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# Marginal Zinc Deficiency during Gestation and Lactation in Rats Affects Oligodendrogenesis, Motor Performance, and Behavior in the Offspring

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A B S T R A C T

**Background:** Oligodendrocytes are responsible for myelin production in the central nervous system (CNS). Hypomyelination may slow saltatory nerve signal conduction and affect motor performance and behavior in adults. Gestational marginal zinc deficiency in rats significantly decreases proliferation of neural stem cells (NSCs) in the offspring brain.
**Objectives:** Given that NSCs are precursors of oligodendrocytes, this study investigated if marginal zinc deficiency during early development in rats affects oligodendrogenesis in the offspring's CNS.
**Methods:** Rat dams were fed an adequate (25 μg zinc/g diet) (C) or a marginal zinc diet (MZD) (10 μg zinc/g diet), from gestation day zero until postnatal day (P) 20, and subsequently all offspring was fed the control diet until P60. Oligodendrogenesis was evaluated in the offspring at P2, P5, P10, P20, and P60, by measuring parameters of oligodendrocyte progenitor cells (OPCs) proliferation, differentiation, maturation, and of myelination.
**Results:** The expression of *1*) proteins that regulate OPC proliferation (Shh, Sox10, Olig2); *2*) OPC markers (NG2, PDGFRα); *3*) myelin proteins (MBP, MAG, MOG, PLP) were lower in the brain cortex from MZD than C offspring at various stages in development. The amount of myelin after zinc replenishment continued to be low in the MZD young adult at P60. Accordingly, parameters of motor performance and behavior [grip strength, rotarod, elevated T-maze (ETM), and open-field tests] were impaired in the MZD offspring at P60.
**Conclusions:** Results support the concept that maternal and early postnatal exposure to MZD affects oligodendrogenesis causing long-lasting effects on myelination and on motor performance in the young adult offspring.

*Keywords*: zinc deficiency, brain development, ERK1/2, oligodendrogenesis, myelination, behavior, motor performance

## Introduction

Pregnant women and children under 10 y of age are particularly at risk of zinc deficiency and its adverse consequences. In developing countries, this risk ranges between 20% and 80% for pregnant women [1]. Prenatal and postnatal zinc deficiency resulting from inadequate zinc intake can have detrimental effects on the development of the central nervous system (CNS). Severe zinc deficiency in rats causes teratogenicity, including neural tube defects, skeletal malformations, growth retardation, and defects of the heart, lung, and brain [2]. In humans, low serum zinc levels in pregnant mothers and neonates are correlated with preterm delivery and neural tube defects [3]. On the other hand, a condition of marginal zinc deficiency that does not

*Abbreviations:* AES, atomic emission spectroscopy; CC, corpus callosum; CNPase, 2',3'-cyclic-nucleotide 3'-phosphodiesterase; CREB, cAMP response element-binding protein; CNS, central nervous system; DAB, 3,3'-diaminobenzidine tetrahydrochloride; ETM, elevated T-maze; ERK1/2, extracellular signal-regulated kinase 1 and 2; GLI1, glioma-associated oncogene homolog 1; LFB, Luxol fast blue; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MEK, mitogen-activated protein kinase; MOG, myelin oligodendrocyte glycoprotein; MZD, marginal zinc diet; NG2, neuron-glial antigen 2; NSC, neural stem cell; OD, optical density; Olig2, oligodendrocytes transcription 2; OPC, oligodendrocyte progenitor cell; PDGFRα, platelet-derived growth factor receptor alpha; PLP, proteolipid protein; PVDF, polyvinylidene difluoride; ROI, region of interest; rpm, revolutions per minute; Shh, sonic hedgehog; Smo, smoothened; Sox10, SRY (sex determining region Y)-box 10; TBST, Tris-buffered saline pH 7.4, 0.1% Tween 20; VZ, ventricular zone.

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cause teratogenicity and/or major adverse impact on pregnancy outcome is more common in pregnant women and young children [4,5]. However, developmental marginal zinc deficiency has been associated with neurological deficits, motor dysfunction, and behavioral abnormalities in rodents, including decreased locomotor activity, learning and memory deficits, depression, and anxiety [6]. In humans, low serum zinc concentration is associated with children's autism spectrum disorders [7] and attention-deficit/hyperactivity disorder symptoms in a middle-class American population [8]. In addition, marginal zinc deficiency is correlated with behavioral disorders including delayed psycho-motor development and social interactions in infants [9], impaired cognitive performance, increased anxiety, and depression in school aged children [10].

Maternal suboptimal zinc nutrition can have long-term effects on fetal/offspring brain development and behavior. Zinc deficiency affects neural stem cells (NSCs) proliferation and neuronal differentiation, maturation, and survival [11–15]. We previously observed that gestational marginal zinc deficiency in rats impairs neurogenesis by decreasing the number of Ki67 positive NSCs (proliferating NSCs) in the fetal brain ventricular zone (VZ) at embryonic day 19 (E19) [16,17]. Maternal marginal zinc diet (MZD) also affects neurogenesis and astrogliogenesis, resulting in a lower number of neurons and astrocytes and altered neuronal specification in the young adult offspring brain cortex [17,18]. Although NSCs are also precursors of oligodendrocytes, there is limited information on the relevance of zinc for oligodendrogenesis and myelination.

NSCs generate radial glial cells, which can differentiate into intermediate progenitors cells (IPCs). In the embryonic brain subventricular zone IPCs can further differentiate into oligodendrocyte progenitor cells (OPCs) [19]. Oligodendrocytes generate the myelin sheath which maintains the integrity of the axon and provides axons with electrical insulation, enabling a faster speed of action potential transmission [20]. Zinc is important to regulate and maintain the oligodendrocyte population and myelin formation [21,22]. Severe zinc deficiency (0.007 μg zinc/g diet) decreased the number of myelinated axons in 21-d-old male rats and myelin sheath thickness in the optic nerve, suggesting that zinc is important for maintaining CNS myelin integrity [23]. Similarly, decreased myelin thickness and axonal degeneration are present in the sciatic nerve from adult rats fed a severe zinc-deficient diet [24]. Maternal marginal zinc deficiency also causes alterations in myelin protein profiles in the developing rat and monkey brain, although individual proteins were not characterized [25].

Our previous findings showed the adverse effects of maternal marginal zinc nutrition on NSCs self-renewal. An impairment of NSC proliferation because of maternal marginal zinc deficiency could also affect oligodendrogenesis. However, there is limited knowledge on the effects of early developmental marginal zinc deficiency on OPC proliferation and differentiation and the cell signaling pathways involved. Thus, this work investigated the potential impact of maternal marginal zinc deficiency during pregnancy and lactation on oligodendrogenesis and its consequences on brain myelination and motor performance in the young adult rat.

# Methods

## Materials

Primary antibodies for β-actin (#12620), phospho-mitogen-activated protein kinase 1 and 2 (MEK1/2) (Ser217/221) (#9154), MEK1/2 (#9126), phospho-extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Thr202/Tyr204) (#4370), ERK1/2 (#9102), myelin-associated glycoprotein (MAG) (#9043), myelin basic protein (MBP), #78896), and glioma-associated oncogene homolog 1 (GLI1) (#2643) were from Cell Signaling Technology. Antibodies for 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) (SC-166558) and myelin oligodendrocyte glycoprotein (MOG) (SC-376138) were from Santa Cruz Biotechnology. Antibodies for oligodendrocytes transcription 2 (Olig2) (AB35128) and neuron-glial antigen 2 (NG2) (AB15894) were from Millipore. Antibodies for proteolipid protein (PLP) (ab28486) and platelet-derived growth factor receptor α (PDGFRα) (ab203491) were from Abcam Inc. The antibody for (sex determining region Y)-box 10 (Sox10) (MBS3201449) was from MyBioSource. Secondary antibodies, IHC Detection Reagents (HRP, Rabbit #8114), 3,3'-diaminobenzidine tetrahydrochloride (DAB) Substrate Kit (#8059), and hematoxylin (#14166) were obtained from Cell Signaling Technology. Polyvinylidene difluoride (PVDF), membranes, and molecular weight standards for Western blot were obtained from BIO-RAD. The enhanced chemiluminescence Western blotting system was from Thermo Fisher Scientific Inc.

## Animals and animal care

All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH 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 female and male rats were purchased from Charles River. Female rats (200–225 g) were housed individually in stainless steel cages in a temperature-controlled (22–23°C) and photoperiod-controlled (12-h light/dark) room. Distilled water was provided through a daily flushed automatic watering system. An egg-white protein-based diet (Supplemental Table 2) with adequate zinc (25 μg zinc/g) was the control diet [26]. Animals were fed the control diet for 7 d before breeding. Males and females were caged together overnight and the following morning, gestation day 0, after the presence of a sperm plug confirmed successful breeding; female rats (11 animals/group) were randomly divided into 2 groups and fed ad libitum a control diet (25 μg zinc/g diet, C group) or a marginal zinc-deficient diet (10 μg zinc/g diet, MZD group). Rat dams were fed the MZD or the control diet from gestation day 0 until postnatal day 20 (P20). Subsequently, offspring from both dietary groups were fed the control diet until P60 (Figure 1A). Food intake was recorded

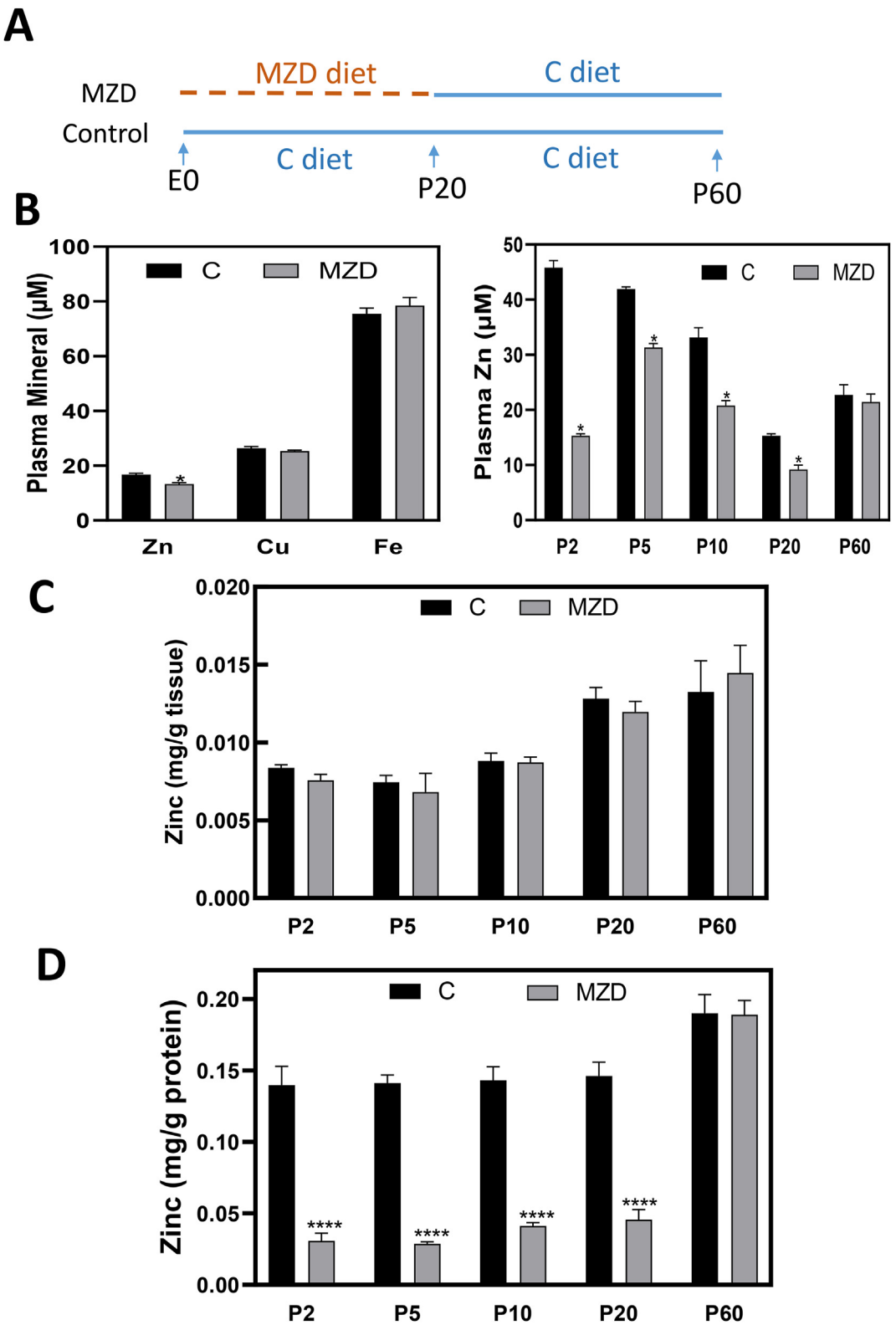


**FIGURE 1.** Maternal and offspring minerals concentration in plasma and/or brain. (A) Experimental design. (B, left panel) Maternal plasma zinc, copper, and iron concentrations at 20 d postpartum and (B, right panel) plasma zinc concentration in the offspring from P2–P60. (C, D) Zinc concentration in the offspring (P2–P60) (C) whole brain and (D) brain 100,000 × g supernatants. Minerals were measured by AES as described in methods. Data are shown as mean ± SEM and are the average of 9 L (1 pup from each litter) per group. *, ****Significantly different from the control group at the same developmental stage (*$P < 0.05$, ****$P < 0.0001$, Student's $t$ test). AES, atomic emission spectroscopy.

daily, and the body weight was measured at 5-d intervals. Offspring at postnatal days 2 (P2), 5 (P5), 10 (P10), 20 (P20), and 60 (P60) were anesthetized with isoflurane (2 mg/kg body weight) and blood and whole brain collected immediately after anesthesia. The whole brain was weighed and processed for immunohistochemistry or frozen in liquid nitrogen and stored at −80°C for further determinations. Blood was collected by cardiac venipuncture into 5-mL mineral-free tubes containing EDTA. Blood was centrifuged at 3000 × g for 15 min at 4°C to separate the plasma, which was subsequently stored at −80°C until analysis.

## Determination of zinc concentration

The concentration of zinc in diets and brain supernatants was measured by inductively coupled plasma atomic emission spectroscopy (AES) as described by Clegg et al. [27]. Brains were weighed, homogenized in ice-cold PBS (1 g/10 mL), and centrifuged for 60 min at 100,000 × g at 4°C. Protein concentration was measured in the 100,000 × g supernatants using the Bradford assay [28]. Whole brain (50–100 mg), 100,000 × g supernatants (1 mL), and diet samples (500 mg) were added with 3 mL of 16 N HNO3 and digested for 72 h at room temperature. Samples were subsequently dried and resuspended in ultrapure water. For plasma zinc concentration analysis, 0.1 N ultrapure HNO3 (100 μL/mL plasma) were added, and samples digested at room temperature for 5 d. Zinc concentration was determined by ICP-AES (Trace Scan; Thermo Elemental). Certified reference solutions (QC 21; Spec CentriPrep) were used to generate standard curves. A sample of a National Bureau of Standards bovine liver (SRM1577; United States Department of Commerce, National Bureau of Standards) was included with the samples to ensure accuracy and reproducibility.

## Western blot analysis

Total brain proteins were extracted as previously described [29]. Protein concentration was measured [28], and aliquots containing 25–50 μg of protein were separated by reducing 10% (w/v) polyacrylamide-SDS gel electrophoresis and electroblotted to PVDF membranes. Colored molecular weight standards were run simultaneously. Membranes were blocked for 1 h in 5% (w/v) nonfat milk and incubated overnight with the corresponding primary antibodies (1:1000–1:5000) in 1% (w/v) bovine serum albumin in Tris-buffered saline pH 7.4, 0.1% Tween 20 (TBST) at 4°C. After incubation with the corresponding peroxidase-conjugated secondary antibodies (1:10,000–1:30,000), proteins were visualized by chemiluminescence detection, and subsequently quantified, using a Phosphoimager 840.

## Assessment of brain myelin volume

Luxol fast blue (LFB) (Fisher, #212170250) was dissolved (1 mg LFB/mL) in 95% (v/v) ethanol and 0.5 μL acetic acid to prepare the working solution for myelin staining in paraffin-embedded brain sections. Slides were deparaffinized, hydrated, and incubated in LFB solution overnight at 37°C and subsequently rinsed in deionized water and incubated with 0.5 g/L lithium carbonate in distilled water. Finally, slides were counterstained with Cresyl Echt violet (Sigma, #C1791) (1 mg/mL in $H_2O$) for 6 min and dehydrated sequentially in 95% (v/v) ethanol, 100% (v/v) ethanol, and xylene, and mounted with mounting medium (Cell signaling, #14177). Images were captured in a Bio Twin CM120 microscope (Philips/FEI). Micrographs were taken with a Bioscan digital camera (Model 792; Gatan). The volume of corpus callosum (CC) stained with LFB stain was measured with ImageJ (ImageJ, National Institutes of Health). Image analysis was performed according to previously described protocols [30]. In brief, blue images were selected by using the Color Deconvolution plugin of ImageJ software with the vector FastRed FastBlue DAB. The threshold intensity was manually set to subtract the staining background and LFB optical density (OD) was determined as previously reported [31,32]. Myelin volume in the CC was calculated by multiplying tissue thickness and the area of CC stained with LFB.

## Immunohistochemistry

Rat offspring brains were dissected and fixed in 4% (w/v) solution of paraformaldehyde in PBS overnight. Preservation was then performed in 70% (v/v) ethanol in PBS before processing and paraffin embedding. Seven-micron-thick paraffin-embedded brain sections were mounted on gelatin-coated positively charged microscope slides. Sections were blocked for 1 h in 5% (w/v) goat serum in TBST at room temperature, then incubated overnight at 4°C with the corresponding primary antibody in blocking buffer (1:500 rabbit anti-MBP). Sections were then washed in TBST and incubated for 30 min at room temperature with detection reagent (HPR-antirabbit). After 3 washes with TBST, sections were incubated with DAB substrate for 5–10 min at room temperature. After immunostaining, cell nuclei were stained with hematoxylin and sections imaged using a Bio Twin CM120 microscope (Philips/FEI). Micrographs were taken with a Bioscan digital camera (Model 792; Gatan). ImageJ software was used to analyze the resulting micrographs. Image analysis was performed according to described protocols [30]. In brief, the selection of the region of interest (ROI), in this case, CC and hippocampus, was cropped from the original image and saved separately, then the digitized ROIs were input to ImageJ software again and the brown images, which represent DAB staining, were selected by using the Color Deconvolution plugin of ImageJ software with the vector H-DAB. The threshold intensity was manually set to subtract the background of staining and DAB OD was determined as described in Khodanovich et al. [31] and Varghese et al. [32]. Briefly, the OD of DAB was calculated on the basis of the formula OD = log (max intensity/mean intensity), where the value of max and mean intensity was measured from ImageJ analysis.

## Rotarod

Rotarod (Rotamex 5, Columbus Instruments) is a widely used test to assess motor performance and neuromuscular coordination in rodents. The apparatus consists of 4 adjacent rods (7-cm diameter and 44.5-cm height) separated with plastic barriers. The duration that rats stay on the rotating rod is associated with neuromotor ability. At P55, the offspring (20 females and 20 males from each group) were pretrained on the rotating rod with constant speed of 4 revolutions per minute (rpm) before testing. At the testing session, animals were placed on the rods at an increasing speed (4–40 rpm). The latency to fall and the speed at which they fell were recorded automatically on the display. Each animal was subjected to the trial 3 times and results were averaged.

### Grip strength test

The grip strength apparatus (San Diego Instrument) was used to measure animals' neuromuscular strength. The apparatus consists of an acrylic base, a force gauge, and an adjustable grip. A total of 40 adult offspring (20 females and 20 males) from each group were tested. During the experiment, the rat was held by the base of the tail about 45° above the bar and was then moved down until its forelimbs grasped the middle of the bar, and the experimenter then pulled the rat horizontally until the grasp was released. During each trial, the maximum force developed by the rat was obtained just before the grasp was released, and the force meter would automatically record the force displayed in the screen. Each testing trial lasted 5–10 s, and the time interval between each trial was 10–30 min to allow animals to rest. Five trials were performed with each animal and the 3 best force readings (in grams) were averaged. The final mean force readings were normalized by animal body weight (force/weight ratio). All procedures were performed by the same operator to avoid interindividual variability.

### Open-field test

The open-field test was conducted to evaluate rat's offspring locomotor activity and anxiety level. A total of 40 adult offspring (20 females and 20 males) from each group were tested in the open field. During the experiment, rats were allowed to explore the open field arena freely for 10 min. The apparatus consists of 4 acrylic square arenas (56 cm × 56 cm) with a wall height of 60 cm. The center zoom was set as 30 cm × 30 cm to evaluate the animal anxiety level. The experiment was performed in a quiet and controlled dimly lit room, and a video camera above the open-field apparatus recorded the experiment. The video camera is connected to a software (Noldus EthoVision XT) that autorecords and analyzes the rat's behavior in the open field. Each rat was placed in the corner of the open field facing the center. The total distance traveled in the open field was measured to assess the locomotor activity and frequency to enter the center zone and duration to stay in the center zone were measured to assess animal's anxiety levels.

### Elevated T-maze

The elevated T-maze (ETM) was used to evaluate rat's offspring anxiety levels. The ETM is a modified version of the elevated plus maze, which is commonly used to test anxiety in laboratory animals. The experimental design was based on published protocols by Graeff et al. [33]. The ETMs consists of 1 enclosed and 2 open arms, being the closed arm perpendicular to the open arms. The maze arm dimensions are 50 cm × 10 cm with a 30-cm height wall, and the whole apparatus was 50 cm above the floor. A total of 40 adult offspring (20 females and 20 males) from each group were tested. Rats were placed at the distal end of the closed arm and allowed to explore it. The time was recorded when rats placed all 4 paws into the open arms. The entire experiment was recorded with a video camera above the ETMs setting for a maximum of 5 min for each animal. Experiments were performed in a dimly lit and quiet room. The experimental operator/observer was isolated from the ETMs setup by a curtain to ensure no human interaction during the trials. All experiments were performed between 8 am and 11 am. The apparatus was cleaned with a 70% (v/v) ethanol solution between subjects. All data analyses were performed by a single examiner who was blinded to the sample group.

### Statistical analysis

Data were analyzed by Student's 2-tailed *t* test using Statview 5.0 (SAS Institute Inc.) and GraphPad Prism. Fisher least significance difference test was used to examine differences between group means. A $P$ value $< 0.05$ was considered statistically significant. Data are shown as mean ± SEM. The litter was the statistical unit.

## Results

### Animal outcome

A marginal zinc nutrition throughout gestation did not affect overall maternal and fetal outcome (Supplemental Table 1). However, maternal weight gain and litter size were significantly lower in the MZD than in the C group (Supplemental Table 1). Maternal plasma zinc concentration at weaning was 21% lower in MZD dams compared with controls, whereas plasma copper and iron concentrations were similar between groups (Figure 1B, left panel). Plasma zinc concentration was significantly lower at P2, P5, P10, and P20 (34%, 25%, 37%, and 40%, respectively) in MZD pups compared with controls (Figure 1B, right panel). Offspring brain weight was similar between groups at all developmental stages (Supplemental Table 1). Whole brain zinc concentration in the P2, P5, P10, P20, and P60 offspring was similar between groups (Figure 1C). Zinc concentration in brain 100,000 × g supernatants from P2, P5, P10, and P20 offspring was significantly lower (79%, 79%, 71%, and 64%, respectively) in the MZD offspring compared with controls (Figure 1D). Iron and copper concentrations in the offspring whole brain and in 100,000 × g supernatants were similar between groups at all developmental stages (Supplemental Figure 1).

### Maternal marginal zinc deficiency affected the levels of transcription factors and other proteins characteristic of oligodendroglial lineage progression, terminal differentiation, and myelination in the P2 rat offspring brain

Our previous work showed that zinc is important in NSC proliferation, self-renewal, and differentiation [17]. We now investigated the effects of maternal MZD on the oligodendroglial lineage progression, terminal differentiation, and myelination at P2. Maternal marginal zinc nutrition affected the protein levels of different markers of OPCs proliferation and progression to differentiation as measured by Western blot (Figure 2). NG2 and PDGFRα are proteins that specifically identify brain OPCs. Maternal marginal zinc deficiency caused a 32% decrease in NG2 and 38% decrease in PDGFRα expression in the P2 offspring brain (Figure 2). Olig2 and Sox10 are 2 transcription factors that act as lineage determinants in oligodendrocytes. Maternal MZD caused a 35% and 50% reduction of Olig2 and Sox10 protein levels, respectively, in the P2 rat offspring brain compared with controls (Figure 2). CNPase is a myelin-associated enzyme present in premyelinating and mature myelinating oligodendrocytes. MAG and PLP are major protein components of myelin expressed in mature oligodendrocytes. MZD caused 17% and 49% reduction in CNPase and PLP protein levels in the P2

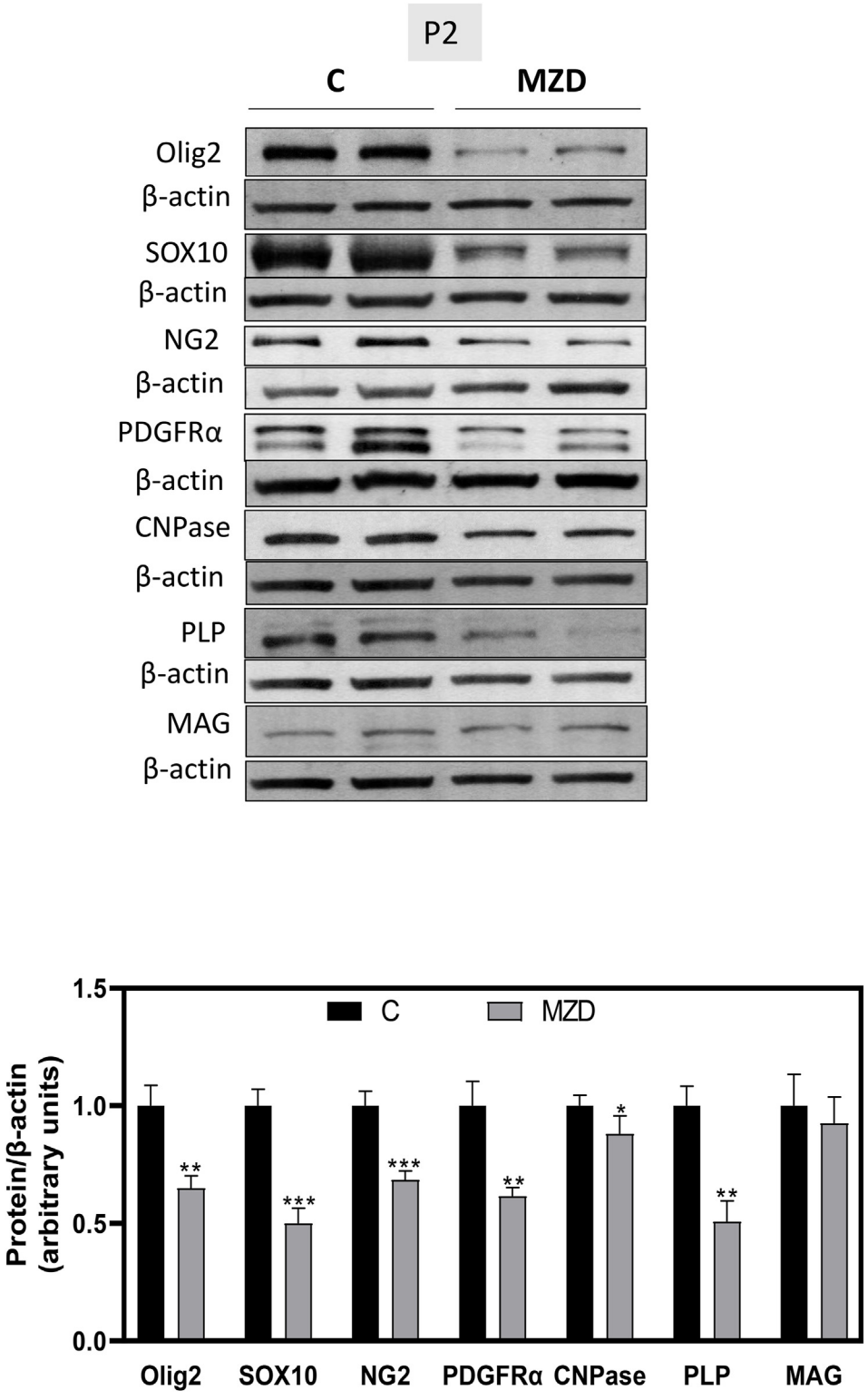


**FIGURE 2.** Maternal marginal zinc deficiency affects the parameters of oligodendrogenesis in the P2 offspring rat brain. Olig2, Sox10, NG2, PDGFRα, CNPase, PLP, and MAG protein levels were measured by Western blot in the P2 offspring rat brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM from 9 litters (1 pup from each litter) per group. *,**,***Significantly different from the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t* test).

offspring brain compared with controls. MAG protein levels were similar between groups (Figure 2).

## Maternal marginal zinc deficiency affected the Shh and ERK1/2 signaling pathways in the P2 offspring rat brain

Sonic hedgehog (Shh) has been implicated in the generation of oligodendrocytes, by increasing the expression of transcription factors Olig1 and Olig2, both necessary for oligodendrocyte production. Thus, we next examined the effects of early developmental zinc deficiency on these signaling pathways. The activation of the Shh pathway was evaluated by measuring the protein levels of cleaved Shh, smoothened (Smo), and GLI1 by Western blot. At P2, cleaved Shh, Smo, and GLI1 protein levels were 61%, 17%, and 54% lower in the MZD offspring brain compared with controls (Figure 3A, B). Our previous work showed that brain ERK1/2 activation was affected by maternal MZD at E19 [16]. We currently observed that the P2 offspring rat brain, ERK1/2 phosphorylation was 34% lower in the MZD group compared with controls, whereas the activation (phosphorylation) of upstream (MEK1/2) and of the downstream target [cAMP response element-binding protein (CREB)] was similar between groups (Figure 3A, C).

## Maternal marginal zinc deficiency affected the level of transcription factors and other proteins characteristics of oligodendroglial lineage progression, terminal differentiation, and myelination in the P5 and P10 offspring rat brain

At P5, a similar pattern as that observed at P2 was observed in the expression of NG2 and PDGFRα. Thus, NG2 and PDGFRα protein levels were 24% and 30% lower in MZD P5 offspring brain compared with controls (Figure 4). Transcription factors Olig2 and Sox10 protein levels were 41% and 38% lower, respectively, in the brain of P5 MZD rat offspring compared with controls (Figure 4). CNPase and PLP protein levels were 30% and 21% lower, respectively in MZD brains compared with controls, whereas MAG protein levels were similar between groups (Figure 4).

At P10, although brain NG2, PDGFRα, and CNPase protein levels were similar between groups, those of Olig2 and Sox10 were 68% and 67% lower, respectively, in MZD compared with the control group (Figure 5). At this stage, oligodendrocytes reach a mature state and actively produce myelin. At P10, PLP, MAG, and MBP protein levels were significantly lower (31%, 51%, and 49%, respectively) in the MZD offspring brain than in controls (Figure 5).

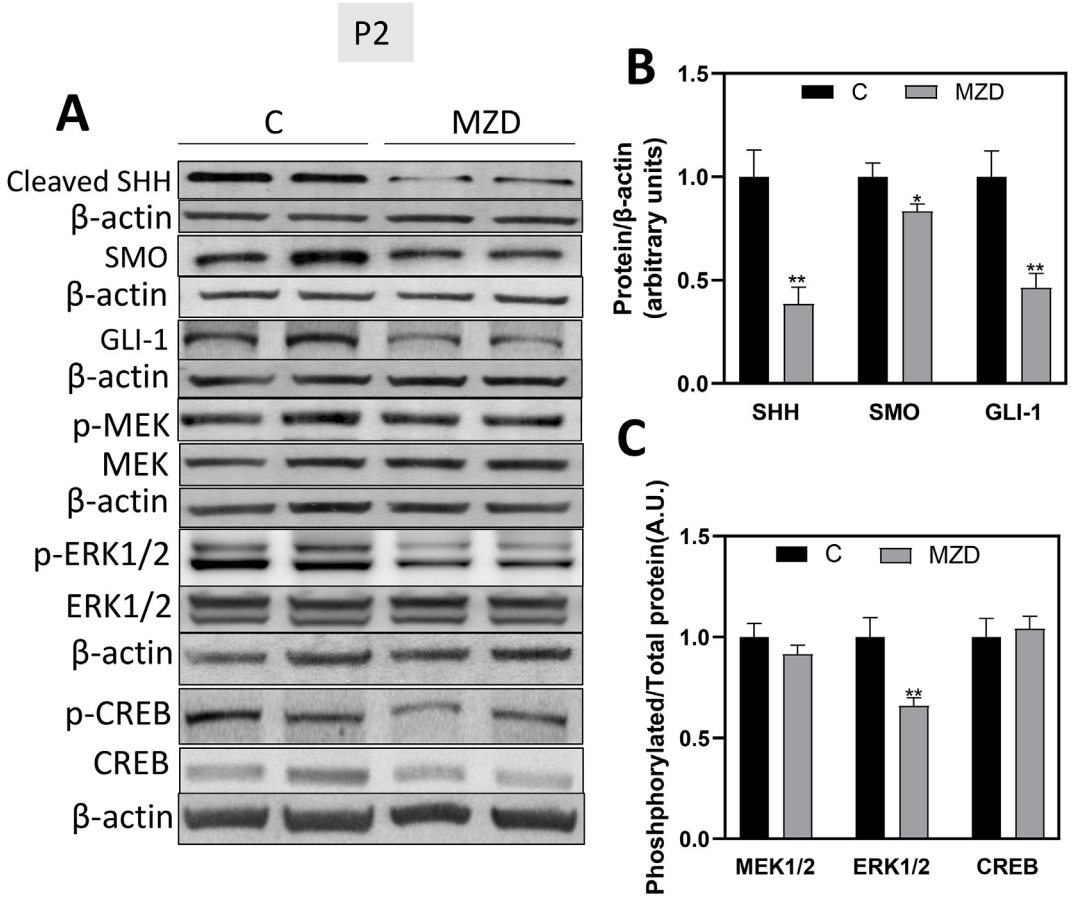


**FIGURE 3.** Maternal marginal zinc deficiency affects the Shh and ERK1/2 signaling pathways in the P2 rat offspring brain. (A) Representative Western blot images, (B) cleaved Shh, Smo, and GLI-1 proteins levels, and (C) phosphorylated and total MEK1/2, ERK1/2, and CREB protein levels were measured by Western blot in P2 rat brain homogenates. After quantification of bands, proteins were referred to (B) β-actin levels, and (C) phosphorylated protein levels were referred to the corresponding total protein content. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM for 9 litters (1 pup from each litter) per group. *,**Significantly different from the control group (*$P < 0.05$, **$P < 0.01$, Student's $t$ test).

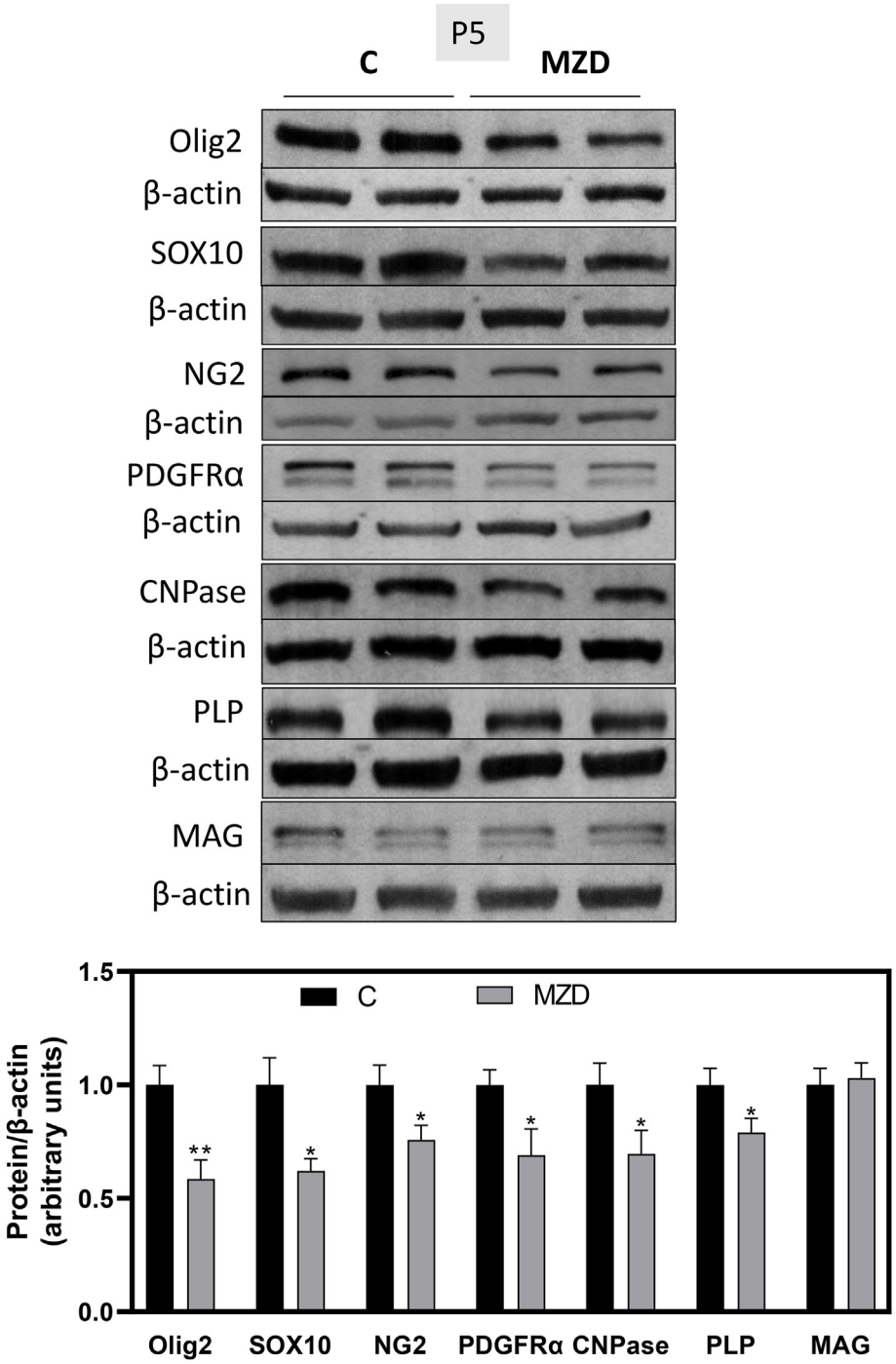


**FIGURE 4.** Maternal marginal zinc deficiency affects the parameters of oligodendrogenesis in the P5 offspring rat brain. Olig2, Sox10, NG2, PDGFRα, CNPase, MAG, and PLP protein levels were measured by Western blot in P5 offspring rat brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM from 9 litters (1 pup from each litter) per group. *,**Significantly different from the control group (*$P < 0.05$, **$P < 0.01$, Student's $t$ test).

## Maternal marginal zinc deficiency affected the level of transcription factors and other proteins characteristic of oligodendroglial lineage progression, terminal differentiation, and myelination in the P20 offspring rat brain

Similarly to the findings at P10, Olig2, Sox10, MAG, MOG, and PLP protein levels in the P20 offspring brain were 28%, 68%, 30%, 70%, and 21% lower, respectively, in the MZD group compared with the control group (Figure 6A). Brain NG2, PDGFRα, and CNPase protein levels were similar between groups (Figure 6A). MBP protein levels, measured by Western blot, were 64% lower in the P20 MZD offspring brain compared with controls (Figure 6A). P15–21 is the period of active myelination in rodents. For this reason, we next characterized myelination using LFB staining and immunohistochemistry for MBP. Myelin LFB staining intensity in the CC was 31% lower in MZD than that in

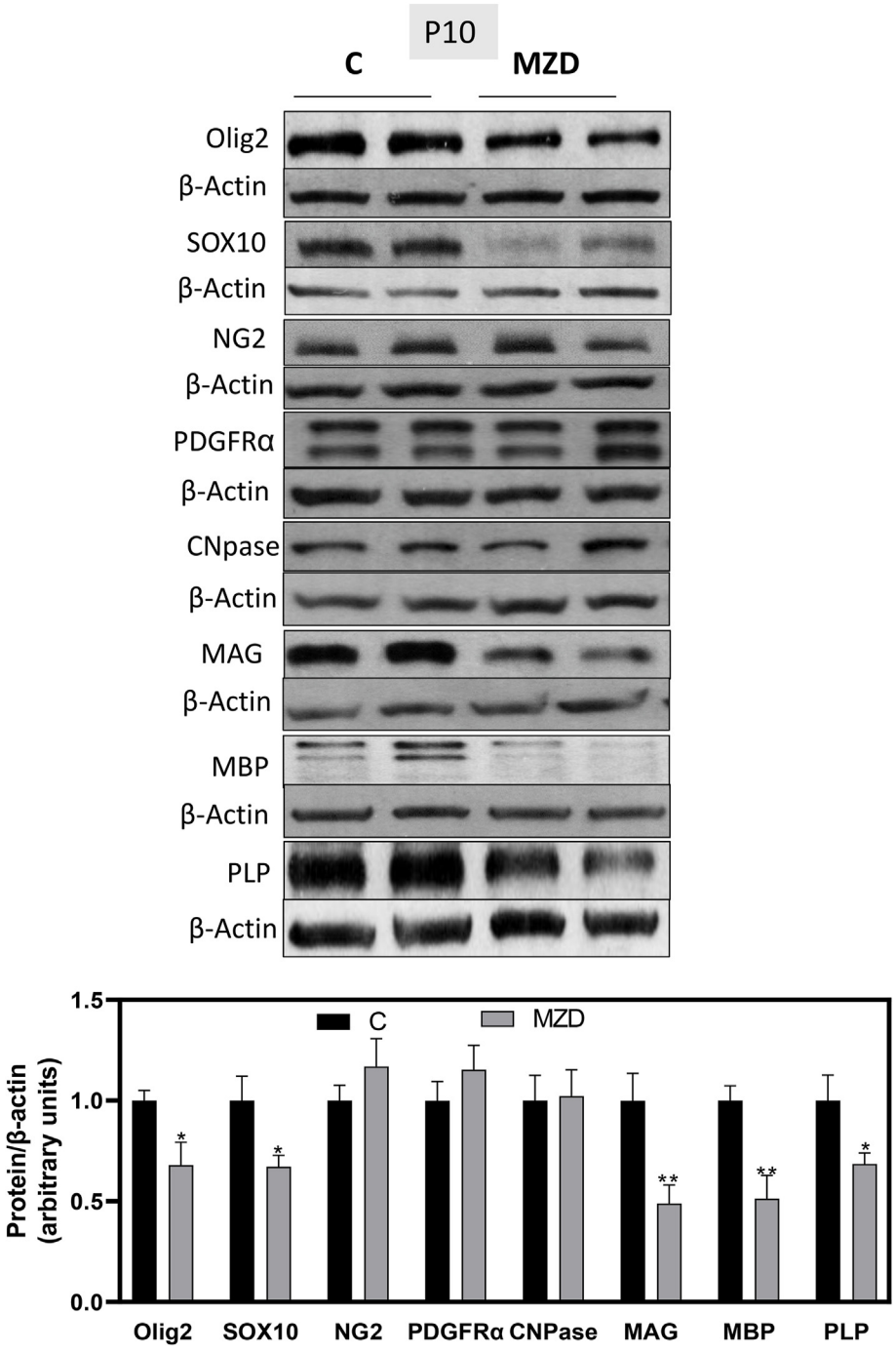


**FIGURE 5.** Maternal marginal zinc deficiency affects the parameters of oligodendrogenesis in the P10 offspring rat brain. Olig2, Sox10, NG2, PDGFRα, CNPase, MAG, MBP, and PLP protein levels were measured by Western blot in P10 offspring rat brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM from 9 litters (1 pup from each litter) per group. *,**Significantly different from the control group (*$P < 0.05$, **$P < 0.01$, Student's *t* test).

control offspring (Figure 6B, F), being the myelin volume 18% lower in MZD than in controls (Figure 6B, E). MBP immunofluorescence intensity was 48% and 31% lower in both the CC (Figure 6C, D, G) and the hippocampus (Figure 6C, D, H), respectively, in the MZD offspring compared with controls.

## Maternal marginal zinc deficiency disrupted oligodendrogenesis, causing long-term effects on myelination in the young adult offspring rat brain

We next assessed the long-term impact of early developmental marginal zinc deficiency on oligodendrogenesis and

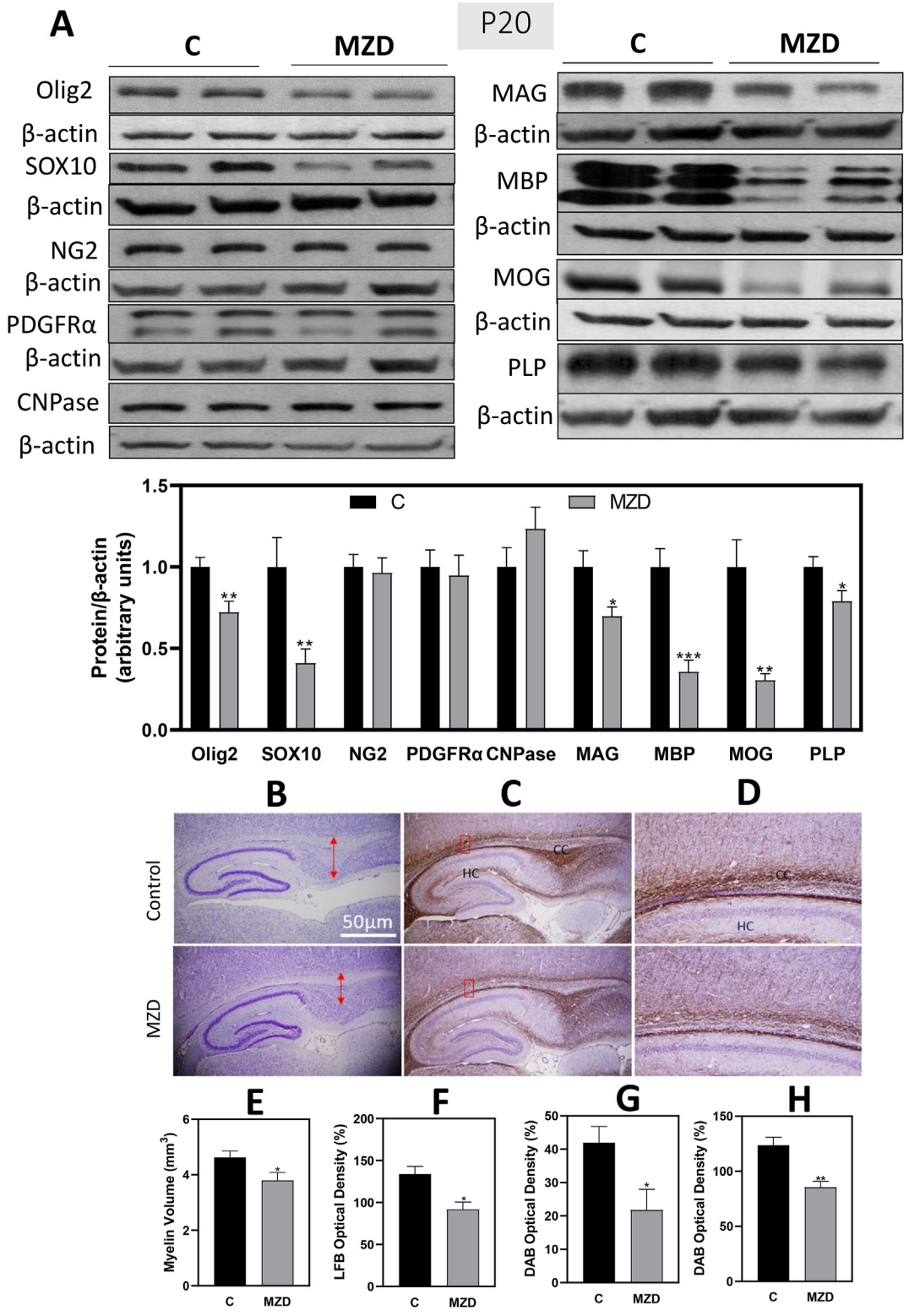


**FIGURE 6.** Maternal marginal zinc deficiency affects the parameters of oligodendrogenesis in the P20 offspring rat brain. (A) Olig2, Sox10, NG2, PDGFRα, CNPase, MAG, MBP, MOG, and PLP protein levels were measured by Western blot in P20 offspring rat brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SE from 9 litters (1 pup from each litter) per group. Significantly different from the control group (*$P < 0.05$, **$P < 0.01$, Student's *t* test). (B–D) Representative photomicrographs of myelin staining using a combination of (B) LFB, and (C, D) anti-MBP immunohistochemistry staining in CC and hippocampus (Scale bar, 50 μm). (E) Quantification of myelin volume was done by measuring the same area of CC multiplied by the thickness of the tissue. (F) Quantification of LFB staining. (G, H) Quantification of anti-MBP immunohistochemistry in (G) the CC and (H) the hippocampus. Quantifications were done as described in methods. Results are shown as mean ± SEM of P20 brains from 5–9 litters (1 pup from each litter) per group. *,**,***Significantly different from the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t* test).

myelination. All offspring were fed the control diet from P20 until P60. At P60, brain NG2, PDGFRα, and CNPase protein levels were similar between groups in males (Figure 7A, C) and females (Figure 7B, D). Even after 40 d in the control diet, lower levels of Olig2 [31% in males (Figure 7A, C) and 33% in females (Figure 7B, D)] and Sox10 [32% in males (Figure 7A, C) and 36% in females (Figure 7B, D)] were observed in the MZD offspring than in controls, suggesting a decrease in the population of oligodendroglial cell lineage. Levels of the myelin proteins MAG, MBP, MOG, and PLP were lower in both male [35%, 72%, 40%, and 31%, respectively, (Figure 7A, C)] and female [27%, 34%, 25%, and 26%, respectively, (Figure 7B, D)] offspring of the MZD group compared with the control group.

Immunohistochemistry analysis also showed lower levels of CC and hippocampus myelination in the MZD group compared with controls in both P60 male and female offspring. Myelin content in CC, measured by LFB staining, was 33% lower in volume and 46% lower in intensity in P60 males from the MZD group compared with controls (Figure 8A, D, E). In female MZD P60 offspring brain, lower CC myelin volume and LFB intensity (25% and 44%, respectively) were observed compared with controls (Figure 8H, K, L). Immunohistochemistry analysis showed lower MBP intensity in both CC and hippocampus (75% and 53%, respectively) in P60 males MZD offspring rat brain (Figure 8B, C, F, G) compared with controls. Similar effects were observed in MZD P60 females, which showed 68% and 38% lower MBP intensity in CC and hippocampus, respectively, compared with controls (Figure 8I, J, M, N). Results indicate that early developmental MZD in rats has long-term effects on oligodendrogenesis and myelination in the mature adult rat CC and hippocampus.

### Early developmental marginal zinc deficiency affects motor performance and behavior in the young adult rat offspring

To assess the potential impact of the observed altered myelination in MZD young adults, P60 offspring were subjected to a grip strength test that evaluates the neuromuscular function and the rotarod test that evaluates neuromuscular strength, coordination, and the ability to maintain balance. The grip force was normalized by the animal body weight. There was an overall 26% reduction in grabbing force in the MZD than that in control offspring (Figure 9A), being 29% and 25% lower in MZD males and females, respectively (Figure 9B). In the rotarod test, the latency to fall from the rotating rod was 21% shorter for the P60 MZD group compared with the control group (Figure 9C). The latency was 26% and 34% shorter in MZD males and females, respectively, compared with controls. (Figure 9D).

Locomotor activity was evaluated using an open-field test. Within 10 min, MZD rats traveled 13% less distance compared with controls (Figure 10A). This overall difference was because of a 71% lower total distance traveled by females MZD compared with controls, whereas for males, it was similar between groups (Figure 10B, G). Anxiety levels were assessed by measuring the frequency to enter and the duration to stay in the center zone of the open field. No significant differences were observed between MZD and control groups (Figure 10C, D, E, F). However, in the ETM test, anxiety levels were 67% higher in MZD rats compared with controls. This was observed when combining both females and males' data (Figure 9E), whereas differences were not significant when analyzing each sex separately (Figure 9F).

## Discussion

This work investigated the effects of a maternal marginal zinc nutrition on oligodendrogenesis and CNS myelination, in particular, in the CC and the hippocampus. We observed that the maternal consumption of a marginal zinc-deficient diet during gestation and until weaning in rats leads to a disruption of oligodendrogenesis in the offspring brain with a consequent hypomyelination that persists into adulthood. This is associated with offspring locomotor and behavioral alterations in adulthood even after dietary zinc replenishment.

Gestational severe zinc deficiency is associated with teratogenicity affecting multiple organs including neural tube defects, decreased brain size, and malformations of eyes and olfactory bulbs [34–36]. In contrast, maternal mild/marginal zinc deficiency does not cause major birth defects in rodents but affects neuroprogenitor cell proliferation, neurogenesis, and astrogliogenesis [16–18], and causes behavioral alterations [6]. Maternal marginal zinc deficiency is associated with deficits in learning and memory [37], impaired social interaction, increase emotionality including anxiety and depression, and aggressiveness [38,39], and decreased locomotor function [40] in the offspring. In addition, lower serum zinc levels have been reported in children suffering from neurological disorders such as autism spectrum disorders [7], Attention-deficit/hyperactivity disorder [8], mood disorders (depression, anxiety, and aggressiveness) [41], schizophrenia [42,43], and altered cognitive and motor function [44].

As previously reported [17], we currently observed that maternal marginal zinc deficiency did not affect maternal and fetal outcomes. The observed lower maternal weight gain at gestational day 19 is probably associated with the lower litter size in the MZD compared with the control group. Maternal marginal zinc nutrition caused a decrease in offspring plasma zinc concentration at P2, P5, P10, and P20. This can be because of a decreased milk zinc content, as previously found in mice fed an MZD [45]. Although maternal marginal zinc deficiency did not affect whole brain zinc concentration in the offspring, it caused a significant decrease in brain cytosolic zinc concentrations, which constitutes the most rapidly accessible zinc pool. Thus, in the marginal zinc offspring brain there would be less of this pool available for the increased zinc requirements associated with the active processes occurring during early development, including cell proliferation and cell signaling regulation.

Zinc deficiency-associated disruption of the ERK1/2 signaling pathway affects NSC proliferation, differentiation, and specification in various stages of neurogenesis [16,17,46]. ERK1/2 is important in regulating the cell cycle [47], neuronal differentiation [48], and survival [49]. We observed that ERK1/2 activation is impaired in the brain of MZD offspring at P2, when OPCs are actively proliferating. Other signaling pathways are also involved in regulating oligodendrogenesis. In this regard, the first oligodendroglial cells arise from the ventral VZ in the developing brain and spinal cord because of Shh-induced

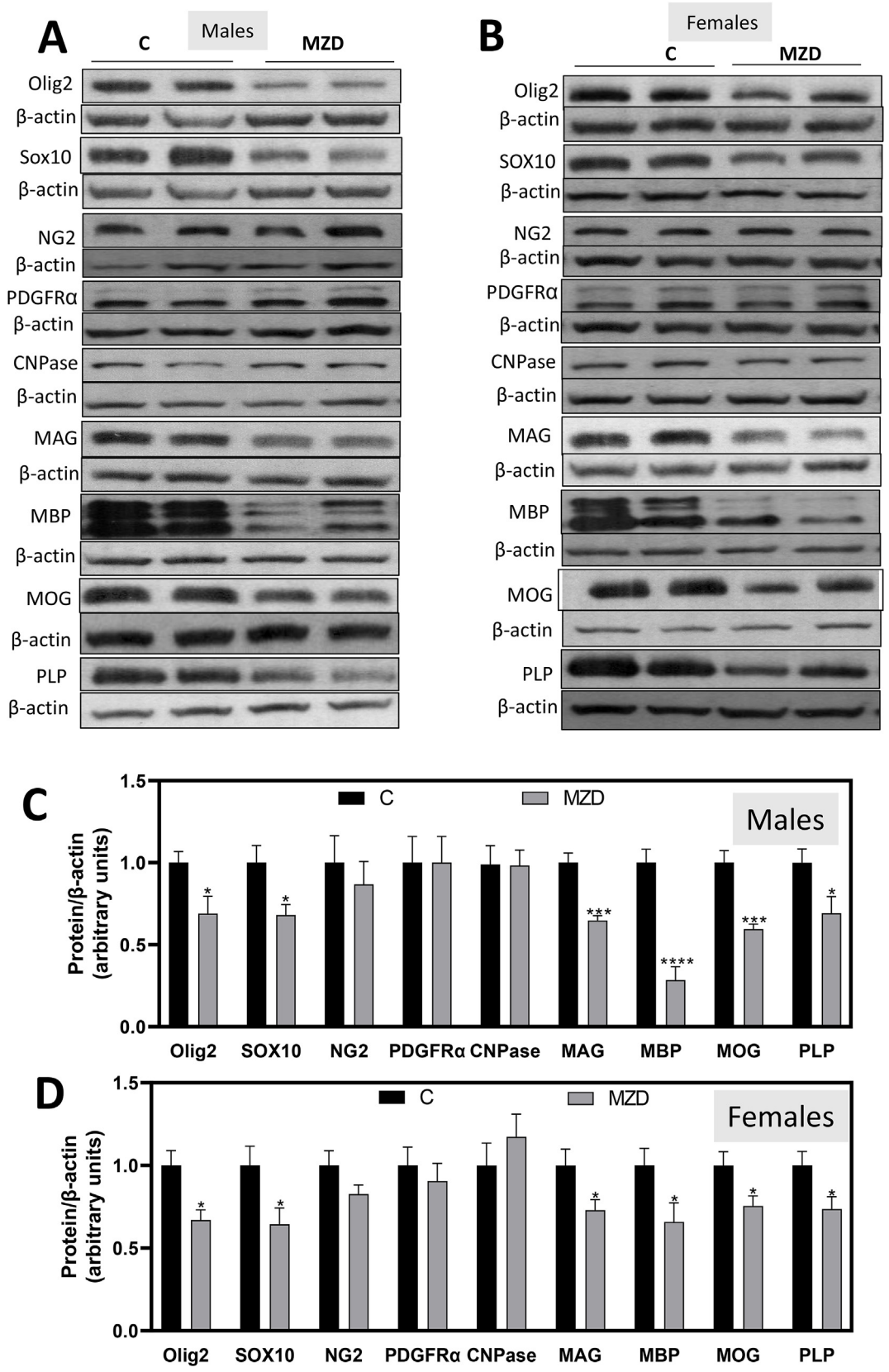


**FIGURE 7.** Maternal marginal zinc deficiency affects the parameters of oligodendrogenesis in the P60 offspring rat brain. (A–D) Olig2, Sox10, NG2, PDGFRα, CNPase, MAG, MBP, MOG, and PLP protein levels were measured by Western blot in P60 (A, C) males and (B, D) female offspring brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM in offspring from 9 litters (1 pup from each litter) per group. *,***,****Significantly different from the control group (*$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, Student's $t$ test).

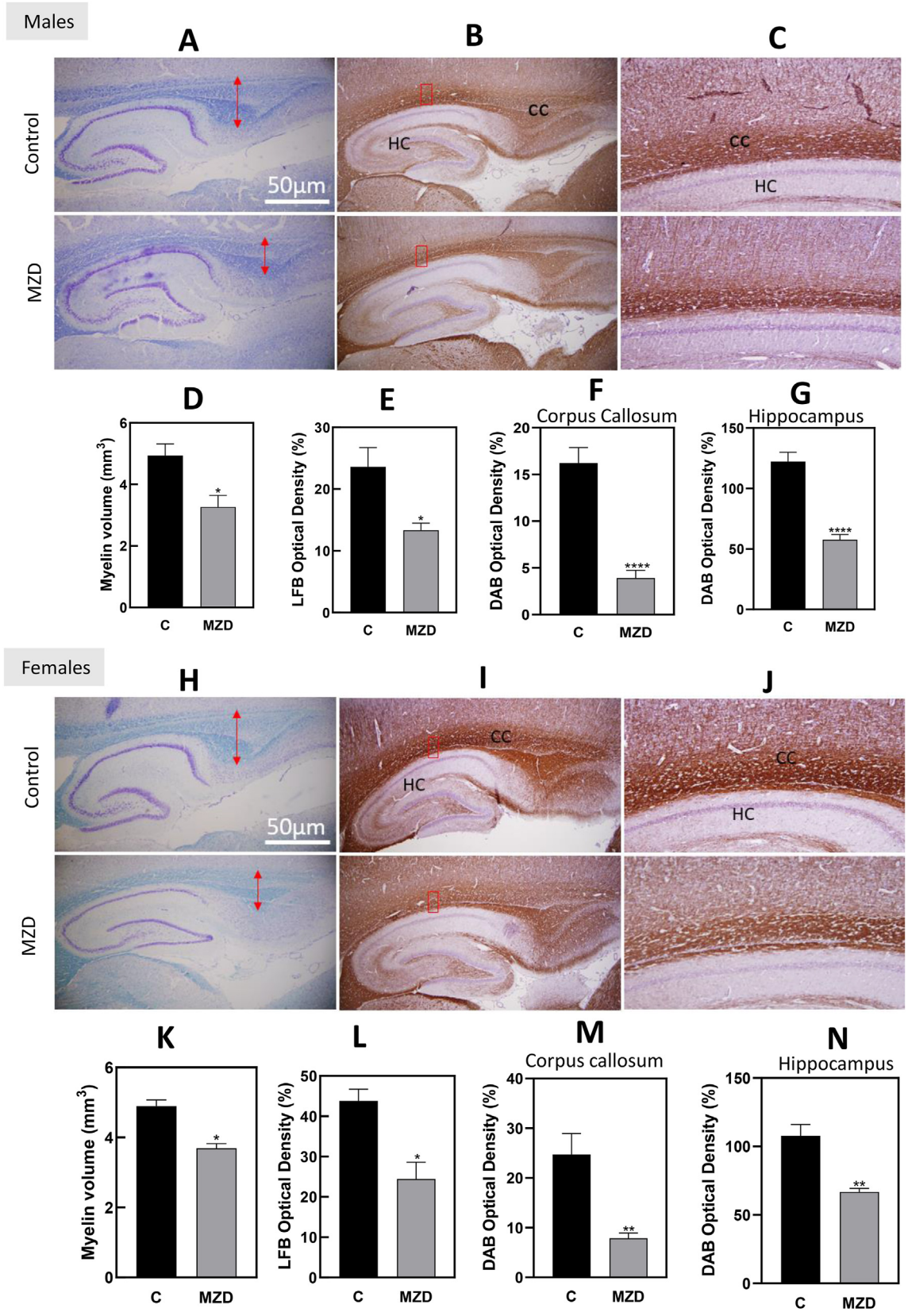


**FIGURE 8.** Maternal marginal zinc deficiency affects myelination in the P60 offspring rat brain. (A–C, H–J) representative photomicrographs of male (A–C) and female (H–J) brains, (A, H) stained with LFB and after (B, C, I, J) immunohistochemistry for anti-MBP staining. (Scale bar, 50 μm). (D, K) myelin volume was evaluated by measuring the area of the CC stained with LFB multiplied by the thickness of the tissue. (E, L) quantification of LFB staining, (F, M) quantification of anti-MBP staining in the CC. (G, N) quantification of anti-MBP staining in the hippocampus (Scale bar, 50 μm). Quantifications were done as described in methods. Results are shown as mean ± SEM in offspring from 5–9 litters (1 pup from each litter) per group. *, **, ***Significantly different from the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's $t$ test).

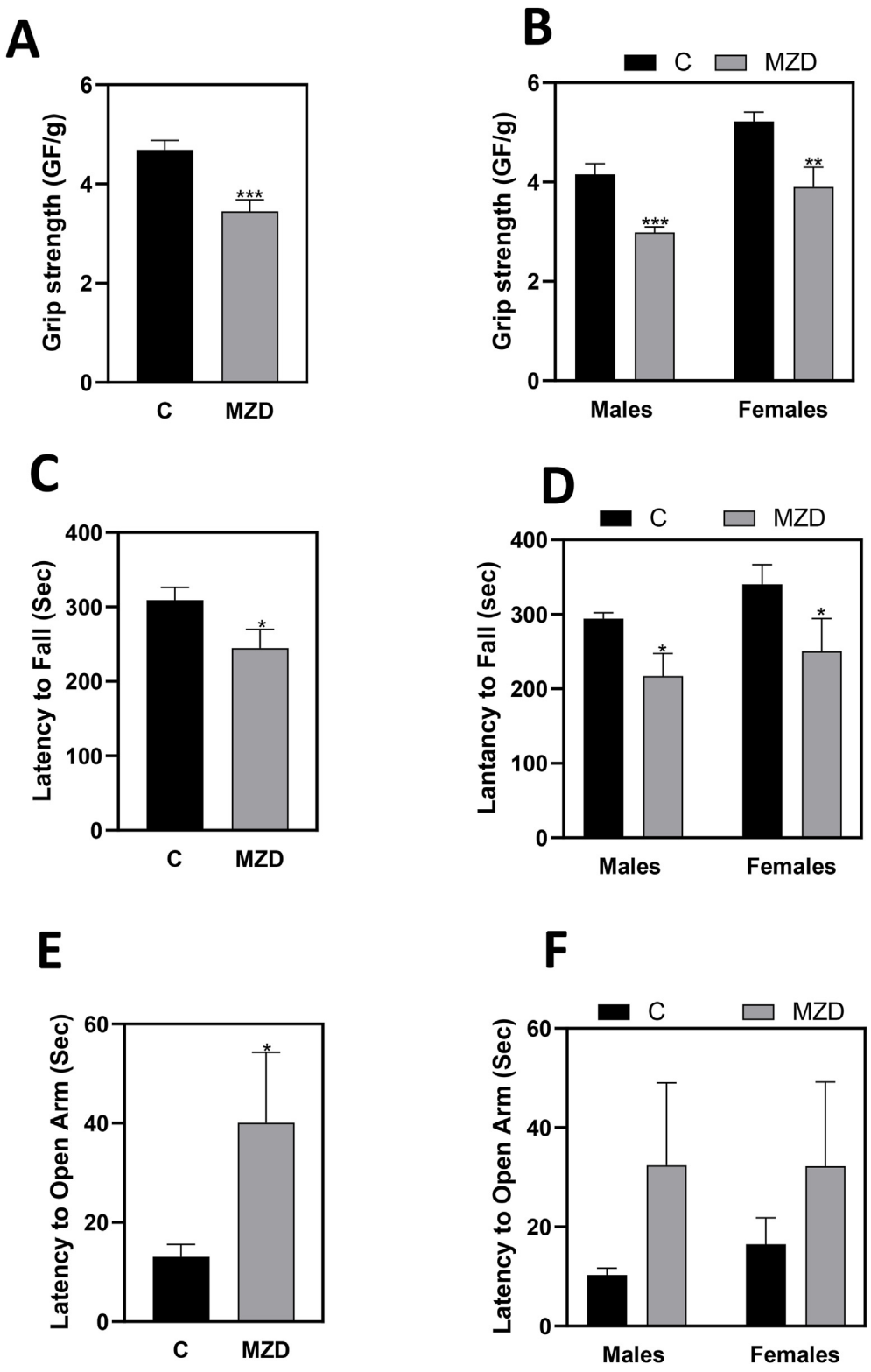


**FIGURE 9.** Maternal marginal zinc deficiency causes behavioral changes in the P60 rat offspring. (A, B) Grip strength in (A) all offspring and (B) males and females separately. Grip strength was measured in gram of force (GF) and normalized per gram of body weight. Results are shown as mean ± SEM of 5–9 litters (1 pup from each litter) per group. (C, D) Latency to fall in the rotarod test in (C) all offspring and (D) males and females separately. (E, F) Latency to enter the open arm in the T-maze in (C) all offspring and (D) males and females separately. Results are shown as mean ± SEM in offspring from 5–9 litters (1 pup from each litter) per group. **,***Significantly different from the control group (**$P < 0.01$, ***$P < 0.001$, Student's $t$ test).

transcription factors Olig1 and Olig2 relevant for oligodendrocyte generation. Olig2 and Sox10 act as lineage determinants during oligodendrogenesis. Shh is a morphogenic protein present throughout the brain during development, being involved in embryonic tissue induction and patterning [50]. Shh is involved in different processes that contribute to the development, maintenance, and repair of the CNS [51]. Shh is also important for OPCs proliferation and migration [52,53], differentiation [54], maturation, and myelination [55]. Thus, the treatment of primary cultures of OPCs with cyclopamine, a potent inhibitor of the Shh signaling activator Smo, decreases MBP and MAG expressions in differentiating oligodendrocytes [54].

Our results show that maternal marginal zinc deficiency decreased Shh activation at P2, which is in agreement with the decreased levels of Olig2 and Sox10, suggesting that ERK1/2 and Shh could crosstalk during oligodendrogenesis. In fact, Shh signaling activates proliferation and migration of fibroblast-like synoviocytes in vitro thought activation of the MAPK/ERK pathway [56]. Overall, Shh in combination with ERK1/2 alterations can, in part, explain the altered offspring brain oligodendrogenesis caused by maternal marginal zinc deficiency.

Postnatally, OPCs are originated from the VZ and then migrate to distant sites, while continuously dividing and differentiating throughout development, persisting into adulthood [57,58]. Maternal marginal zinc deficiency also disrupted offspring OPC differentiation into mature/myelinating oligodendrocytes as indicated by a decreased expression of CNPase and PLP at P2 and P5, and MBP, MAG, and MOG at P10 and P20, the most active myelination period. The decreased myelination in the marginal zinc-deficient offspring P20 brain was confirmed by histological stain and immunohistochemistry. Overall, results suggest that maternal marginal zinc deficiency affects oligodendrogenesis in the offspring's early stages of development (P2 and P5) causing hypomyelination at P10 and P20.

The observed alterations in oligodendrogenesis and myelination caused by early developmental marginal zinc deficiency had long-lasting adverse effects in the young male and female adult brain. Thus, consumption of a zinc-sufficient diet from weaning to P60 did not reverse or alleviate the hypomyelination and negative neuromuscular and neurocognitive outcomes in the offspring from MZD dams. These alterations were reflected in an impaired motor performance as measured using the rotarod and grip strength tests. Studies show that the number of falls from the rotating rod is increased in hypomyelinated/demyelinated [59,60] and multiple sclerosis mouse models [61]. Alterations in the grip strength test are observed in mouse models of white matter injury [62] and cuprizone-induced demyelination [60]. In terms of motor activity assessed in the open-field test maternal marginal zinc deficiency affected more females that males. However, mixed results are observed in animal models of demyelination, which showed no alterations [63,64], increased [59,64], or decreased [60] locomotor activity measured by total distance traveled in the open-field test. In terms of sex differential effects, previous evidence also showed sex differences in the behavioral and cognitive adverse responses to zinc deficiency. In this regard, female offspring from mouse dams exposed to zinc deficiency throughout gestation were more severely affected than males in terms of nest building, marble burying, and social novelty [65]. Also, in a model of maternal zinc deficiency induced by lipopolysaccharide injection at gestational days 15 and 16, only the male offspring showed impaired working memory and decreased cortical GAD67 expression [66]. Differences associated with the sex of the offspring can be attributed to an effect of maternal zinc deficiency on estrogen and progesterone metabolism. In support of this, we previously observed that maternal marginal zinc deficiency causes higher levels of liver estradiol only in the rat E19 male fetus [67].

Using the T-maze tests, higher [60] and lower [68] anxiety levels were reported in mouse models of demyelination. Although this test indicated higher levels of anxiety in the MZD offspring, how this can relate to the observed hypomyelination is not clear. However, the previously observed alterations in neurogenesis and neuronal specification in the young adult offspring from rat dams fed an MZD throughout gestation can, in part, contribute to the higher anxiety levels. Relevant to the current results, demyelination in mouse models is associated with schizophrenia-type behavior [69]. A decreased number of OPCs and mature oligodendrocytes, and consequently hypomyelination, have been observed in a mouse model of autism spectrum disorder [70,71]. Patients with multiple sclerosis, characterized by demyelination, have co-existing mental disorders such as schizophrenia [72]. The above evidence suggests that the rat offspring's hypomyelination caused by early maternal exposure to insufficient zinc intake may have similarities to the behavioral abnormalities observed in humans with diseases that affect the myelin sheath.

Other mechanisms can be involved in the deleterious effects of developmental zinc deficiency on motor performance, learning, and memory later in life. Among them, alterations in the regulation of the NMDA receptor expression are found in the rat offspring exposed developmental zinc deficiency [73]. Not only the expression of NMDA receptor subunits (NR1, NR2A, and NR2B) was lower in the offspring from dams fed zinc-deficient diets compared with controls, but they also showed lower expression of brain nerve growth factor and brain-derived neurotrophic factor. In addition, developmental zinc deficiency can lead to epigenetic mechanisms, which will have transgenerational impact, given the regulation by zinc of several enzymes involved in epigenetic modifications [74]. The process of oligodendrogenesis is, in part, regulated by the histone-modifying enzymes HDAC1 and HDAC2, which are zinc-dependent proteins [75]. Also, HDAC3 interaction with p300 histone acetyltransferase controls oligodendrocytes-specification gene Olig2 expression, and HDACs mutants exhibit fewer oligodendrocytes and OPCs and cause myelination defects [76].

In summary, current findings provide evidence that even a mild maternal zinc deficiency during early development can have long-lasting consequences on myelination in the adult offspring. This agrees with our previous report of an irreversible effect of maternal MZD on NSC proliferation that affects the number of neurons and astrocytes and neuronal subtype specification in the young adult brain [17,18]. A high susceptibility of the developing brain to decreased zinc availability and the associated disruption of both the neuronal and oligodendroglial lineages can have a major and irreversible impact on motor performance and behavior later in life.

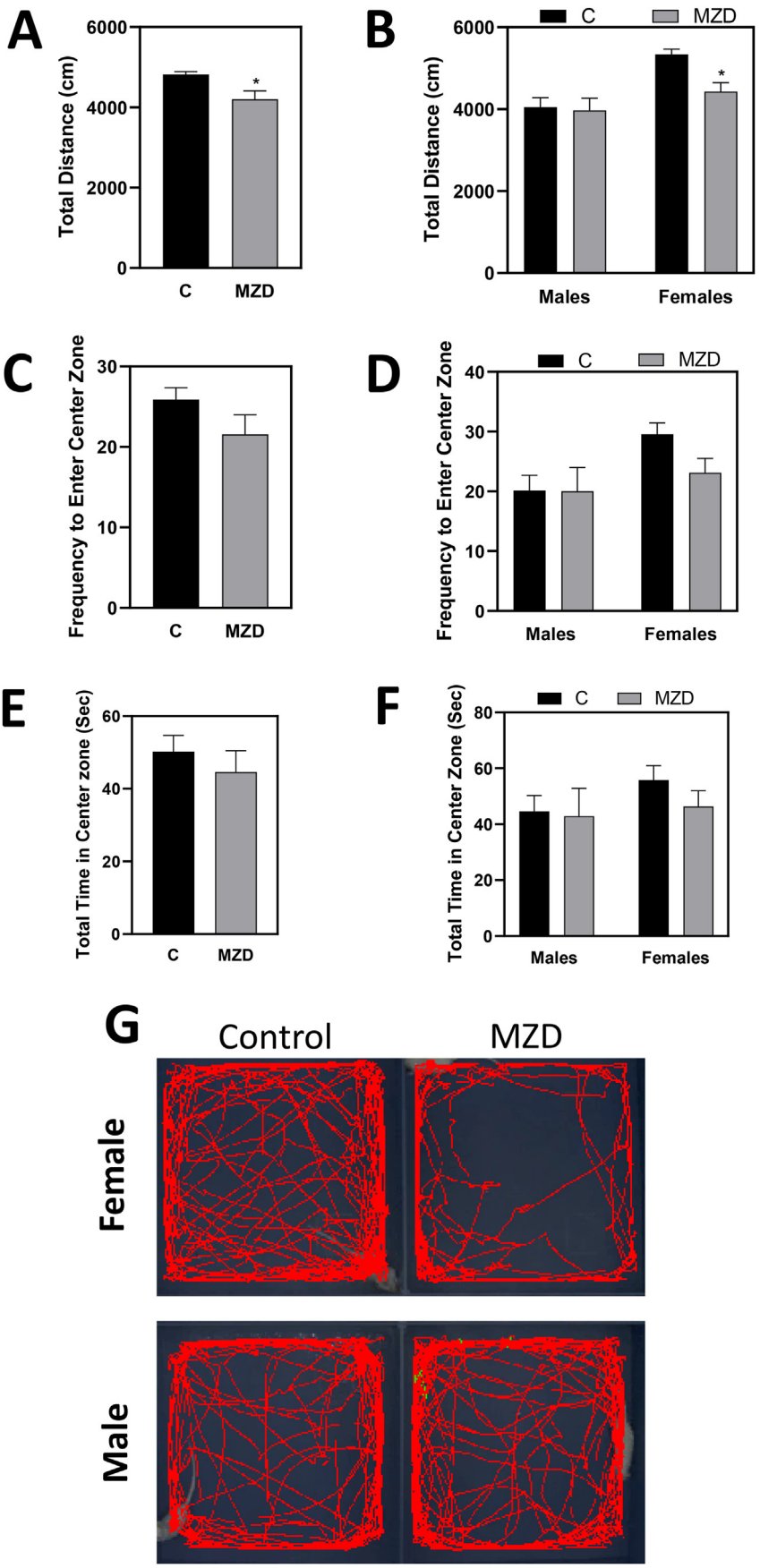


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**FIGURE 10.** Maternal marginal zinc deficiency causes behavioral changes in the P60 rat offspring. (A, B) Locomotor activity was measured as total distance traveled in an open-field test in (A) all offspring and (B) males and females separately. Offspring anxiety level was measured in the open-field test as (C, D) the frequency to enter center zone and (E, F) time spent in the center zone for (C, E) all offspring and (D, F) males and females separately. Results are shown as mean ± SE from 9 litters (1 pup from each litter) per group. (G) Representative open field track paths for control and MZD rats. Each track represents the total distance traveled by the animal during the 10-min period of the test.

## Author contributions

The authors' responsibilities were as follows—XL, PIO: designed the research; XL: conducted the research; XL: analyzed the data and performed the statistical analysis; XL: wrote the original draft; PO, AMA: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

## Conflict of interest

The authors report no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.08.029.

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