, MD, USA) was used to merge and analyze the resulting micrographs. The proportion of Ki67+ cells was counted in one field per slice located within the dorsomedial ventricular zone on coronal sections. Four slices per animal and four animals from each group were analyzed.

2.7. Immunocytochemistry

RCN and IMR-32 cells were cultured on coverslips and incubated for 6 h in control or zinc-deficient medium. After washing, cells were fixed with 4% (wt/vol) paraformaldehyde, containing 0.12 M sucrose, in PBS for 1 h at room temperature. Fixed cells were permeabilized by incubation with 0.01% (vol/vol) Triton X-100 in PBS for 5 min at room temperature. Samples were then blocked with 1% (wt/vol) BSA in PBS for 2 h and

incubated overnight at 4°C with primary antibodies for phosphorylated ERK1/2 diluted (1:100 vol/vol) in 1% (wt/vol) BSA. Coverslips were rinsed three times in PBS then incubated for 2 h at room temperature with Cy2-conjugated donkey anti-mouse IgG (1:200) (Jackson Immuno Research Co. Laboratories). After immunostaining, cell nuclei were stained with Hoechst 33342 [22] and coverslips were examined by laser confocal microscopy using an Olympus FV 300 microscope.

2.8. PP2A activity assay

E19 brain cortex or cells were homogenized on ice in 20 mM Imidazole–HCl, 1 mM PMSF and 1× protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). PP2A was immunoprecipitated from lysates using a monoclonal antibody against the C subunit of PP2A. PP2A activity in the immunoprecipitates was measured using a synthetic phosphopeptide substrate and a malachite green detection system for released phosphate (Millipore, Bedford, MA, USA). PP2A activity was expressed as pmoles of phosphate released in 30 min and normalized to total PP2A C subunit recovered within individual immunoprecipitates measured by quantitative immunoblot.

2.9. Live dead cell staining

After treatment with chelated medium for 24 h, 0.015 mg/ml fluorescein diacetate and 2 mg/ml propidium iodide were added to the medium. Cells were incubated at room temperature for 3 min, washed with PBS and immediately imaged with a Leica DMI 3000B epifluorescent microscope. Image pro software (Rockville, MD, USA) was used to merge and analyze the resulting micrographs.

2.10. DNA fragmentation

Cytoplasmic mono- and oligonucleosomes were measured with the Cell Death Detection ELISA (Roche Applied Science). Absorbance measurements were then normalized to protein concentration, and results were represented as fold expression relative to the control (50 μM zinc).

2.11. Cell proliferation

The proportion of IMR-32 cells in S-phase was measured by BrdU (BD Bioscience) labeling. Briefly, cells were incubated in control or zinc-deficient medium with or without 5 nM OA for 24 h. Medium containing unchelated serum was used as an internal control to normalize the data from each experiment. Following the incubation, cells were pulse labeled with 10 μM BrdU 30 min prior to harvesting by trypsinization. Following washing twice with 1% (wt/vol) BSA in PBS, cells were fixed in ice-cold 70% (wt/vol) ethanol for 30 min. Following treatment with 2N HCl for 30 min and posterior neutralization with borate buffer, samples were incubated with a monoclonal anti-BrdU antibody conjugated with FITC (BD Biosciences) and analyzed by flow cytometry.

2.12. Statistical analysis

Data were analyzed using GraphPad Prism software (La Jolla, CA, USA). The Student's t test was used to compare data from two groups. One-way analysis of variance (ANOVA) test was used to compare data from three groups, and Dunnett's post hoc test was used to compare each group to the control. For all experiments, α was set at .05.

3. Results

3.1. Gestational marginal zinc deficiency decreased NPC proliferation and impaired ERK1/2 phosphorylation in the ventricular zone of the fetal rat brain during a critical period for cortical neurogenesis

At E19 maternal plasma zinc was 35% lower in the MZD group than in controls (Table 1), which reflects a state of marginal zinc deficiency in rats [14]. As previously described [14], marginal zinc nutrition throughout gestation did not affect dam food intake and body weight or fetal body and brain weight (Table 1). Phosphorylated and total ERK1/2 were measured with quantitative immunoblots of total cell lysates from the E19 cerebral cortex. Maternal marginal zinc deficiency caused approximately a 40% decrease in the ratio of phosphorylated ERK1/2 relative to total ERK1/2 (P<.05, Fig. 1A). Decreased ERK1/2 phosphorylation was confirmed by immunohistochemistry, with the greatest difference found at the ventricular surface where radial glia are in M-phase (Fig. 1B, C). Considering the role of ERK1/2 signaling in the stimulation of NPC proliferation, this pattern of decreased ERK1/2 signaling at the ventricular surface suggests that NPC proliferation may be affected by marginal zinc deficiency. To investigate this hypothesis, immunofluorescent staining was used to measure the number of proliferative cells in the ventricular zone of the frontal cortex. Marginal zinc deficiency resulted in a 28% decrease (P<.01) in the proportion of cells expressing the mitotic marker Ki67 in the ventricular zone (Fig. 2). A decreased number of Ki67 expressing cells could result from decreased proliferation or increased cell death. However, marginal zinc deficiency did not affect the levels of the apoptotic marker cleaved caspase-3 in the frontal cortex at E19 (data not shown). Together these results suggest the decrease in ERK1/2 signaling resulting from marginal zinc deficiency during gestation leads to decreased proliferation of NPCs in the ventricular zone of the frontal cortex.

Table 1
Outcome parameters in dams fed control or MZD diets

Parameter	Control	MZD
Food intake (g)	438±24	412±23
Maternal weight gain (g)	152±9	145±9
Fetal body weight (mg)	2419±80	2364±56
Fetal brain weight (mg)	92±6	90±3
Fetal brain: body weight	0.04±0.04	0.04±0.02
Maternal plasma zinc (μM)	14.6±0.9	9.5±0.7 *

Dams were fed a control (25 μg zinc/g) or MZD (10 μg zinc/g) diet from E0 until E19. Values are shown as means ± standard error, n=5.
* Significantly different from control (P<.05; unpaired t test).

3.2. Zinc deficiency increased ERK1/2 directed phosphatase activity

To investigate the mechanism underlying the decrease in ERK1/2 phosphorylation associated with zinc deficiency; phosphorylation of the upstream mitogen-activated ERK1/2 kinases (MEK1/2) was measured in the cerebral cortex at E19. MEK phosphorylation was not affected by marginal zinc deficiency (Fig. 3A). Considering that MEK1/2 are the only kinases known to phosphorylate ERK1/2, these data suggest dephosphorylation is most likely responsible for the decreased ERK1/2 phosphorylation. Previous work found that zinc regulates the major ERK-directed phosphatase PP2A [23]. To investigate the potential role of PP2A in the decreased ERK phosphorylation resulting from marginal zinc deficiency, PP2A activity was measured in cortical homogenates from the fetal rat brain. Marginal zinc deficiency led to 48% higher PP2A activity in the E19 fetal cortex compared to controls (Fig. 4A). To further investigate the regulation of ERK1/2 signaling by zinc, RCN cultures obtained from the fetal rat brain at E19 were exposed to chelated medium containing 1.5 (ZD) or 15 μM zinc (C) for 6 h. Immunostaining for phosphorylated ERK1/2 was observed in the cytoplasm and nucleus of RCN and IMR-32 cells (Fig. 3B). Quantification of ERK1/2 phosphorylation by immunoblot of cell lysates from RCN revealed a 45% decrease resulting from zinc deficiency after 6 h incubation (P<.01, Fig. 3C). Similar results were observed in IMR-32 neuroblastoma cells exposed to chelated medium containing 5 (ZD) or 50 μM zinc (C) for 6 h (P<.05, Fig. 3B, C). Lending further support to the hypothesis that ERK1/2-directed phosphatase activity is increased by zinc deficiency; MEK phosphorylation was not affected in RCN or IMR-32 cells cultured for 6 h in zinc-deficient medium (Fig. 3C), and PP2A activity was increased in IMR-32 cells after exposure to zinc-deficient medium for 6 h (P<.05, Fig. 4A).

3.3. Inhibition of PP2A rescued ERK1/2 phosphorylation and proliferation of zinc-deficient IMR-32 cells

IMR-32 cells were incubated with an inhibitor of PP2A (okadaic acid) to test the hypothesis that increased PP2A activity associated with zinc deficiency contributes to decreased ERK1/2 signaling and

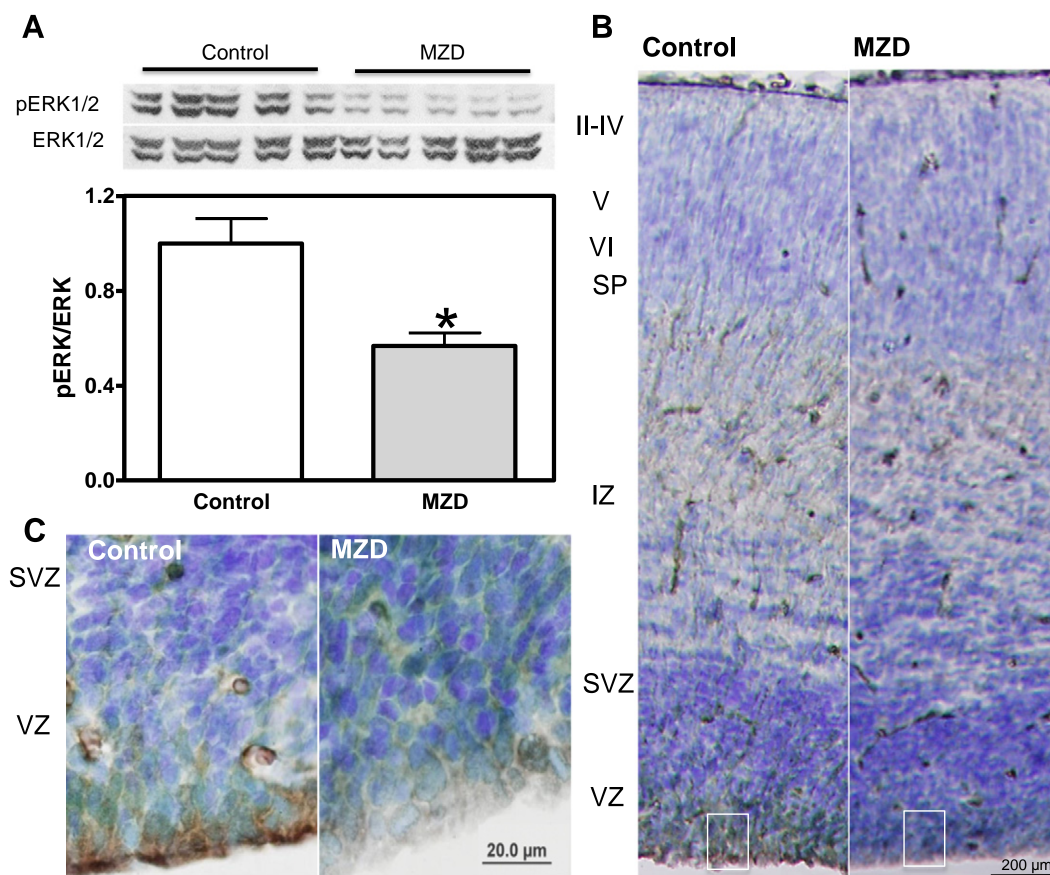

Fig. 1. Marginal zinc deficiency (MZD) decreased ERK1/2 phosphorylation in the developing rat brain. (A) Total and phosphorylated ERK1/2 were measured by immunoblot in the rat cortex at E19. Data represented as fold expression relative to control are shown as means±standard error (*t-test $P<.05$, $n=5$). (B) Phosphorylated ERK1/2 (brown DAB stain) was measured at E19 by immunohistochemistry. Bright-field micrographs show cortical plate layers II–VI, subplate (SP), intermediate zone (IZ), subventricular zone (SVZ) and ventricular zone (VZ) at 100-fold magnification (B) and the ventricular zone at 1000-fold magnification (C).

cell proliferation. Inhibition of PP2A prevented the decrease in ERK1/2 phosphorylation measured by immunoblot of cell lysates from IMR-32 cells after 6 h incubation in zinc-deficient medium ($P<.01$, Fig. 4B). Furthermore, inhibition of PP2A prevented the decrease in cell viability resulting from 24 h exposure to zinc-deficient medium (Fig. 4C). To test if this was related to an increase in cell survival or cell proliferation, live-dead cell staining was performed after 24 h exposure to zinc-deficient medium supplemented with okadaic acid (Fig. 4E). Zinc deficiency resulted in a 24% decrease in the number of viable cells stained by fluorescein diacetate ($P<.01$), and this was prevented by inhibition of PP2A with okadaic acid. Exposure to zinc-deficient medium for 24 h resulted in a 16% decrease in the proportion of S-phase IMR-32 cells labeled by BrdU ($P<.05$), and this was prevented by inhibition of PP2A with okadaic acid (Fig. 4D). However, exposure to zinc-deficient medium for 24 h with or without okadaic resulted in approximately a twofold increase in the proportion of dead cells positive for propidium iodide staining ($P<.01$, Fig. 4E) and a twofold increase in cytosolic mono and oligonucleosomes measured by ELISA ($P<.05$, Fig. 4F). Thus, the protective effect of okadaic acid was related to increased proliferation rather than survival. Together these results supported a model where zinc deficiency disrupts NPC proliferation by increasing the dephosphorylation of ERK1/2 by PP2A.

4. Discussion

This study found that marginal zinc nutrition throughout gestation in rats decreases proliferation of cortical NPCs at E19. This is associated with a disruption of the ERK1/2 signaling pathway, which is central to the regulation of cell proliferation. A decreased ERK1/2 phosphorylation as a consequence of zinc deficiency is not due to impaired upstream events, but to the activation of the ERK-directed phosphatase PP2A. In a previous study, we observed that decreased ERK1/2 phosphorylation resulting from zinc deficiency in IMR-32 neuroblastoma is associated with cell cycle arrest and apoptosis [15]. Likewise, apoptotic cell death is associated with decreased ERK1/2 phosphorylation following depletion of intracellular zinc by 24-h exposure to the cell permeable zinc chelator *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine in cultured hippocampal neurons [24]. Here we report that the inhibition of PP2A in zinc-deficient neuroblastoma cells: (i) rescues ERK1/2 phosphorylation and cell proliferation, but (ii) does not prevent the increased DNA fragmentation and membrane permeability. Thus, a decreased ERK1/2 signaling can underlie the decrease in cortical NPC proliferation associated with developmental zinc deficiency.

Several mechanisms could account for the regulation of ERK1/2 signaling by zinc. For example, zinc can activate receptors that stimulate ERK1/2 and inhibit phosphatases that inactivate ERK1/2 [10]. Most of this research has focused on high levels of zinc found in the hippocampal mossy fiber synapses. Zinc directly binds to G-protein coupled receptor-39 on CA3 pyramidal neurons in the mouse hippocampus leading to activation of phospholipase C, calcium influx, and stimulation of the Ras-ERK1/2 cascade [25]. Zinc also stimulates activation of the BDNF receptor tyrosine kinase, TrkB, through a BDNF independent mechanism [26]. While both of these

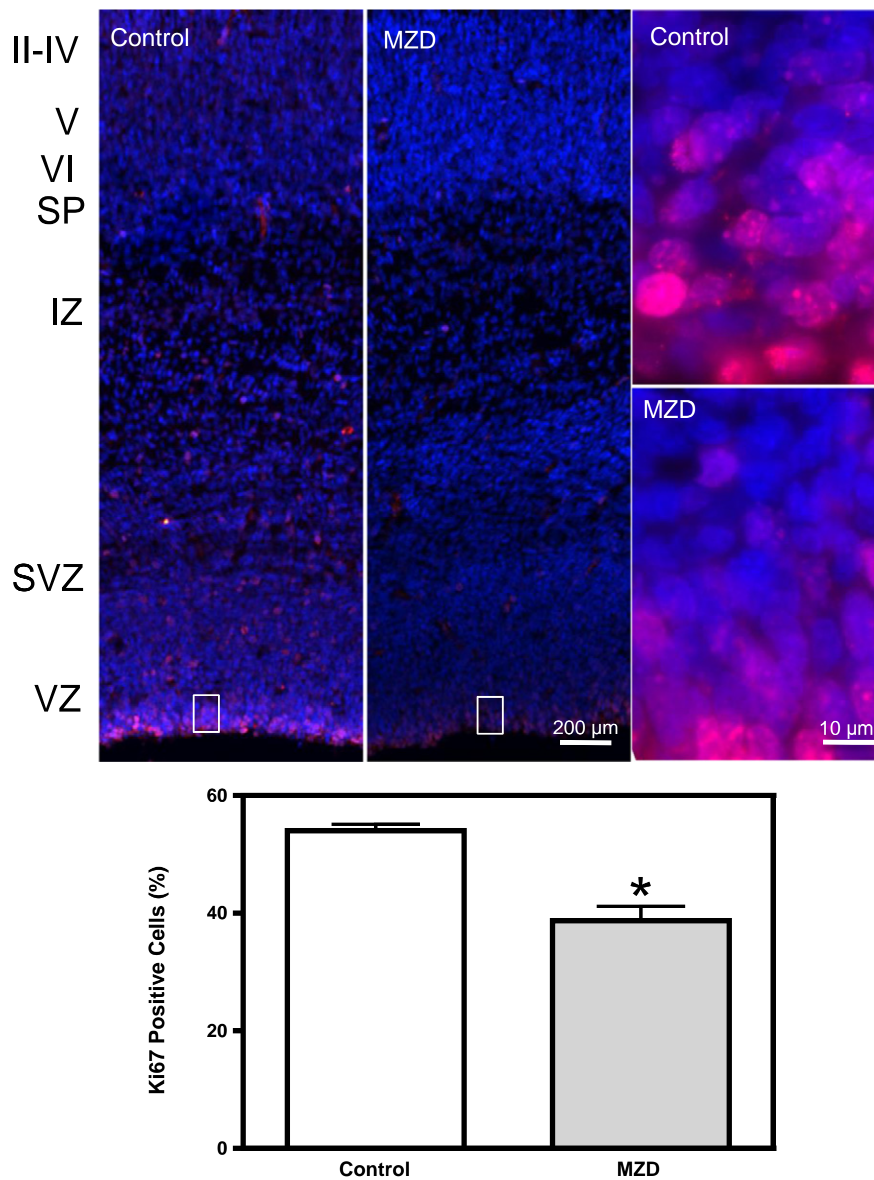

Fig. 2. Marginal zinc deficiency (MZD) decreases immunostaining for Ki67 in the ventricular zone of the fetal rat brain at E19. Immunofluorescence staining for Ki67 is shown in red and Hoescht nuclear counterstain in blue. Fluorescent micrographs show cortical plate layers II–VI, subplate (SP), intermediate zone (IZ), subventricular zone (SVZ) and ventricular zone (VZ) at 100-fold and 1000-fold magnification. Ki67-positive cells were counted as described in Materials and Methods, and results are shown as means±standard error (**t* test, $P<.01$, $n=4$).

mechanisms involve activation of receptors leading to increased MEK1/2 phosphorylation, Znt3 knockout mice show increased ERK1/2 directed phosphatase activity leading to decreased ERK1/2 phosphorylation without affecting MEK1/2 [27]. Our observations suggest that marginal zinc deficiency increases ERK directed phosphatase activity in the fetal rat brain because the decrease in ERK1/2 phosphorylation resulting from marginal zinc deficiency is not associated with a decrease in MEK1/2 phosphorylation.

Increased PP2A activity provides a mechanism for decreased ERK1/2 phosphorylation resulting from zinc deficiency. PP2A is an important regulator of ERK1/2 [28]. In this regard, ERK1/2 phosphorylation is increased in mice expressing a dominant negative PP2A gene under a neuronal promoter [29], and toxic levels of zinc decrease PP2A activity leading to ERK1/2-dependent apoptosis in cultured neuronal cells [23]. Although iron or magnesium is required for the catalytic activity of PP2A, zinc is the predominant metal found in PP2A isolated from the pig brain [30]. This suggests that zinc can inhibit PP2A by competing with iron and magnesium. *In vitro*, 1 μM zinc leads to 87% inhibition of PP2A activity [31], and zinc released during synaptic activity of glutamatergic neurons inhibits PP2A in cultured slices from the rat hippocampus [32]. PP2A refers to an important family of heterotrimeric serine/threonine phosphatases that represents a major contribution of the serine/threonine phosphatase activity in eukaryotic cells participating in a variety of signaling pathways involved in diverse cellular processes such as control of the cell cycle, apoptosis and differentiation. PP2A is enriched in the distal axons of hippocampal neurons where it promotes axon growth by regulating cytoskeletal dynamics [33]. PP2A is necessary for neurite growth in cultured neuro-2a neuroblastoma cells and primary hippocampal neurons [34]. PP2A is expressed in NPCs of the ventricular zone and RNA interference to knockdown PP2A increased NPC proliferation and decreased the rate of neuronal differentiation [35]. The current study found that zinc deficiency was associated with increased PP2A activity in cultured IMR-32 cells. Supporting a model where zinc regulates

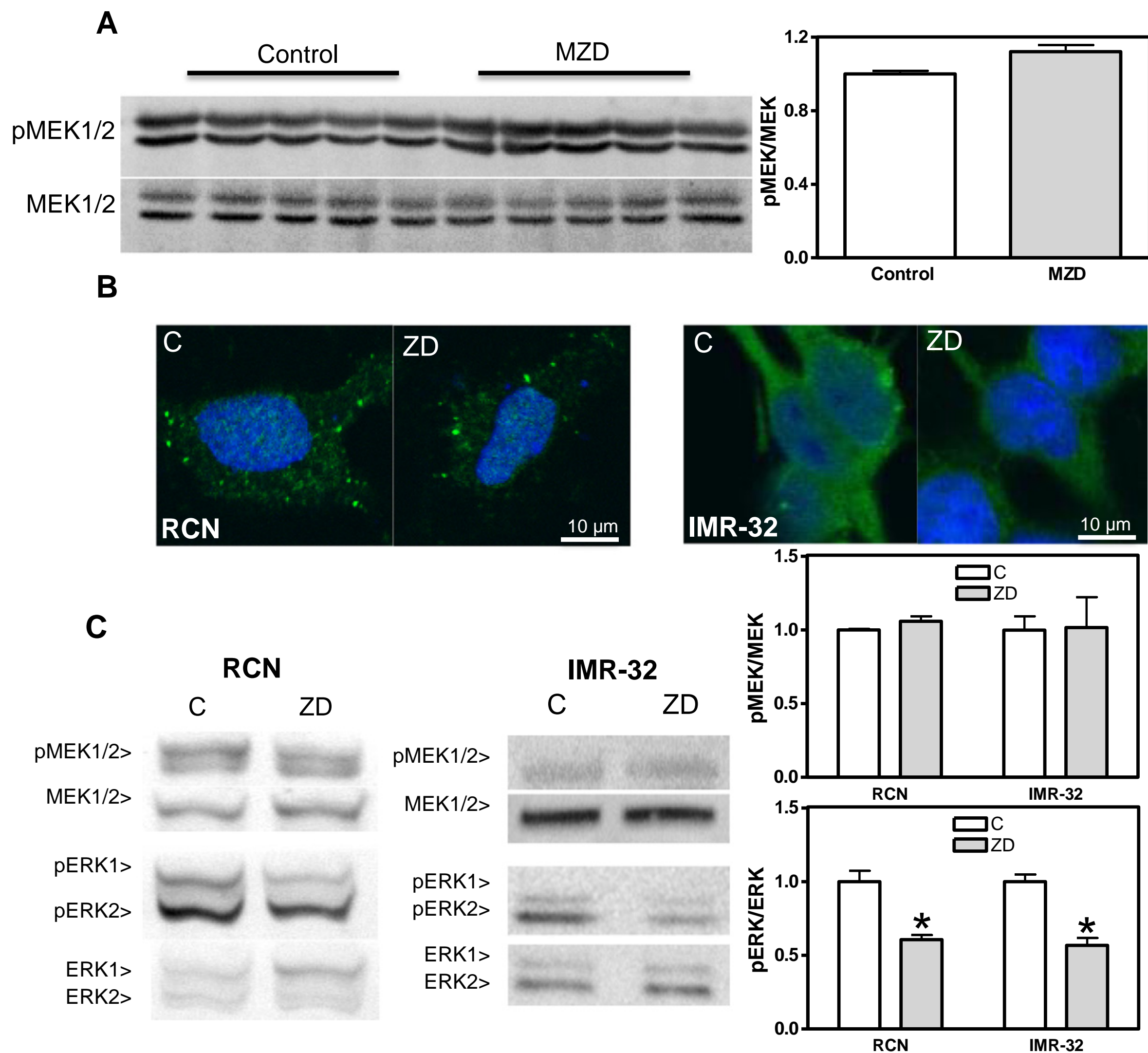

Fig. 3. Decreased ERK1/2 phosphorylation resulting from zinc deficiency is not associated with impaired phosphorylation of MEK1/2. (A) Total and phosphorylated MEK1/2 were measured by immunoblot in the fetal rat cortex at E19. Data represented as fold expression relative to control are shown as means±standard error (**t* test, *P*<.05, *n*=5). (B) Phosphorylated ERK1/2 shown in green was measured in RCNs and IMR-32 neuroblastoma by immunocytochemistry after 6-h incubation in control or zinc-deficient medium. (C) Total and phosphorylated MEK and ERK1/2 were measured by immunoblot in RCN and IMR-32 neuroblastoma after 6-h incubation in control or zinc-deficient medium. All data represent fold expression relative to control shown as means±standard error of at least three independent experiments (**t* test, *P*<.05). C: control, MZD: marginal zinc deficiency, ZD: zinc deficiency.

dephosphorylation of ERK1/2 through inhibition of PP2A to regulate NPC proliferation, inhibition of PP2A with okadaic acid prevents the decrease in ERK1/2 phosphorylation and proliferation of IMR-32 cells resulting from incubation in zinc-deficient medium.

Reduced ERK1/2 signaling and cell proliferation in marginal zinc deficiency could serve as a homeostatic mechanism; however, inhibition of mitosis during a critical period of neurogenesis could lead to structural defects and contribute to neurodevelopmental diseases. The rate of cell division may need to be regulated in proportion to nutrient availability. Zinc is required for structural and enzymatic functions in a wide variety of proteins including many key enzymes involved in DNA replication and repair. Cellular zinc deficiency increases single-strand DNA breaks and leads to activation of DNA repair enzymes like p53 [15,36]. Zinc is a key regulator of cellular redox homeostasis [37], and zinc deficiency leads to accumulation of reactive oxygen species that damage DNA and oxidize protein thiols including those in tubulin resulting in disruption of the cytoskeleton [38]. Reduced proliferation and increased apoptosis may be important mechanisms to conserve zinc and prevent the formation of neurons with damaged DNA and dysfunctional proteins.

In addition to decreasing NPC proliferation, disruptions in ERK1/2 signaling resulting from zinc deficiency may also affect neuronal differentiation, neurite growth and synaptogenesis. Dietary zinc deficiency decreased BDNF levels in the developing rat brain [39], and a constitutively active form of the cyclic AMP response element-binding protein (CREB) rescued the decrease in neurite growth resulting from knockdown of the neuronal zinc importer ZIP12 [40]. While ERK1/2 is activated by neurotrophic stimuli, ERK1/2 can also regulate the transcription of BDNF by activating CREB. In this way, ERK1/2–CREB–BDNF signaling stimulates a positive feedback loop to promote neuronal growth. Considering the decrease in ERK1/2 phosphorylation resulting from zinc deficiency is not associated with decreased MEK phosphorylation, it is likely that disruption of ERK1/2-CREB signaling contributes to decreased BDNF and impaired neuronal growth resulting from zinc deficiency.

Prolonged zinc deficiency during development can lead to structural malformations and behavioral consequences including lethargy, memory impairment and altered emotional behavior [10]. Some of these effects may be irreversible because marginal zinc deficiency during gestation and lactation leads to impaired working

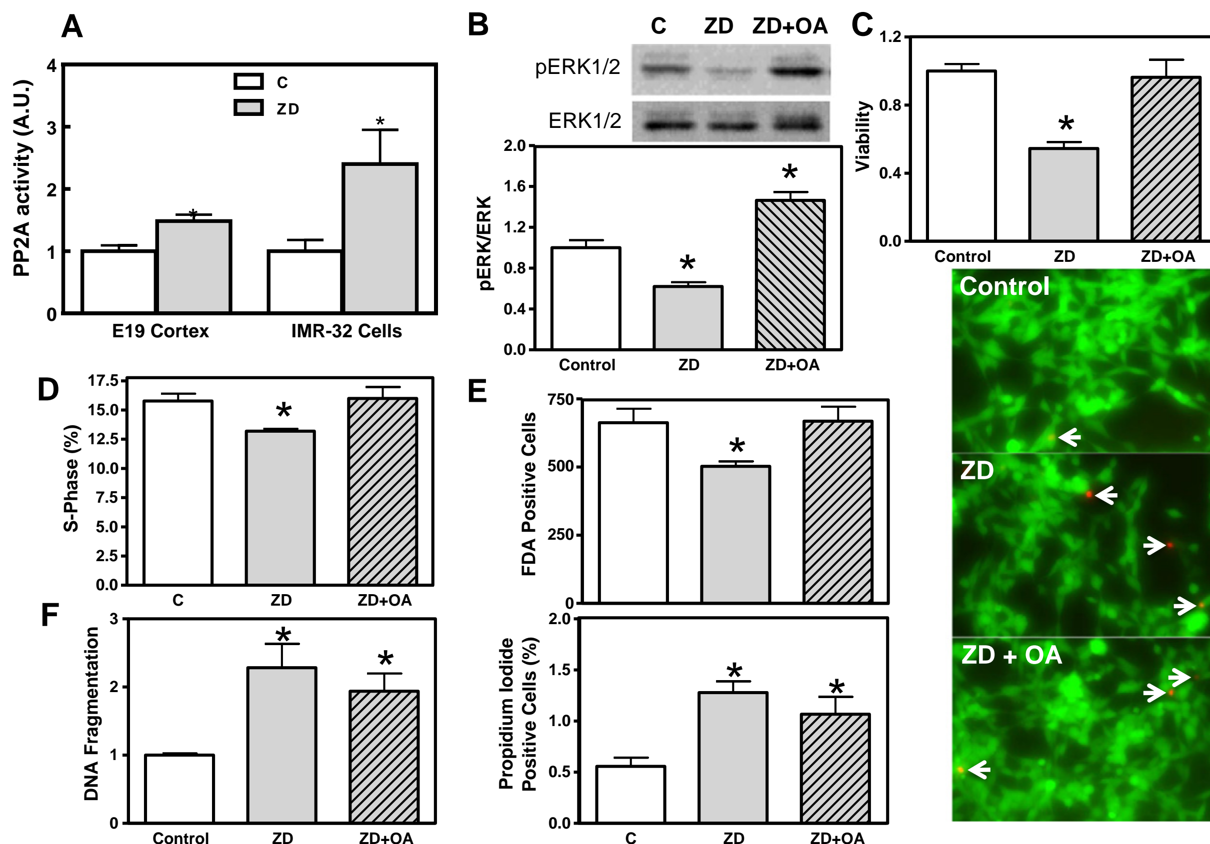

Fig. 4. Zinc deficiency increases PP2A activity and inhibition of PP2A with okadaic acid rescues ERK1/2 phosphorylation and proliferation but does not prevent apoptosis. IMR-32 cells were incubated in control (C) or zinc-deficient medium (ZD) with vehicle or 5 nM okadaic acid (ZD+OA). (A) PP2A activity was measured *in vitro* after immunoprecipitation in E19 cortex or IMR-32 cell lysates with a monoclonal antibody for the PP2A C subunit. Activity was normalized relative to PP2A protein levels measured by immunoblot (**t* test, *P*<.05). (B) After 6-h incubation in C, ZD, and ZD+OA media, total and phosphorylated ERK1/2 were measured by immunoblot. After 24-h incubation in C, ZD and ZD+OA media: (C) viability was evaluated by measuring cellular ATP levels, (D) the proportion of cells in S-phase after labeling with BrdU was measured by flow cytometry, (E) live-dead cell staining was performed with fluorescein diacetate (FDA, green) and propidium iodide (red) (images shown in right panels), and (F) DNA fragmentation (mono- and oligonucleosomes) was measured in cytosolic factions by ELISA for mono- and oligonucleosomes. Bar graphs represent means±standard error of at least three independent experiments (*one-way ANOVA with Dunnett's post hoc test; *P*<.05 relative to control).

memory performance on the radial arm maze after dietary zinc repletion [41]. Similarly, gestational zinc deficiency with repletion at birth leads to an impairment in social behavior consistent with models of autism in mice [42]. Although neurogenesis is not limited to the developmental period and continues in the hippocampus and the subventricular zone throughout life, most of the neurons in the adult brain are born during the embryonic stage. During early fetal brain development in the rat, for example, on E14, most NPCs in the cerebral cortex are going through a symmetrical self-duplication process to increase the size of the ventricular zone. Some of these NPCs differentiate into intermediate progenitor cells (IPCs) that populate the subventricular zone. Both NPCs and IPCs differentiate into neurons [43]. The first neurons formed are the Cajal-Reitzus cells, populating the most superficial marginal zone/layer I of the developing cortex, and subplate neurons that populate the deepest layers. Subsequently, newborn neurons migrate past the subplate to populate layers VI, V, IV, III and finally II. During late fetal development in the rat, for example E19, newborn neurons are incorporated into the most superficial layers (e.g., II/III). Meanwhile the processes of axon growth and synaptogenesis are beginning. Although critical developmental processes including synapse formation, gliogenesis, myelination and pruning will continue to shape the developing cortex through adolescence; neurogenesis is completed in the rat neocortex by birth. Therefore, disruptions of NPC proliferation during fetal brain development could contribute to irreversible consequences. For example, rats exposed to an inhibitor of mitosis on E17 have behavioral alterations consistent with rodent models of schizophrenia [44]. Similarly, conditional deletion of ERK2 in NPCs disrupts neurogenesis, leading to altered cortical excitability, and behavioral alterations consistent with rodent models of autism [12]. Therefore, impaired ERK1/2 signaling and NPC proliferation during critical periods of neurogenesis might contribute to the persistent cognitive deficits resulting from developmental zinc deficiency.

In conclusion, our findings reveal that even marginal zinc nutrition during gestation can decrease fetal NPC proliferation, which could affect neurogenesis and contribute to irreversible physiological and behavioral consequences in the offspring. An increased activity of the ERK1/2-directed phosphatase PP2A, as a consequence of zinc deficiency, impairs the ERK1/2 signaling pathway which can lead to a decreased rate of NPC proliferation. Future work will, on one hand, investigate the effect of marginal zinc deficiency on the number and distribution of neurons in the cerebral cortex after the completion of neurogenesis and, on the other hand, attempt to clarify the mechanisms downstream from ERK1/2 signaling which regulate NPC fate.

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