Effects of Developmental Zinc Deficiency on Neurogenesis and Oligodendrogenesis in the Offspring Rat Brain

By

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Table of Contents

Abstract

Adequate zinc intake during prenatal and postnatal period is important for brain development. During development, severe zinc deficiency can cause teratogenic effects including neuronal tube defects and multiple organ malformations. Marginal zinc deficiency during pregnancy and in the early postnatal period can have irreversible adverse effects on offspring neurodevelopment which can persist into adulthood. This can result in behavioral abnormalities including cognitive function, mood disorders, and motor dysfunctions. This thesis focused on investigating the effects of a decreased availability of zinc, primarily due to low zinc intake or secondarily to di-2-ethylhexyl phthalate (DEHP)-induced alterations in zinc homeostasis, during early development in rats on the genesis of the neuronal and/or oligodendroglial lineages. Cell signaling pathways that are involved in these processes were investigated. The impact of maternal marginal zinc deficiency on neurogenesis was evaluated in chapter one of the thesis. The progression of neurogenesis was evaluated at different developmental stages: embryonic day (E) 14, E19, postnatal day (P) 2, and P56. Results showed that the expression of protein markers neural stem cells (NSCs) (SOX2, PAX6), neuronal differentiation (TBR1, TBR2), mature neurons (NeuN), neuronal subtype glutamatergic neuron (VGLUT1) were significantly decreased due to maternal marginal zinc intake, whereas GABAergic neurons (GAD65) were not affected. This was associated with the downregulation of ERK1/2 signaling pathway, which participates in the regulation of NSCs self-renewal, proliferation, and differentiation. In chapter two we investigated the effects of maternal exposure to the environmental toxicant DEHP on neurogenesis. DEHP altered zinc homeostasis, decreasing zinc availability to the fetus in the developing E19 brain. Results

indicated that DEHP had similar adverse effects on neurogenesis as the nutritional marginal zinc deficiency, causing decreased NSCs proliferation and differentiation into neurons and particularly affecting the glutamatergic neuron subtype but not markers of GABAergic neurons. In *vivo and vitro* mechanistic studies showed that the ERK1/2 signaling pathway was inhibited (decreased phosphorylation) in part through the activation of protein phosphatase 2A. Finally, chapter three investigated the effects of maternal marginal zinc deficiency on oligodendrogenesis and myelination and the consequences on behaviors in the offspring rat. The progression of oligodendrogenesis was evaluated at different developmental stages: P2, P5, P10, P20 and P60. Results showed that the expression of markers for oligodendrocytes progenitor cells (OPCs) (NG2, PDGFRα), OPCs differentiation (CNPase), mature oligodendrocytes (MBP, MAG, MOG, PLP) and transcription factors (Olig2, SOX10) were significantly decreased in the marginal zinc deficient offspring rat brain at certain stages of oligodendroglial development. Behavioral assessment results showed that early stages maternal marginal zinc nutrition had long-term impact on neuromuscular functions, locomotor activity and anxiety in the offspring rat. In summary, results from this thesis stress the need of an adequate zinc availability during early development to prevent alterations in neurogenesis and oligodendrogenesis and the long-lasting consequences on brain structure and function.

Introduction

Prenatal and early postnatal zinc deficiency in humans have irreversible adverse effects on neurodevelopment [1]. The worldwide risk of zinc deficiency is estimated to be 17% in the general population [2]. The prevalence of zinc deficiency for pregnant women and children under 10 years of age is greater than 20% and can reach to 80% in some developing countries [2, 3]. Zinc is an important micronutrient during the processes of embryogenesis, fetal growth and development, and for mammary gland function, milk synthesis and secretion [4, 5]. Zinc plays essential roles in cell division, differentiation, survival, and function. Zinc-dependent enzymes, zinc-binding factors, and zinc-regulated signal molecules are involved in cell proliferation, differentiation, and maturation. Specifically, zinc is necessary for DNA and RNA synthesis, signal recognition and transduction, and gene expression [6-8]. Gestational severe zinc deficiency has teratogenic effects which affect multiple organs. Among them, severe zinc deficiency increases the frequency of neural tube defects (NTDs), decreases brain size, and decreases the content of lipids, DNA, RNA, and proteins in the brain [9-11]. Marginal/mild zinc deficiency (MZD) is more common at reproductive age and in young children [12, 13]. MZD is associated with poor pregnancy and fetal outcomes and neurocognitive development both in human and animals [1, 14-17]. MZD during pregnancy causes a reduction in both, near term fetal and placental weights and disruption in placental development and function [18]. A large body of research has shown that prenatal/early postnatal exposure to MZD have negative effects on brain development that can lead to behavioral changes. In this regard, developmental MZD causes altered behavior, cognitive and motor performance [19-21], inattentive symptoms [22], and depression [23].

Supplementation with zinc improves pregnancy outcomes, birth weights and children cognitive function, intelligence scores, motor activities, and emotional performance [24-26].

Zinc deficiency and neurodevelopment

Zinc regulates a variety of cellular events such as the proliferation, differentiation, maturation and survival of neurons during brain development [27]. In the fetal brain, MZD inhibits the activation of transcription factors, i.e., activating protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Nuclear factor of activated T-cells (NFAT), that are important for neural cells proliferation, differentiation, and survival [28]. Female mice fed a marginal zinc deficient diet from gestational day one (GD1) significantly decreases the number of proliferating neural stem cells (NSCs) [29], whereas zinc supplementation promotes NSCs proliferation in the subventricular zone (SVZ) of the newborn offspring [30]. Zinc deficiency decreases the expression of the NSCs marker nestin during the prenatal and postnatal period, suggesting that zinc is important for both embryonic and postnatal NSCs proliferation [29]. Zinc deficiency also impairs neuronal differentiation. Human induced pluripotent stem cells (hiPSCs) incubated in zinc deficient medium have high apoptotic rate and fail to differentiate into motor neurons [31]. Moreover, Zinc deficiency reduces the activation of transcription factors that participate in retinoic acid-stimulated neuronal differentiation, specifically to, zinc finger proteins such as the retinoid X receptor (RXR) and activating transcription factor 2 (ATF2) and non-zinc finger proteins such as paired box protein 3 (PAX3), signal transducer and activator of transcription 1 and 3 (STAT1 and 3), and SRY (sex

determining region Y)-box 9 (SOX9) [32]. Furthermore, zinc deficiency alters gene expression in hippocampal neuron, decreases markers for neuronal differentiation including doublecortin and β-tubulin type III (Tuj1) and down-regulates the transforming growth factor-beta (TGF-β) signaling pathway that plays a key role in NSCs differentiation [33]. Moreover, zinc regulates NSCs proliferation and neuronal cell survival. Zn deficiency inhibits cell proliferation through the arrest of the cell cycle at the G0/G1 phase and induces apoptosis by increasing pro-apoptotic and decreasing anti-apoptotic proteins expression in both, human neuroblastoma IMR-32 cells and primary cultures of rat cortical neurons [34]. Zinc deficiency significantly diminishes NSCs proliferation and increases NSCs apoptosis in the sub-granular zone (SGZ) and granular cell layer of the dentate gyrus in part through a p53-dependent mechanism [35]. Although neuronal proliferation and differentiation and survival have been studied in a variety of aspects, the effects of zinc deficiency on neuronal migration, maturation, and specification remain to be investigated. Additionally, there is currently limited experimental evidence on whether deficits associated with maternal zinc deficiency affect long-term learning and memory, behavioral and emotional changes.

Zinc deficiency and cell signaling, cell signaling involved in neurogenesis

Zinc deficiency affects signal transduction pathways that regulate NSCs proliferation, differentiation, apoptosis and axon outgrowth including transcription factors AP-1, NF-κB and NFAT [36-38]. In the developing fetal brain, zinc deficiency causes tubulin oxidation and impaired polymerization, which affects the nuclear translocation of transcription factors AP-1, NF-κB, NFAT and STAT3. This consequently decreases their DNA binding

activity, which ultimately leads to a lower transcription of target genes, including many involved in neurogenesis [28, 39-41]. AP-1, NF-κB, NFAT, and STAT3 transcription factors regulate several cellular processes including neuronal cell proliferation, differentiation, apoptosis, and axonal outgrowth during brain development [34, 36-38, 41-47]. Additionally, zinc deficiency causes neuronal cell death by promoting apoptosis, increasing the activation of caspase 3 in primary culture of cortical neurons, human neuroblastoma IMR-32 cells [34] and in the hippocampus [48]. According to Izsak et al., TGF-β is a signaling protein that promotes neuronal differentiation, and a low TGF-β receptor expression is associated with altered differentiation in the developmental rat brain [33, 49]. Neuronal migration is an important process in brain development, and it requires cytoskeleton integrity [50]. Thus, a dysfunctional cytoskeleton caused by zinc deficiency-induced oxidative stress could potentially disrupt neuronal migration [39].

The disruption of cell signaling by zinc deficiency is in part due to the associated alterations in redox homeostasis. Zinc is a component of the body's antioxidant defense system, acting at different levels and through several mechanisms during neurodevelopment [51]. In this regard, zinc is required for: i) the synthesis of the major antioxidant, glutathione [52, 53], ii) indirectly inhibition for oxidant (reactive oxygen/nitrogen species) generation [54], iii) preventing oxidative product formation by competing the binding site with redox-active metals ($Fe^{2+,}$ Pb^{2+}) [55, 56], iv) upregulation of signal pathway (NF-E2 related factor 2 (Nrf2)) that promotes antioxidant defensive response [57, 58], v) regulation of glutathione metabolism and association/dissociation with protein thiols group to regulate redox status [53, 59]. As a result, dysbalanced

neuronal oxidant/antioxidant system induced by zinc deficiency could affect neurogenesis during brain development.

Neurogenesis is a process of neuronal cells generation that occurs in embryo and adulthood. The adult vertebrate central nervous system (CNS) contains four major cell types including neurons, myelin-producing oligodendrocytes, astrocytes, and ependymal cells [60]. During early development in vertebrate embryo, neural fate specification originates from ectoderm by the underlying notochord. Next, the neural plate goes through processes of formation, bending and closure, eventually structuring into neural tube. The neural tube wall is made up neuroepithelial cells that can switch their identity to radial glial cells (RGCs) which give rise to all neurons, directly or indirectly, and later, to glial cells, astrocytes and oligodendrocytes (**Fig. 1**) [61]. In this way, RGCs switch from symmetric to asymmetric divisions, generating an RGC daughter cell and, in certain areas of the brain, the intermediate progenitor cells (IPs). IPs can undergo symmetric divisions yielding two neurons in VZ [62-64]. In the VZ, RGCs can directly differentiate into immature neurons and migrate to different layer of the neocortex, where neurons become mature and specify into different subtypes [65]. The peak of neurogenesis occurs from embryonic day (E) 14 to birth in rodents and from week 4 to birth in humans [64]. There are several molecules that are specific to neuronal proliferation, differentiation, and survival in the developing brain. Sox2, Pax6, and Empty Spiracles Homeobox 1 (EMX1) are transcription factors present in NSCs and are involved in NSCs proliferation [66-68]. The transcription factors PAX6, TBR2, TBR1 expressed by RGCs, IPs and postmitotic neurons, respectively, regulate different stages of neuronal process including NSCs proliferation, NSCs differentiation into IPs, and from IPs differentiation into cortical

neurons. Tbr1 and Tbr2 belong to the T-box transcription factor subfamily Tbr1 and are involved in the differentiation of glutamatergic neuron in cerebral cortex, olfactory bulbs, hippocampal dentate gyrus, cerebellum and adult SVZ [69, 70]. In later stages of neurogenesis, all the mature neurons express neuronal nuclei protein (NeuN), and neurons start to specify into different subtypes [71]. The glutamatergic (excitatory) neurons express vesicular glutamate transporters (VGLUTs) [72], and GABAergic (inhibitory) neurons express glutamate decarboxylase (GAD) [73, 74].

ERK1/2 downstream proteins (e.g., EGR1, TGF-β) are also linked to cell proliferation, differentiation, and survival [77]. Moreover, in embryonic rat brain, the markers of proliferating NSCs Sox2 and Nestin are found in the same brain region where EGR1 is expressed [75], supporting the concept that ERK1/2 is involved in early neuronal development, particularly to proliferation events. However, the relationship between MZD induced ERK1/2 inactivation and the expression of transcription factors, i.e., Sox2, Tbr2, Tbr1 and NeuN that are specific to the neurogenic lineage during embryogenesis remains unknown and further investigation is needed.

The ERK1/2 and SHH signaling pathways and neurogenesis

The extracellular signal-regulated kinase 1 and 2 (ERK1/2) signal pathway regulates cell growth, differentiation, and survival [76]. Mitogens or growth factors, including brain-derived neurotrophic factor (BDNF) insulin growth factor I (IGF-I), and vascular endothelial growth factor (VGFR) [77], epidermal growth factor (EGF) and platelet derived growth factor (PDGF) [78] activate ERK1/2. Upon binding of growth factors to their receptors, receptors are dimerized and lead to a conformational change of rat sarcoma

virus (RAS) from guanosine diphosphate (GDP)-bound RAS to guanosine triphosphate (GTP)-bound RAS, GTP-bounded RAS then activates proto-oncogene serine/threonine protein kinase RAF. Activated RAF phosphorylates and activates mitogen-activated protein kinase kinase (MAPKK) 1 and 2 **(**MEK1/2)**.** Activation of MEK1/2 directly leads to the phosphorylation of ERK1/2. Activated ERK1/2 phosphorylates multiple substrates including ribosomal S6 kinase (RSK) and MAPK interacting Kinase (MNK) and transcription factor E26 transformation-specific (ETS) Like- 1 (ELK1) and ultimately lead to gene expression of many proteins that regulate multiple cellular processes [76]. Phosphorylation of ERK1/2 is a key step to promote the translocation of ERK1/2 to the nucleus to activate one of its downstream transcription factor ELK1. ELK1 activation promotes the expression of genes involved in cell growth (protein components of the NF-κB and AP-1 pathways), cell survival (B-cell lymphoma 2 (BCL2), myeloid cell leukemia 1(MCL1)), cell cycle (cyclin D1 (CCND1), cyclin dependent kinases (CDKs), p21) and neuronal growth factors (TGF-α, TGF-β, early growth response-1 (EGR-1) [79, 80] ERK1/2 can be de-phosphorylated by protein phosphatase 2A (PP2A), which is a key protein that inhibits ERK1/2 activation and the downstream expression of target genes (**Fig. 2**) [81]. ERK1/2 has been extensively linked to synaptic plasticity, learning and memory [82], cognitive behavior [83], and emotional modulation [84]. In neuronal cells, ERK1/2 phosphorylation is initiated when BDNF binds to a tyrosine kinase receptor, which leads to the transcription of genes involved in neuronal differentiation [85]. ERK1/2 also plays an important role in regulating neurodevelopment by promoting NSCs proliferation [86-88], astrocyte proliferation and differentiation [89], and neuronal cell survival [90]. Developmental MZD is associated to ERK1/2 deactivation. Gestational MZD decreases

the level of phosphorylated ERK1/2 with a lower number of proliferating NSCs in the SVZ of developing fetal rat brain [91]. ERK1/2 is important for neurodevelopment by upregulating neuronal cell proliferation, differentiation, and survival. *In vitro*, zinc deficiency causes ERK1/2 inactivation in human neuroblastoma IMR-32 cells leading to a decreased cell proliferation [92]. However, there is limited information on how zinc depletion affects ERK1/2 modulation during early development and how this is associated with alterations in neuronal and glial cells proliferation, differentiation, and survival.

Sonic hedgehog (SHH) signal pathway regulates neuronal cell proliferation, differentiation, and maturation in the CNS [93]. Briefly, the SHH signal pathway is activated when cleaved SHH binds to its receptor patched 1 (PTCH1). Upon SHH binding, PTCH1 relieves its inhibition on the G-protein-coupled receptor smoothened (SMO). SMO is an intracellular regulatory protein that acts on mesenchymal Suppressor of Fused **(**SUFU), which will release the Glioma-associated oncogene homolog (GLI) and promote its translocation to the nucleus. When the SHH ligand is not present, PTCH1 inhibits SMO, consequently SUFU binds directly to the GLI protein and prevents its translocation to the nucleus, inhibiting the pathway. GLI is a zinc finger protein that regulates the expression, among other genes, of SOX2 and Olig2 [94] (**Fig. 3**). Numerous studies showed that the SHH signaling pathway is essential for NSCs proliferation, differentiation, and maturation [93]. Specifically, SHH has mitogenic effect that regulates hippocampus NSCs proliferation in the adult brain [95]. During embryonic brain development, SHH expresses in different brain regions at different time points regulating NSCs proliferation. At early stages, SHH appears in the ventral forebrain later in the dorsal and cerebellar areas where NSCs are actively dividing [96, 97]. Additionally, SHH promotes the

differentiation to motor neurons under the stimulation of neurotrophin 3 (NT3) in the spinal cord [98] and regulates neuronal subtype specification, specifically, promoting dopaminergic neurons formation in the hypothalamus [99].

Zinc deficiency affects myelin structure

Previous studies showed that zinc plays important roles in maintaining oligodendrocyte integrity and myelination [100, 101]. Rats fed a zinc deficient diet for 4-7 weeks starting at 3 weeks of age, have significantly thinner myelin sheath in the optical nerve, suggesting that zinc is essential for maintaining CNS myelin sheath structure [102]. A similar study showed that 3 weeks old rats fed a zinc deficient diet for 4 weeks have lower number of myelinated axons in the sciatic nerve [103]. Moreover, young rats (27 days old) fed a zinc deficient diet for 4 weeks have significantly lower levels of myelin basic protein (MBP), indicating a decreased of myelin content [104]. Furthermore, MBP is a zinc binding protein [105], and MBP can act as a myelin-stabilizing protein by interacting with microtubules in the myelin sheath during the oligodendrocyte differentiation process [106]. Thus, by affecting MBP expression, zinc deficiency could destabilize myelin and potentially induce demyelination. Maternal MZD from E0 or from E14 until postnatal day (P) 20 have long term effect on myelin protein profiles, with a higher percentage of high molecular weight proteins and a lower percentage of small and large MBP in myelin in the offspring rat brain [107], however, the underlying mechanism is unknown. There is very limited published evidence on the effects of maternal zinc deficiency on oligodendrogenesis, specifically on oligodendrocyte progenitor cells (OPC) proliferation, differentiation, and ultimately oligodendrocyte maturation and myelination.

Oligodendrogenesis

In the developing brain, NSCs give rise to neurons, OPCs, astrocyte progenitor cells (APC) and ependymal cells [60] (**Fig. 1**). Oligodendrocytes are the responsible cells for myelin sheath formation and axonal development and integrity in the CNS [108, 109]. Oligodendrocytes appear around E12.5 in rodents during embryonic CNS development and complete their maturation after birth when myelination takes place. Oligodendrocytes from spinal cord originate from a restricted ventral area under the regulation of the SHH signaling. A second wave of oligodendrogenesis starts at E15 in dorsal areas, where RGCs differentiated into IPs and then from IPs to OPCs, and these processes are not influenced by SHH signaling [110, 111]. In the telencephalon, there are three waves of OPC generation. The first wave occurs at E12.5 in the medial and ventral medial ganglionic eminence and then migrate into all parts of telencephalon and the second one takes place in the lateral ganglionic eminence (LGE) at E15. Next, OPCs from LGE migrate dorsally to the cortex at E18 and the last wave of oligodendrogenesis occurs in the cortex locally after birth [112, 113], The development of oligodendroglial lineage progresses through different consecutive stages starting from early OPC, late OPC or preoligodendrocyte, immature oligodendrocyte and finally mature or myelin-forming oligodendrocyte. Each of these stages is characterized by the expression of specific proteins markers and exhibiting different morphological features (**Fig. 4**) [114]. Nerve/glial-antigen **2** (NG2) and platelet-derived growth factor receptor-alpha (PDGFα) are expressed in OPCs [115]. NG2 is an integral membrane chondroitin sulfate proteoglycan that plays a role in inflammatory demyelination diseases and may implicate in re-myelination [116]. PDGF is a potent mitogen that specifically binds to PDGFα at the

cell membrane of OPCs to induce OPCs proliferation [117]. Oligodendrocyte transcription factors 1 (Olig1) and 2 (Olig2) are markers of oligodendrogenesis, both can be found along the progeny, in OPCs, immature oligodendrocytes and mature oligodendrocytes [118]. Transcriptional factor Sox10 functions as a signaling molecule that directs IPs differentiation into OPCs in the developing and adult brain [119]. 2',3'-Cyclic-nucleotide 3'-phosphodiesterase, also known as CNPase, is an enzyme found in the CNS, playing a role in oligodendrocytes differentiation and myelination [120]. In mature oligodendrocytes, myelin-specific proteins are present in the cell membrane or the cytoplasm. MBPs are located in the cytoplasm of mature oligodendrocytes playing a critical role in maintaining myelin integrity by adhering opposing cell lipid bilayer [121]. Proteolipid protein (PLP) is the most abundant cell membrane integral protein of oligodendrocytes, and it is claimed to participate in cell signal transduction pathways involved in myelin formation [122]. Myelin oligodendrocyte glycoprotein (MOG) is less abundant than other CNS myelin proteins and are located on the outermost of myelin sheath and are thought to play a role in adhering myelin fibers together in the CNS [123].

The ERK1/2 and SHH signaling pathways and oligodendrogenesis

ERK1/2 signaling pathway regulates oligodendrogenesis. Xiao et al. demonstrated that BNDF induces oligodendrocyte myelination which is in part regulated by ERK1/2, whereas *in vitro* inhibition of ERK/1/2 pathway significantly decrease the level of myelin proteins such as MBP and myelin associated glycoprotein (MAG) in the OPC culture [124]. Similar findings indicate that the inhibition of MEK1/2, an upstream kinase of ERK1/2 signaling, causes a reduced expression of myelin proteins (MBP, CNPase),

suggesting that ERK1/2 activation is essential for oligodendrocyte differentiation [125]. Fyffe-Maricich et al. showed that deletion of ERK2 in embryonic mouse brain has no effects on the proliferation and generation of OPCs, whereas oligodendrocyte differentiation and MBP expression are impacted [126]. A similar study demonstrated that ERK1/2 knockout mice have a decreased myelin thickness but the number of NG2 positive cells is not affected, indicating that ERK1/2 is important for myelination but not for OPC proliferation [127]. The ERK1/2 signal pathways may also have a role facilitating OPCs migration with the stimulation of chemokine C-X-C motif chemokine ligand 12 (CXCL12) production, which is also a regulatory protein of OPC proliferation. Inhibition of ERK1/2 significantly decreased OPCs migration [128].

In early research, SHH derived from the notochord and floor plate was found to be required for the development of the oligodendroglia lineage [129]. SHH is necessary and sufficient to trigger the expression of genes encoding for Olig1 and Olig2, which are associated with the oligodendroglial cell lineage in the ventral region of the neural tube and dorsal SVZ [130]. SHH appears to promote OPCs differentiation towards mature oligodendrocytes, whereas bone morphogenetic protein 4 (BMP4) has inhibitory effects on oligodendrocyte maturation [131]. In addition, SHH inhibitors alter OPCs differentiation and decrease the expression of myelin proteins such as MBP and MAG, in primary oligodendrocytes culture [132, 133]. While the signaling pathways that regulate OPCs proliferation, differentiation, and maturation specifically towards oligodendrocytes lineage have been characterized, there is negligible evidence on the effects of maternal zinc deficient diet on ERK1/2 and SHH signal pathways and their consequences on oligodendrogenesis during prenatal and early postnatal neurodevelopment.

Secondary zinc deficiency and DEHP

Zinc deficiency can be primary due to insufficient intake or secondary to several physiology and pathological conditions. Secondary zinc deficiency can be induced by decreased zinc absorption, which can be affected by age [134], gastric intestinal surgeries [135], intestinal disease (Celiac disease) [136, 137], vegetarian diets [138] and excess alcohol consumption [139, 140]. Zinc deficiency can also occur due to increased zinc losses in the urine in individuals with kidney disease [141] or diabetes [142]. Zinc availability can decrease due to infections and inflammation [143], obesity and diabetes [144], and exposures to toxicants such as di(2-ethylhexyl) phthalate (DEHP) [145]. These conditions can induce an acute phase response, which results in an increased expression of the zinc binding protein metallothionein in the liver. As a consequence, zinc accumulates in the liver and the amount of zinc available in the plasma and other tissues decreases [143]. In the case of DEHP, gestational exposure to DEHP in mice alters the expression of genes that are involved in zinc metabolism, increasing metallothionein and decreasing zinc transport proteins (ZNTs) levels in maternal liver, resulting in increased maternal zinc storage in the liver and less zinc is delivered to the embryo [145, 146]. Previous study from our group shows that rat dams exposed to DEHP throughout gestation have increased circulating levels of interleukin-6 which activates the STAT3 signaling pathway in the liver leading to increased metallothionein expression, which could contribute to maternal liver zinc accumulation. As a result, less zinc is availability to conceptus and fetus [147]. Importantly, DEHP caused dysbalanced of zinc distribution have potential effects on fetal neurodevelopment.

DEHP and Neurodevelopment

DEHP is one of the plasticizers that are widely used in the production of polyvinyl chloride (PVC). DEHP can be found in many plastics products such as medical intravenous bags, medical tubing, blood bags, artery catheters and many household products, cosmetics, baby bottles, and toys [148-150]. Early studies showed that DEHP is a potential endocrine disruptor [151], causing reproductive dysfunction, decreasing sperm production and promoting obesity [152, 153]. DEHP exposure can have other adverse health effects including liver disease [154] and cardiovascular disease [155], renal fibrosis [156], and impaired lung development and growth restriction in newborns [157]. Moreover, DEHP is a potent neurotoxin that has detrimental effects on neurodevelopment [158]. Exposure to high amounts of DEHP during pregnancy has teratogenic effects including low maternal weight gain, increased frequency of resorptions and fetal NTDs, increased frequency of pre-term delivery and infant/children growth retardation [145, 159]. Cognitive deficits and lower numbers of neurons and synapses have been observed in adult offspring rats after perinatal exposure to DEHP [160], suggesting that early developmental DEHP exposure has long-term effects on neurodevelopment. Maternal exposure to DEHP is associated with altered children's intellectual quotient scores [161], increased risk of autism spectrum disorders [162], decreased infant attention and social interactions [163], impaired cognitive function [164], decreased learning and memory, and increased anxiety and depression [165, 166].

Summary of current knowledge and future perspective

Maternal zinc deficiency imposes a potential risk for normal brain development, in general. However, more research is needed to understand the effects of zinc deficiency,

in particular, on neurogenesis and oligodendrogenesis. Given that different molecular and signaling pathways could be differentially regulated by zinc, the effects of primary zinc deficiency caused by insufficient zinc intake on the neurogenesis and oligodendrogenesis should be compared with secondary zinc deficiency induced by maternal exposure to toxicants. Our hypothesis is that: 1) low maternal zinc intake affect cellular processes of neurogenesis, specifically at the level of cell proliferation, differentiation, maturation, and specification, in the fetal brain, 2) zinc deficiency secondary to the maternal exposure to DEHP affects zinc homeostasis and consequently impairs neurogenesis in the offspring, 3) low maternal zinc intake affects oligodendrogenesis impairing OPCs proliferation, differentiation, maturation, and, consequently, myelination, in the offspring and 4) maternal MZD causes long-term alternations on behavior in the adult offspring.

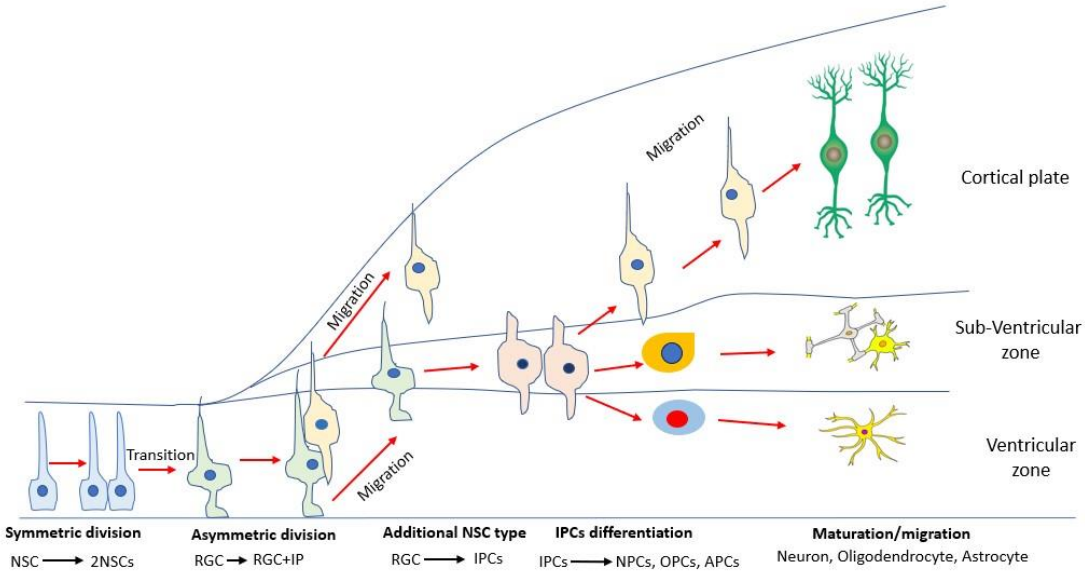

Figure 1. Embryonic brain development. After neural tube formation, neural stem cells (NSCs) first undergo symmetric division from one NSC to two NSCs, next, NSCs transit to radial glial cells (RGCs), which undergo asymmetric division from one RGC to another RGC and neural intermediate progenitor cell (nIPC) that can differentiate into neurons in the ventricular zone. RGCs can migrate to the subventricular zone and further differentiate into intermediate progenitor cells (IPCs), which further differentiate into nIPCs, oligodendrocyte intermediate progenitor cells (OPCs), and astrocyte intermediate progenitor cells (APCs) in the subventricular zone, and these progenitor cells can undergo differentiation and maturation further into neurons, oligodendrocytes, and astrocytes, respectively. Adapted from [63].

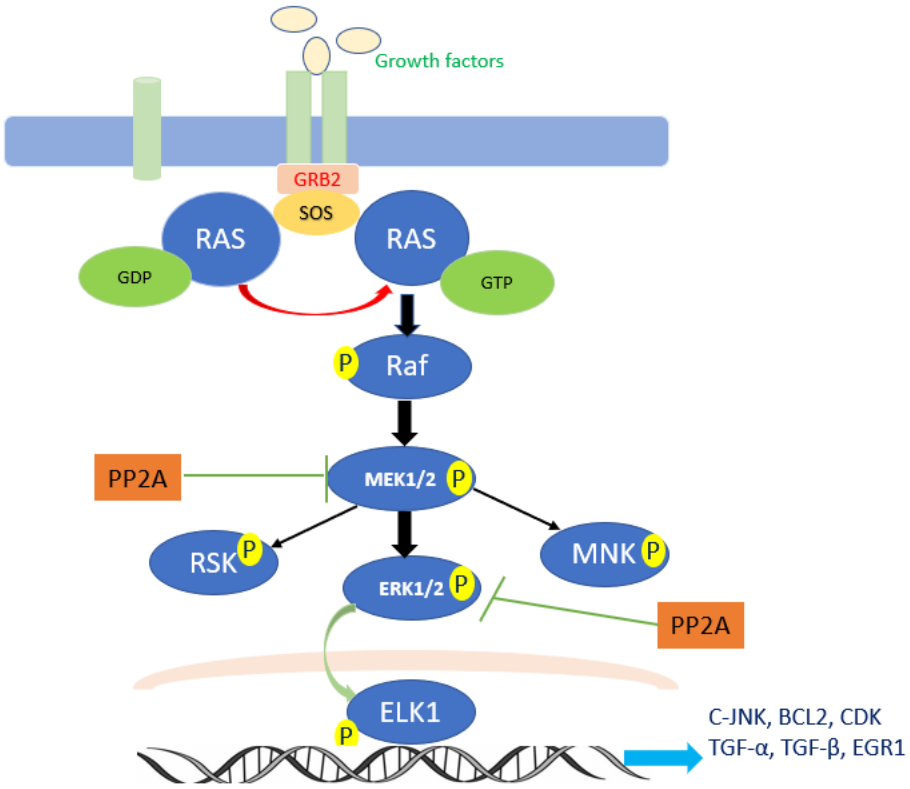

Figure 2. Extracellular signal-regulated kinase (ERK)1/2 pathway. The ERK1/2 pathway is activated when mitogens and growth factors bind to upstream receptor tyrosine kinases (RTK). Binding to the receptor triggers recruitment of adaptor proteins (e.g., Growth factor binding protein 2 (GRB-2)) and exchange factors son of sevenless (SOS), which causes a conformational change of the rat sarcoma virus (RAS) protein from RAS-GDP to RAS-GTP, which cause phosphorylation and activation of Raf. This leads to the phosphorylation of mitogen-activated protein kinase kinase (MAPKK) 1 and 2 (MEK1/2), which activates ERK1/2 by phosphorylation. Activated ERK1/2 then translocate to the nucleus where it phosphorylates, among many targets, E26 transformation-specific (ETS) Like- 1 (ELK1). The ERK 1/2 pathway is inhibited by protein phosphatase 2A (PP2A) in the cytosol. ERK1/2 also phosphorylates many other cytosolic and nuclear targets. Adapted from [79].

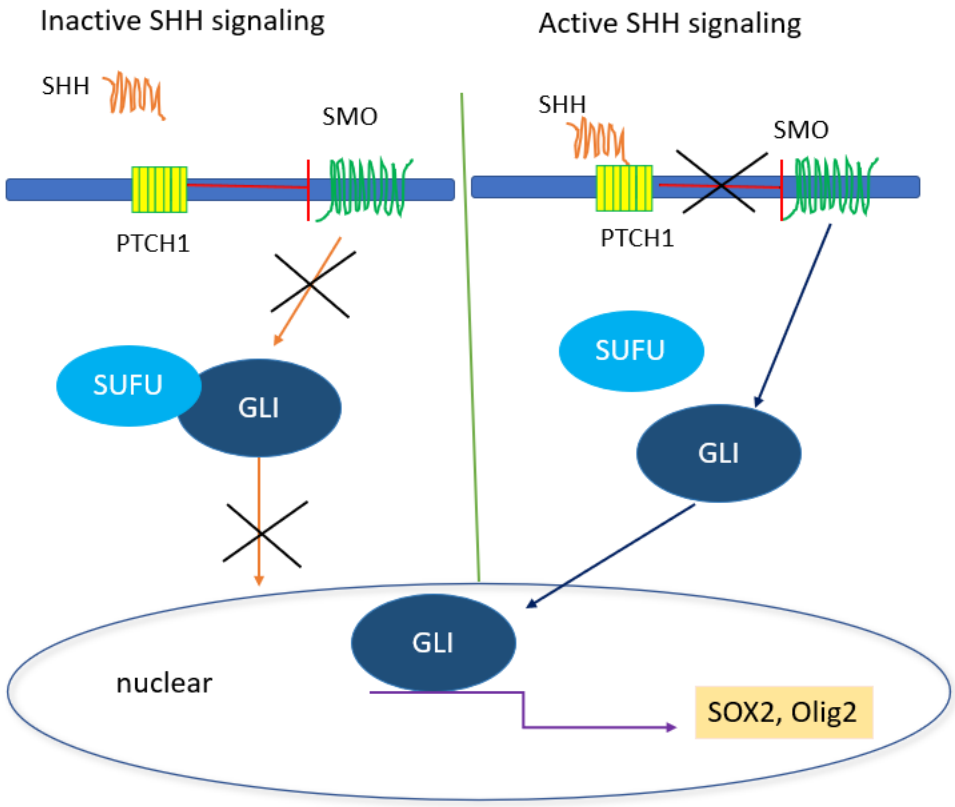

Figure 3. Sonic Hedgehog (SHH) signaling pathway. Inactive signaling (left) occurs when the SHH ligand is not bound to Patched 1 (PTCH1), which inhibits smoothened (SMO), resulting in Glioma-associated oncogene homolog (GLI) sequestration in the cytoplasm by mesenchymal Suppressor of Fused **(**SUFU). In the presence of SHH (right), PTCH1 suppression of SMO is released, resulting in the nuclear accumulation of GLI and activation of target genes that promote oligodendrogenesis. Adapted from [93].

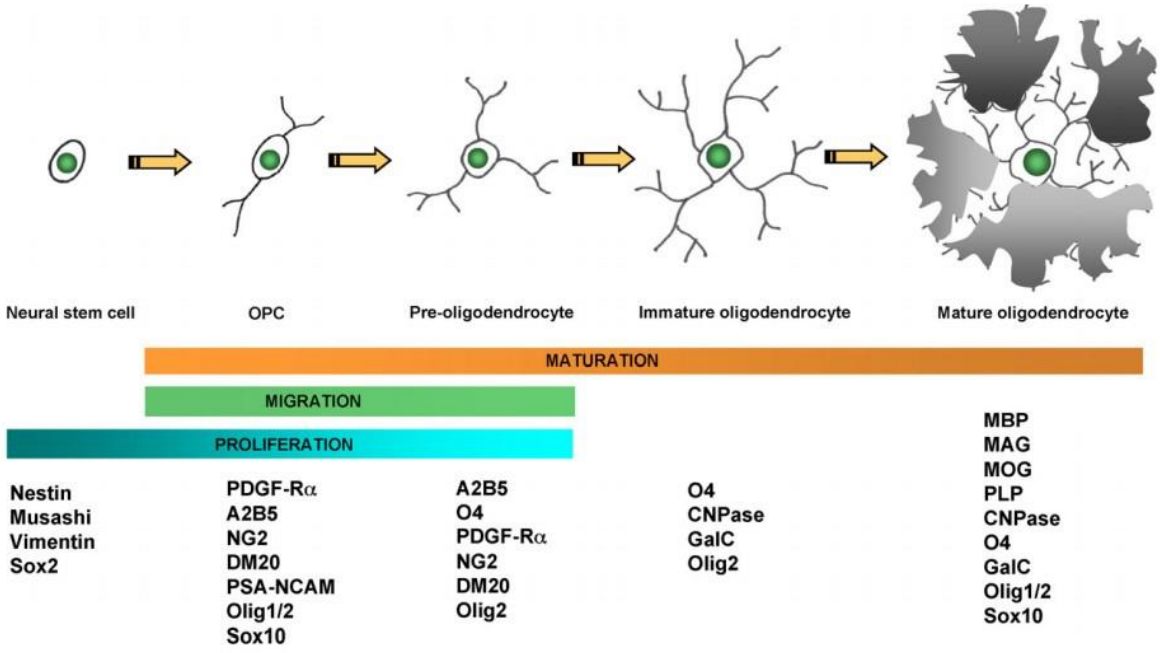

Figure 4: Schematic representation of the development of the oligodendroglial lineage (OL) from neural stem cell (NSC). The morphology (top) of NSC, oligodendrocyte progenitor cell (OPC), pre-oligodendrocyte, immature oligodendrocyte and mature oligodendrocyte and specific cell markers (bottom) expressed by each developmental stage of OL. Adapted from [114].

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Chapter one

Early Developmental Marginal Zinc Deficiency Affects Neurogenesis Decreasing Neuronal Number and Altering Neuronal Specification in the Adult Rat Brain

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Early Developmental Marginal Zinc Deficiency Affects Neurogenesis Decreasing Neuronal Number and Altering Neuronal Specification in the Adult Rat Brain

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Abbreviations: ERK1/2; Extracellular signal-regulated kinase 1 and 2; GAD65, glutamic acid decarboxylase 65; NeuN, neuronal nuclei protein; PAX6, Paired box protein 6; PP2A, protein phosphatase 2A; SOX 2, SRY (sex determining region Y)-box 2; TBR1 and TBR2, T-box brain 1 and 2; VGLUT1, vesicular glutamate transporter 1.

Abstract

During pregnancy, a decreased availability of zinc to the fetus can disrupt the development of the central nervous system leading to defects ranging from severe malformations to subtle neurological and cognitive effects. We previously found that marginal zinc deficiency down-regulates the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway and affects neural progenitor cell (NPC) proliferation. This study investigated if marginal zinc deficiency during gestation in rats could disrupt fetal neurogenesis and affect the number and specification of neurons in the adult offspring brain cortex. Rats were fed a marginal zinc deficient or adequate diet throughout gestation and until postnatal day (P) 2, and subsequently the zinc adequate diet until P56. Neurogenesis was evaluated in the offspring at embryonic day (E)14, E19, P2, and P56 measuring parameters of NPC proliferation and differentiation by Western blot and/or immunofluorescence. At E14 and E19, major signals (i.e., ERK1/2, Sox2, and Pax6) that stimulate NPC proliferation and self-renewal were markedly downregulated in the marginal zinc deficient fetal brain. These alterations were associated to a lower number of Ki67 positive cells in the ventricular and subventricular zones. Following the progression of NPCs into intermediate progenitor cells and into neurons, Pax6, Tbr2 and Tbr1 were affected in the corresponding areas of the brain at E19 and P2. The above signaling alterations led to a lower density of neurons and a selective decrease of glutamatergic neurons in the young adult brain cortex exposed to maternal marginal zinc deficiency from E14 to P2. Current results support the concept that marginal zinc deficiency during fetal development can disrupt neurogenesis and alter cortical structure potentially leading to irreversible neurobehavioral impairments later in life.

1. Introduction

Prenatal zinc deficiency resulting from insufficient dietary intake, absorption, or transport can compromise development of the central nervous system leading to a spectrum of defects ranging from severe congenital malformations to subtle neurological and cognitive impairments. Severe zinc deficiency during fetal development has been implicated as a mechanism contributing to neural tube defects (NTDs). Severe dietary zinc deficiency in rats during pregnancy leads to NTDs in association with decreased cell proliferation in the ventricular zone of the fetal brain [1]. In humans, supplementation with dietary zinc and adequate plasma zinc concentrations are related to a reduced risk of NTDs [2, 3]. Although developmental marginal zinc deficiency does not cause gross malformations like NTDs, it is associated with neurological morbidity such as impairments in learning, working memory, and social behavior [4]. Similar cognitive defects result from secondary zinc deficiency caused by gestational exposure to infection in rats [5].

Developmental exposure to a decreased zinc availability could have a long-term and irreversible impact on the offspring's brain leading to neurological and behavioral disorders later in life. In rats, severe postnatal zinc deficiency impairs neurogenesis in the cerebellum [6], and decreases the expression of genes related to proliferation and neuronal differentiation in the hippocampus [7]. In cultured cells, zinc deficiency impairs human IMR-32 neuroblastoma cell proliferation and induces apoptosis [8], and inhibits retinoic acid-induced neuronal differentiation [7]. Furthermore, zinc deficiency alters brain redox regulation and affects signaling pathways involved in neurogenesis [9-12]. Thus, we previously observed that gestational marginal zinc deficiency in rats decreases the number of neuronal progenitor cells (NPCs) expressing Ki67 in the ventricular zone at

embryonic day (E) 19 [13]. NPCs give rise to all neuronal types present in the brain and play a pivotal role in regulating the balance between cell self-renewal and neurogenesis. Cortical excitatory neurons are directly generated from radial glial progenitors (RGPs) during embryonic neurogenesis or indirectly through intermediate progenitor cells (IPCs) derived from the ventricular zone (VZ) [14, 15]. Radial glia cells proliferate at the ventricular surface and IPCs divide at a non-surface area in the VZ and subventricular (SVZ) zone. The transcription factor cascade involving Pax6, Tbr2 and Tbr1 regulates the different stages of differentiation from NPCs to IPCs to cortical neurons. Tbr1 and Tbr2 are part of the T-box transcription factor subfamily Tbr1, and play key roles in glutamatergic neuron differentiation in the cerebral cortex, olfactory bulbs, hippocampal dentate gyrus, cerebellum and adult SVZ [16]. The extracellular signal-regulated kinase (ERK1/2) pathway is also a key regulator of NPC proliferation and neuronal differentiation [17]. Marginal zinc deficiency during pregnancy in rats causes a decreased phosphorylation of ERK1/2 in the frontal cortex of the fetal rat brain at E19 [13], which is associated with a decrease in NPC number. However, it remains unclear if this decrease in NPCs can affect the number of neurons in the adult offspring brain as well as the process of neuronal differentiation and specification.

A complex and tightly regulated cascade of events lead to the differentiation of NPCs into neurons. Our previous results showed impaired NPC proliferation in association with maternal zinc deficiency. Thus, the goal of the present work was to evaluate whether a marginal zinc diet fed throughout gestation and until postnatal day (P) 2 can: i) affect, in the offspring brain, the transcription factor cascade involved in NPC and IPC proliferation and differentiation into glutamatergic neurons, and ii) cause long-

term effects in the number and specification of cortical neurons in the offspring young adult brain. To such end, the temporal activation/expression of ERK1/2 and transcription factors Sox2, Pax6, Tbr2 and Tbr1 were characterized in the E14, E19 and P2 offspring brain. Alterations in cortical neuronal number and specification to glutamatergic and GABAergic neurons secondary to maternal marginal zinc deficiency were assessed in the adult offspring brain after postnatal repletion of dietary zinc.

2. Materials and methods

2.1. Materials

Primary antibodies for β-actin (#12620), phospho (Thr202/Tyr204) ERK (#4370), and ERK (#9102), were from Cell Signaling Technology (Danvers, MA). Antibodies for GAD65 (SC-377154) and Tbr1 (SC-376258) (Western blot) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for Neu-N (MAB377) and Tbr2 (AB15894) were from Millipore (Burlington, MA, USA). The antibody for Ki67 (550609) was obtained from BD Pharmingen (San José, CA, USA). Antibodies for Sox2 (ab97959), Tbr1 (ab31941) (immunofluorescence), VGLUT1 (ab77822) and Pax6 (ab5790) were from Abcam Inc. (Cambridge, MA). Secondary fluorescent antibodies were obtained from Jackson ImmunoResearch Co. Laboratories (West Grove, PA, USA). Polyvinylidene difluoride (PVDF), membranes and molecular weight standards for Western blot were obtained from BIO-RAD (Hercules, CA, USA). The Enhanced chemiluminescence (ECL) Western blotting system was from Thermo Fisher Scientific Inc. (Piscataway, NJ). Zinquin, the antibody for gamma amino butyric acid (GABA) (A2052), and all other reagents were of

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 [18]. Animals were fed the control diet for 1 week before breeding. The overall experimental design is shown in **Fig. 1A**. 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 (six animals/group for E14, E19, and P2/P56) were divided into two groups and fed ad libitum a control diet (25 μg zinc/g diet, control group) or a diet containing a marginal concentration of zinc (10 μg zinc/g diet; MZD group). Food intake was recorded daily, and body weight was measured at 3-day intervals. On E14 and E19, dams were anesthetized with isoflurane (2 mg/kg body weight) and laparotomies were performed. The gravid

uterus was removed and fetuses were weighed. Fetal brains were removed, weighed and immediately either processed for immunohistochemistry, or removed and kept on ice to microdissect regions enriched in cortical tissue (CT), which were then frozen in liquid nitrogen and stored at -80°C. In the E14 offspring, CT included the cortical neuroepithelium with the SVZ and VZ; in E19 offspring, CT included the cortical plate with the SVZ and VZ. At P2, litters were adjusted to 8 pups/litter, and brain/brain cortices were dissected from the euthanized pups and processed as described before. Dams from both the control and the MZD groups were subsequently fed the control diet. Pups were weaned at P21 and were all fed the control diet until P56 when euthanized; blood and brain/brain cortex collection was carried out as described before.

With regard to the time of exposure of the offspring to zinc deficiency, it should be considered that milk zinc content does not decrease in conditions of marginal zinc nutrition both in rodents and humans, even when maternal plasma zinc levels are low [19]. Thus, in the current experimental model, it is expected that the offspring would have access to similar amounts of zinc in milk starting at birth in both control and MZD groups.

2.3. Determination of zinc concentrations

The concentration of zinc in diets and brain supernatants was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) as described by Clegg et al. [20]. E14 and E19 brain CT and P2 and P56 cortices were weighed, homogenized in ice-cold PBS (1:10), and centrifuged for 60 min at 100,000 x g at 4°C. The supernatant was collected and protein concentration was measured using the Bradford assay [21]. 3 ml of 16 N HNO_3 were added to the 100,000 x g supernatants and

diet samples and allowed to digest for 72 h. Samples were dried and resuspended in ultrapure water. Zinc concentration was determined by ICP-AES (Trace Scan; Thermo Elemental, Franklin, MA, USA). Certified reference solutions (QC 21; Spec CentriPrep, Metuchen, NJ, USA) were used to generate standard curves. A sample of a National Bureau of Standards bovine liver (SRM1577; U.S. Department of Commerce, National Bureau of Standards, Washington, DC, USA) was included with the samples to ensure accuracy and reproducibility.

2.4. Zinquin staining

Labile zinc was measured in coronal sections from the fetal rat brain at E19. Slides were overlaid with a solution of 25 μM zinquin in Hank's balanced salt solution (HBSS) and incubated at 37 °C for 40 minutes. After washing in HBSS, coverslips were mounted with a solution of 90% glycerol and 10% HBSS and imaged on an Olympus BX50 epifluorescence microscope provided with a Cool-Snap digital camera. Image pro software (Rockville, MD) was used to analyze the resulting micrographs. Three randomly selected fields were measured per animal and experimental condition (n= 3).

2.5. Western blot analysis

Extraction of total cellular protein from CT and cortex homogenates was done as previously described [9]. Protein concentration was measured using the Bradford assay [21], and aliquots containing 25–50 μg of protein were separated by reducing 10% (w/v) SDS-PAGE 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 TBST (20 mM Tris, 150 mM NaCl, pH 7.4, 0.1% Tween 20) at 4ºC. After incubation for 1h at room temperature 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 (Amersham, Piscataway, NJ).

2.6. Immunofluorescence

E14, E19, and P2 rat brains were dissected out and fixed in a 4% (w/v) solution of paraformaldehyde in PBS overnight. P56 rats were deeply anesthetized as described above and perfused transcardiacally with PBS followed by 4% (w/v) solution of paraformaldehyde in PBS, after which brains were collected. In all cases, tissue cryoprotection was subsequently performed in 30% (w/v) sucrose until the tissue sank down, after which, brains were submerged in Cryoplast freezing medium (Biopack, Buenos Aires, Argentina), frozen, cut into 18 µm coronal sections on a Leica CM 1850 cryotome (Leica Microsystems, Nussloch, Germany), and mounted on positively charged microscope slides. Sections were blocked for 45 min in 1% (v/v) donkey serum in 0.1% (v/v) Triton X-100 in PBS, and incubated with the corresponding dilution of primary antibody in blocking solution (1:200 rabbit anti-Sox2, 1:100 rabbit anti-Pax6, 1:200 rabbit anti-Trb1, 1:100 chicken anti-Tbr2, 1:200 mouse anti-NeuN, 1:200 rabbit anti-VGLUT1, 1:200 rabbit anti-GABA, 1:100 mouse anti-Ki67) overnight at 4 °C. Sections were then washed once in 0.1% (v/v) Triton X-100 in PBS and once in 0.1 M phosphate buffer, pH 7.4, and incubated with the corresponding dilution of secondary antibody (1:500 Cy3-

conjugated donkey anti-rabbit, 1:500 Alexa 488 donkey anti-mouse IgG, and 1:500 Alexa 488 donkey anti-chicken) for 2 h at room temperature. After immunostaining, cell nuclei were stained with 1 µg/ml Hoechst 33342 and sections were imaged using an Olympus FV 1000 laser scanning confocal microscope or an Olympus BX50 epifluorescence microscope provided with a Cool-Snap digital camera. Image pro software (Rockville, MD) was used to merge and analyze the resulting micrographs. Marker-positive cells were counted using ImageJ (National Institutes of Health, Bethesda, MD, USA) and results were expressed as the number of positive cells per area. At least 300 cells were counted for each cell marker, analyzed in three independent experiments that were performed in triplicates for 4 animals per group. Alternatively, fluorescence intensity was measured for VGLUT1 and GABA.

2.7. Statistical Analysis

Data for the control and MZD groups at each developmental stage were analyzed by Student's t test using Statview 5.0 (SAS Institute Inc., Cary, NC). The litter was the statistical unit. A p value < 0.05 was considered statistically significant. Data are shown as mean ± SE.

3. Results

3.1. Animal outcome

Pregnant dams were fed a marginal zinc or a control diet from E0 until P2. Subsequently all dams until P21, and the offspring, until P56, were fed the control diet (**Fig. 1A**). As previously described [9, 13], we observed that a marginal zinc nutrition throughout gestation does not affect overall maternal and fetal outcome (data not shown).

Furthermore, consumption of the marginal zinc diet throughout gestational and perinatal period did not affect fetal/offspring brain weight between E19 and P56 (**Fig. 1B**). Zinc concentration in E14 and E19 CT and P2 brain cortex 100,000 x g supernatants was significantly lower (72%, 58% and 56%, respectively) in MZD offspring compared to the control group (**Fig. 1C**). Loosely bound zinc in the E19 brain was evaluated by Zinquin staining and subsequent fluorescence microscopy. In the control group, Zinquin fluorescence appeared strongest in the VZ and surrounding blood vessels. In the MZD offspring VZ, the intensity of Zinquin fluorescence was 34% lower than in controls (**Fig. 1D**).

3.2. Maternal marginal zinc deficiency affects markers of neurogenesis at E14

Given our previous work showing a decrease in ERK1/2 activation together with a reduction in NPC proliferation, we aimed to investigate the impact of these events on cortical excitatory neurons. Signaling cascades involved in NPC proliferation, self-renewal, and progression to differentiation were evaluated at E14 (**Fig. 2**). ERK1/2 phosphorylation was 66%, lower in the MZD E14 brain CT compared to controls (**Fig. 2**). Maternal marginal zinc nutrition also affected different markers of NPC proliferation and progression to differentiation as evaluated by Western blot (**Fig. 2**). Sox2 is a transcription factor that controls the self-renewal of NPCs throughout development. Maternal marginal zinc deficiency caused a 95% decrease in E14 cortical Sox2 levels compared to controls. Pax6, a transcription factor that is central to the development of the brain cortex, showed 63% lower levels in the E14 cortex from MZD compared to the control group (**Fig. 2**). Marginal zinc nutrition also affected the abundance of Tbr2, a transcription factor that regulates the specification of the intermediate neural progenitors (INPs) that will differentiate into

excitatory neurons. Tbr2 levels were 55% lower in MZD compared to control E14 CT. Protein levels of Tbr1 and of the marker of post-mitotic mature neurons NeuN were similar between groups.

3.3. Maternal marginal zinc deficiency affects markers of neurogenesis in the offspring brain at E19 and P2

At E19, all measured markers of cortical neurogenesis, from NPC proliferation to fully differentiated neurons, were affected in the MZD embryo brain (**Fig. 3**). ERK1/2 phosphorylation was lower (36%) in the E19 CT from MZD compared to controls. Sox2, Pax6, Tbr2, Tbr1 and NeuN protein levels were 57, 37, 57, 43 and 42% lower in MZD compared to control embryo CT as measured by Western blot (**Fig. 3A**). Immunofluorescence analysis also showed lower levels of total proliferative cells in the E19 SVZ of MZD compared to control offspring (**Fig. 3B**). Thus, NPC Sox2 and IPC Tbr2 positive cells in the SVZ of MZD embryos were 47 and 71%, respectively, compared to controls (**Fig. 3B**). The ratio Sox2/Ki67 and Tbr2/Ki67 positive cells were 56 and 59% lower, respectively, in MZD than in control embryo SVZ. The number of Tbr1 positive cells in E19 cortices were 33% lower in MZD compared to controls.

Markers of cortical neurogenesis were measured next at P2 by Western blot (**Fig. 4A**). Tbr2 and Tbr1 levels were significantly lower in MZD than in control brain cortices (32 and 51%, respectively). A decrease in mature neurons is supported by a 64% decrease in NeuN levels in MZD compared to controls. Furthermore, alterations in neuronal specification were already evident at P2. Thus, a 58% decrease in VGLUT1, a glutamate/proton exchanger indicator of glutamatergic neuron abundance, was observed in MZD offspring brain cortices. On the other hand, the protein abundance of Gad65

(glutamic acid decarboxylase) an enzyme involved in GABA synthesis, was similar between groups. Immunofluorescence analysis (**Fig. 4B**) showed no significant differences between groups for Sox2 and Tbr2. Although values were 44% lower for Tbr2, differences were not significant ($p<0.15$). The number of Tbr1 positive cells was 30% lower in MZD cortices than in controls. The number of NeuN positive cells and of VGLUT1 fluorescence intensity were 42 and 47% lower in MZD compared to control P2 offspring brains, while GABA fluorescence intensity was similar between groups.

3.4. Early developmental marginal zinc deficiency disrupted neurogenesis leading to a decreased neuronal number and altered neuronal specification in the adult brain (P56)

We next investigated if the effects of gestational marginal zinc deficiency on fetal brain development have long lasting repercussions, even following repletion with a diet containing adequate zinc. The total number of mature neurons (NeuN positive cells) in the brain cortex of the P56 offspring was evaluated by immunohistochemistry. Marginal zinc deficiency from E0-P2 resulted in a 29% decrease in the number of cells expressing NeuN in the offspring frontal cortex (**Fig 5**). While no differences in the fluorescence intensity for GABA was observed between groups, 26% lower levels of fluorescence for VGLUT1 were observed in the P56 brain cortex from MZD offspring compared to controls (**Fig. 5**).

4. Discussion

Results from this study demonstrate that a decreased proliferation of NPCs resulting from gestational marginal zinc deficiency leads to the disruption of cortical neurogenesis in the rat offspring brain. This is associated with a decreased number of neurons in the MZD

young adult brain cortex, and an altered specification that results in a reduced number of excitatory glutamatergic neurons, not affecting GABAergic neurons.

Severe nutritional zinc deficiency, both due to low zinc content or high content of zinc-binding phytates, during early development causes NTDs and brain/organ teratology [3, 22]. Also maternal infections, diabetes, and toxicant exposures that stimulate an acute-phase response causes a decreased transport of zinc to the developing fetus [23], which can lead to increased risk of NTDs [24, 25]. On the other hand, a marginally low dietary zinc intake during pregnancy is associated with decreased fetal heart rate variability, suggesting impaired regulation of the autonomic nervous system [26]. Accordingly, prenatal zinc supplementation improves the regulation of the autonomic nervous system later in life [27]. Mild developmental zinc deficiency, both in humans and rodents, impairs learning, working memory, and social behavior [4]. Similar cognitive defects result from secondary zinc deficiency in a rat model of maternal infection [5]. Importantly, these cognitive defects are consistent with rodent models of autism spectrum disorder, suggesting that disruption of fetal brain development resulting from marginal zinc deficiency could contribute to the risk of developing autism [5, 28, 29]. Moreover, reports in humans have established an association between zinc deficiency and Phelan McDermid Syndrome, a genetic disorder characterized by features of autism spectrum disorders [30]. Thus, although not teratogenic, marginal zinc deficiency can have long-lasting effects on the nervous system.

We currently observed that consumption of a marginal zinc deficient diet during pregnancy did not affect the overall pregnancy and fetal/offspring outcome. On the other hand, the concentration of zinc in the offspring brain cytosolic fraction was markedly affected from E14 through P2. This finding stresses the major impact that a mild decrease

in zinc availability can have on zinc homeostasis in the developing brain. Cytosolic zinc and Zinquin-reactive zinc largely reflect loosely bound and rapidly available zinc pools, which are highly relevant to the regulation of cell signaling. For example, and stressing the relevance of available zinc pools, both the decrease in ERK1/2 phosphorylation and cell proliferation observed in zinc-deficient IMR-32 cells are rapidly restored upon zinc supplementation and via the inhibition of the ERK1/2-directed phosphatase PP2A [13].

Zinc deficiency affects signaling pathways that can contribute to altered brain development. In this regard, we previously observed a downregulation of transcription factors NF-κB and NFAT, the activation of MAPKs p38 and JNK, and a downregulation of ERK1/2 both in zinc deficient IMR-32 cells and E19 fetal rat brain [9, 12, 13]. In particular, ERK1/2 phosphorylation showed a major decrease in the MZD brain CT at E14 and E19. This can in part explain maternal zinc deficiency-associated decrease in VZ NPC number and proliferation and the observed alterations in neuronal specification. In this regard, ERK1/2 is not only important for NPC proliferation but also for neuronal differentiation [31, 32]. In humans, mutations that disrupt ERK1/2 signaling impair brain development leading to cognitive dysfunction in neuro-cardio-faciocutaneous syndromes and autism spectrum disorders [33, 34]. Mice with a conditional deletion of ERK1/2 in NPCs have decreased NPC proliferation leading to abnormal distribution of neurons in the cortical plate, increased excitability of cortical neurons, increased anxiety-like behavior, reduced memory, and impairments of social behavior [31, 35]. Overall, ERK1/2 can be a key signal underlying the altered neurogenesis and behavior associated with maternal marginal zinc deficiency [17].

Transcription factor Sox2 functions by regulating NPC self-renewal in both the developing and mature brain, and inhibiting NPC differentiation [36, 37]. A major decrease

of Sox2 was observed in the MZD E14 and E19 embryos, suggesting that Sox2 downregulation can contribute to the observed decrease in NPC proliferation. On the other hand, Sox2 downregulation causes premature neuronal differentiation [37], which was not observed in the MZD embryonic brain. Pax6 expression at E14 was also impaired in the MZD group. Pax6, a homeobox and paired domain transcription factor, promotes the expression of Tbr2, inducing the transition of NPC-like radial glial progenitors (RGPs) to IPCs [38]. Pax6 regulates the proliferation of cortical progenitors being expressed in RGPs at the VZ, but not in IPCs [39]. Thus, not only ERK1/2 but also Sox2 and Pax6 downregulation at a period of active progenitor proliferation can contribute to the decrease in the number of fetal brain NPCs as a consequence of maternal marginal zinc deficiency.

We next evaluated the potential disruption of the process of neuronal differentiation and specification that could be associated with an impaired NPC proliferation. Similarly to Sox2 and Pax6, Tbr2 protein levels were markedly low in E14 and E19 MZD brain CT. Tbr2 is expressed in the mouse brain as early as E10.5 in proliferative areas, where neuronal progenitors reside [40]. Tbr1 expression is located in the cerebral cortex and other brain areas populated by postmitotic neurons, decreasing the expression postnatally [41]. Tbr2 is a marker of IPCs, being required for the progression of RGPs to IPCs, a process that is in part mediated by the downregulation of Pax6 by Trb2 [42]. Most glutamatergic neurons in the brain cortex are originated from a Tbr2 positive lineage [43]. We observed a reduction in the number of Tbr2 positive IPCs in the SVZ and of Tbr1 positive postmitotic neurons in the cortical plate. Thus, while the decrease in NPCs in the MZD embryonic brain may explain a decreased number of neurons, the observed downregulation of Tbr2 during the

period of active differentiation suggests that the specification of neurons may also be affected.

As IPCs are necessary to expand the population of cortical glutamatergic neurons, we next analyzed the population of mature NeuN positive neurons and the populations of VGLUT1 and GABA positive neurons at P2 and P56. We observed not only a lower number of mature neurons in the MZD mature brain but also, in agreement with alterations in Tbr2 and Tbr1 expression, an impairment in the generation of glutamatergic neurons. Very importantly, even after postnatal zinc repletion, VGLUT1 expression in the MZD P56 brain cortex remained markedly affected. The mammalian neocortex has two types of neurons, glutamatergic pyramidal cells and GABAergic non-pyramidal cells [44, 45]. Glutamatergic projection neurons give rise from progenitor cells in the VZ of the pallium from where they migrate radially into the neocortex. On the other hand, GABAergic interneurons are born in the subpallium and migrate tangentially into the neocortex [46]. They arise from the ventral telencephalon [47], in particular the medial and caudal ganglionic eminences [48-50] from Nkx2.1-expressing progenitors [51], in a process modulated by Sonic hedgehog [52]. The described diverse origin and regulation of glutamatergic and GABAergic neurons can explain the differential effect of developmental zinc deficiency on these neuronal populations.

The current findings stress the concept that the adverse effects of limited zinc availability during early development can have irreversible consequences in the number and specification of neurons in the mature brain. These results add to previous evidence showing that severe zinc deficiency can disrupt NPC proliferation and neuronal differentiation [1, 6, 7], but in a condition of marginal zinc availability which can be

extrapolated to human populations. The disruption observed in neurogenesis could contribute to the described persistent effects of marginal zinc deficiency on behavior. In this regard, marginal zinc deficiency throughout gestation and lactation decreases working memory in rats after dietary repletion [53]. In mice, marginal zinc deficiency through gestation leads to increased anxiety-like behavior, abnormal social behavior, and impaired motor learning even after dietary repletion [29, 54]. Very relevant to the current results, a dysbalance in excitatory/inhibitory systems has been proposed as a developmental precursor of autism spectrum disorders [55] and a possible target for restoring functional connectivity even in adulthood [56].

In summary, this work demonstrates that gestational marginal zinc deficiency affects neurogenesis in the fetal rat brain leading to a disruption of the cortical excitatory/inhibitory balance that persists into adulthood even after dietary repletion. While earlier studies focused on severely deficient animal models, this is the first study to report disruption of fetal neurogenesis resulting from marginal zinc deficiency that affects the cellularity of the mature brain cortex. These findings stress the relevance of an adequate zinc nutrition during pregnancy to prevent irreversible effects on the offspring cortical structure and ultimately on behavior and cognition.

Author contributions

P.I.O. and A.M.A. designed the research. X.L., A.M.A, S.S., J.N. and P.M. performed the research. All authors participated in the analysis and discussion of data. P.I.O., A.M.A and J.N. wrote the paper.

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Conflict of interest disclosure

Although unrelated to this project, P. Oteiza has received funding and is a member of the Advisory Board of Pharmanex Research, NSE Products Inc., Provo, UT, USA. PIO has also received research grants from other food companies and government agencies with an interest in health and nutrition.

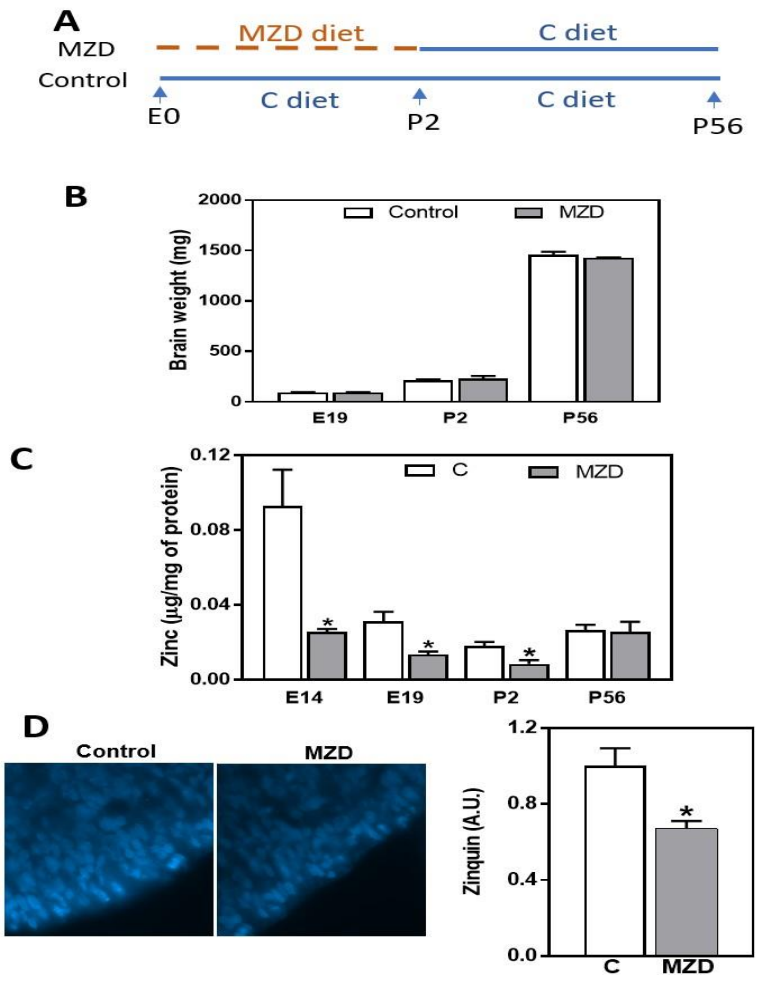

Figure 1. Fetal/offspring brain weight and zinc concentration after maternal consumption of a control or a marginal zinc diet throughout gestation and until P2. (A) Experimental design. **(B)** embryonic day (E)19-P56 offspring brain weight. **(C)** zinc concentration in fetal/offspring CT and brain cortex 100,000 x g supernatants were measured by atomic emission spectroscopy (AES). **(D)** Labile zinc was measured in the ventricular zone (VZ) at E19 by zinquin staining (blue fluorescence). Micrographs show VZ of the dorsomedial frontal cortex at 1000-fold magnification. Fluorescence was quantified as described in "Materials and Methods" section. **(B-D)** Data are shown as mean ± SEM and are the average of 4-6 litters per group. *Significantly different from the control group at the same developmental stage (t-test, $p<0.05$).

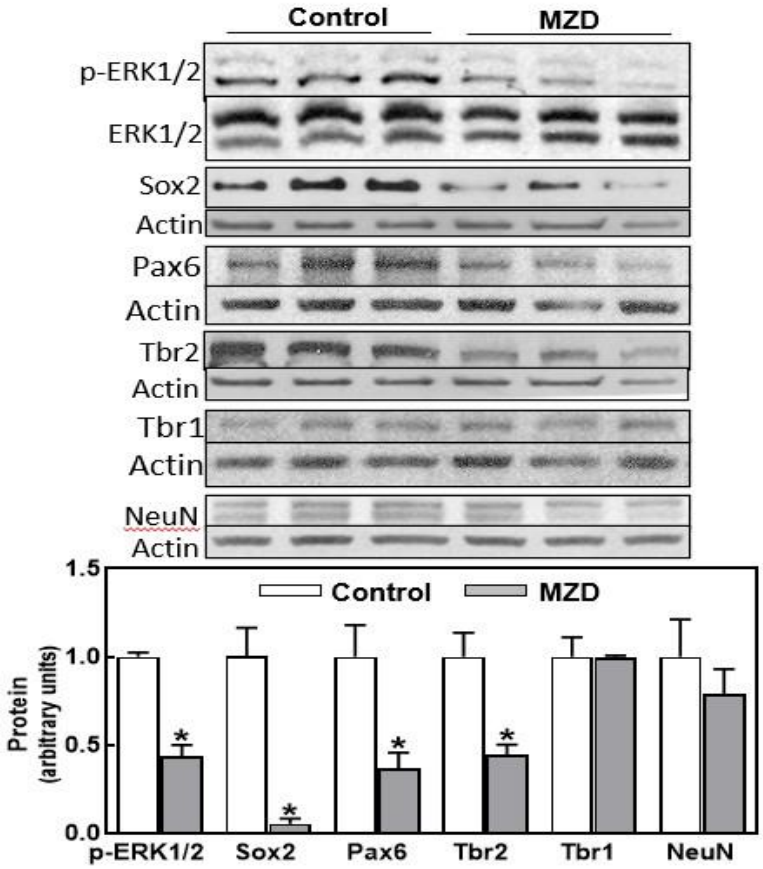

Figure 2. Maternal marginal zinc deficiency affects parameters of neurogenesis in the E14 rat brain. Phospho ERK1/2, ERK1/2, Sox2, Pax6, Tbr2, Tbr1 and NeuN protein levels were measured by Western blot in E14 brain CT homogenates. After quantification of bands, phospho-ERK1/2 levels were referred to total ERK1/2 content and the other proteins were referred to β-actin levels. Values (A.U.: arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM of E14 brain cortices from 6 litters/group. *Significantly different from the control group ($p<0.05$, Student's t-test).

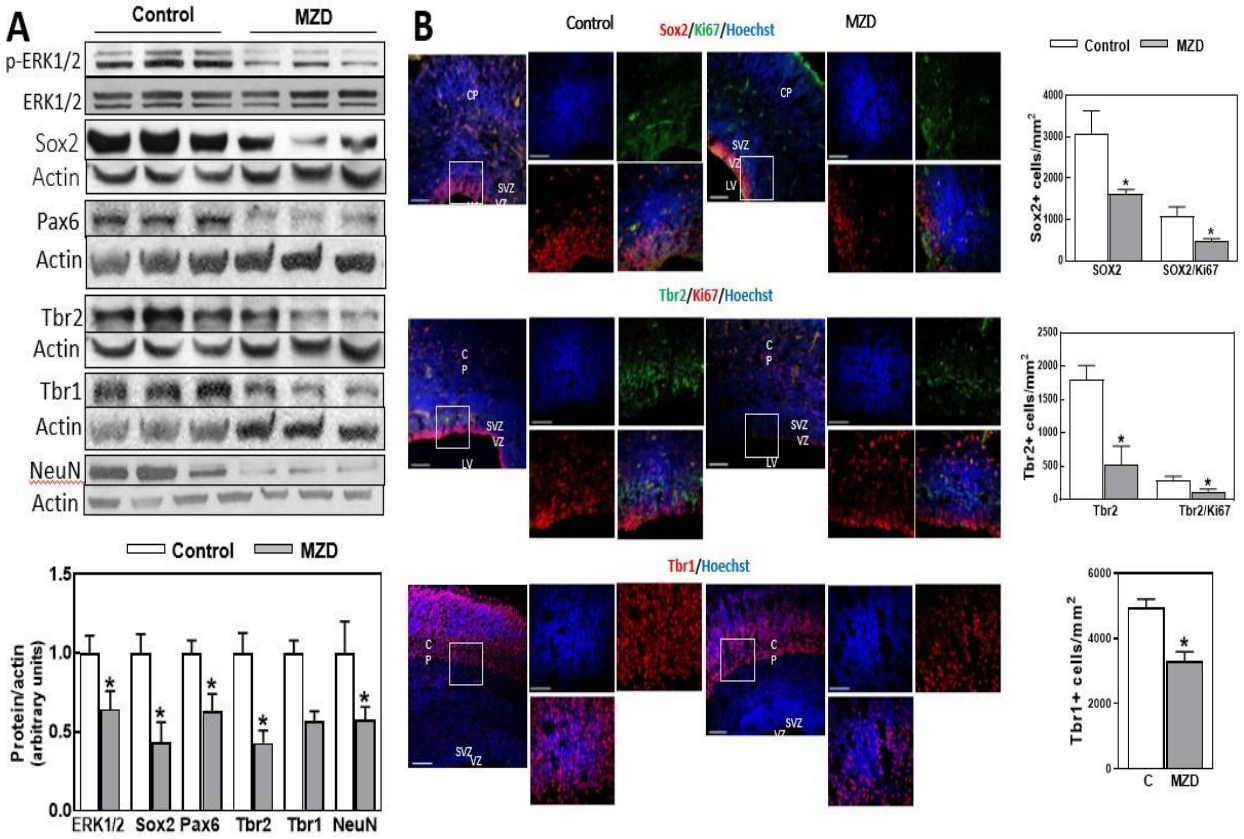

Figure 3. Maternal marginal zinc deficiency affects parameters of neurogenesis in the E19 rat brain. **(A)** Phospho ERK1/2, ERK1/2, Sox2, Pax6, Tbr2, Tbr1 and NeuN protein levels were measured by Western blot in E19 CT homogenates. After quantification of bands, phospho-ERK1/2 levels were referred to total ERK1/2 content and the other proteins were referred to β-actin levels. Values (A.U.: arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM of fetal brain cortices from 6 litters/group. *Significantly different from the control group ($p<0.05$, Student's t-test). **(B)** Immunofluorescence for Sox2 (red fluorescence) and Ki67 (green fluorescence); Tbr2 (green fluorescence) and Ki67 (red fluorescence); Tbr1 (red fluorescence) and Ki67 (green fluorescence). Nuclei were visualized with Hoechst staining (blue fluorescence; Scale bar, 100 µm). Images in boxes are shown at a higher

magnification (Scale bar, 50 µm). Quantifications were done as described in methods. Results are shown as mean ± SEM of E19 brains from 4 litters/group. *Significantly different from the control group ($p<0.05$, Student's t-test).

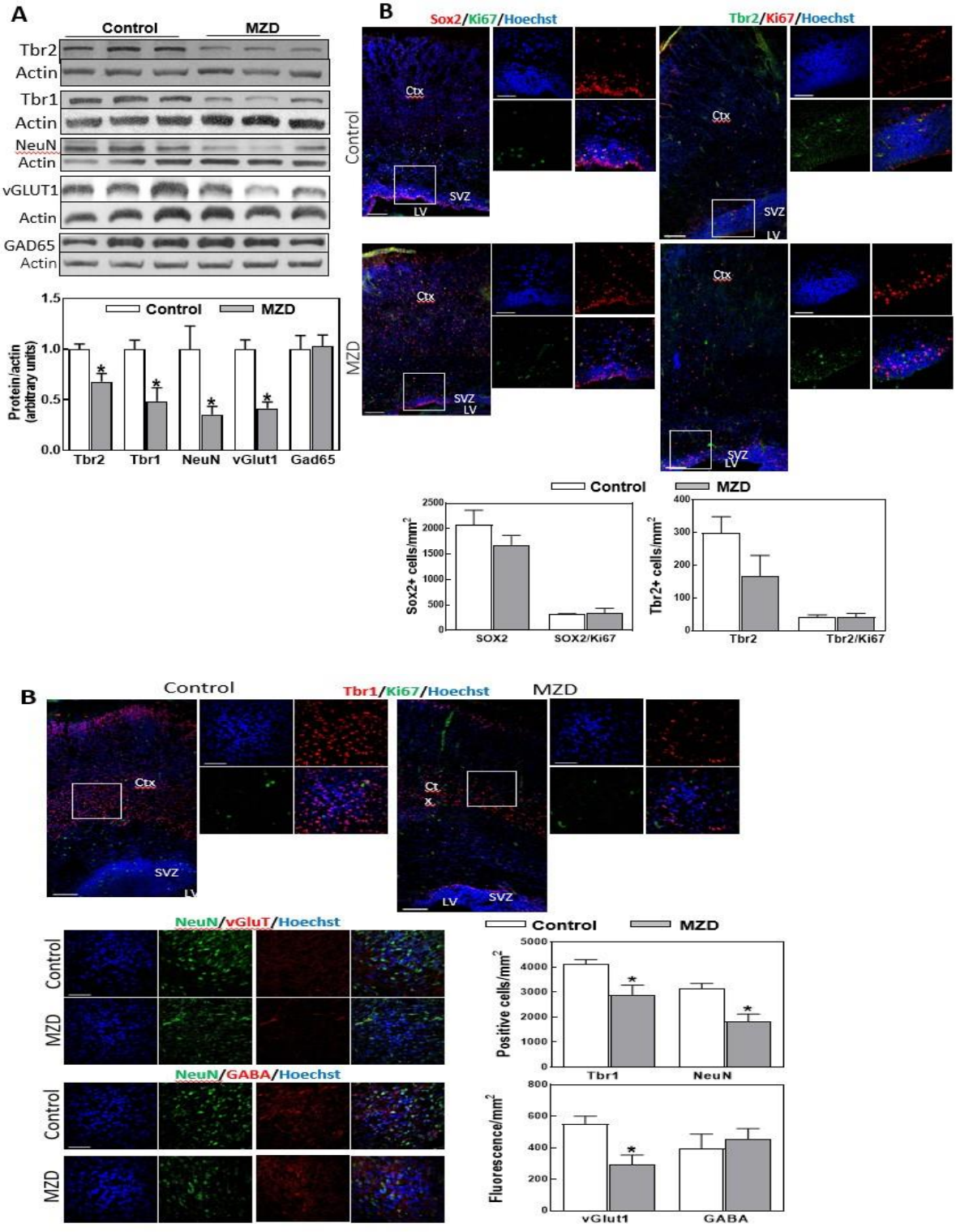

Figure 4. Maternal marginal zinc deficiency affects parameters of neurogenesis in the P2 fetal rat brain. (A) Tbr2, Tbr1, NeuN, VGLUT1 and GAD65 protein levels were

measured by Western blot in P2 brain cortex homogenates. After quantification of bands, values were referred to β-actin levels and values (A.U.: arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM of fetal brain cortices from 6 litters/group. *Significantly different from the control group ($p<0.05$, Student's t-test). **(B)** Immunofluorescence for Sox2 (red fluorescence) and Ki67 (green fluorescence); Tbr2 (green fluorescence) and Ki67 (red fluorescence); Tbr1 (red fluorescence) and Ki67 (green fluorescence); NeuN (green fluorescence) and VGLUT1 (red fluorescence); and NeuN (green fluorescence) and GABA (red fluorescence). Nuclei were visualized with Hoechst staining (blue fluorescence; Scale bar, 100 µm). Images in boxes are shown at a higher magnification (Scale bar, 50 µm). Quantifications were done as described in methods. Results are shown as mean ± SEM of offspring brains from 4 litters/group. *Significantly different from the control group ($p<0.05$, Student's t-test).

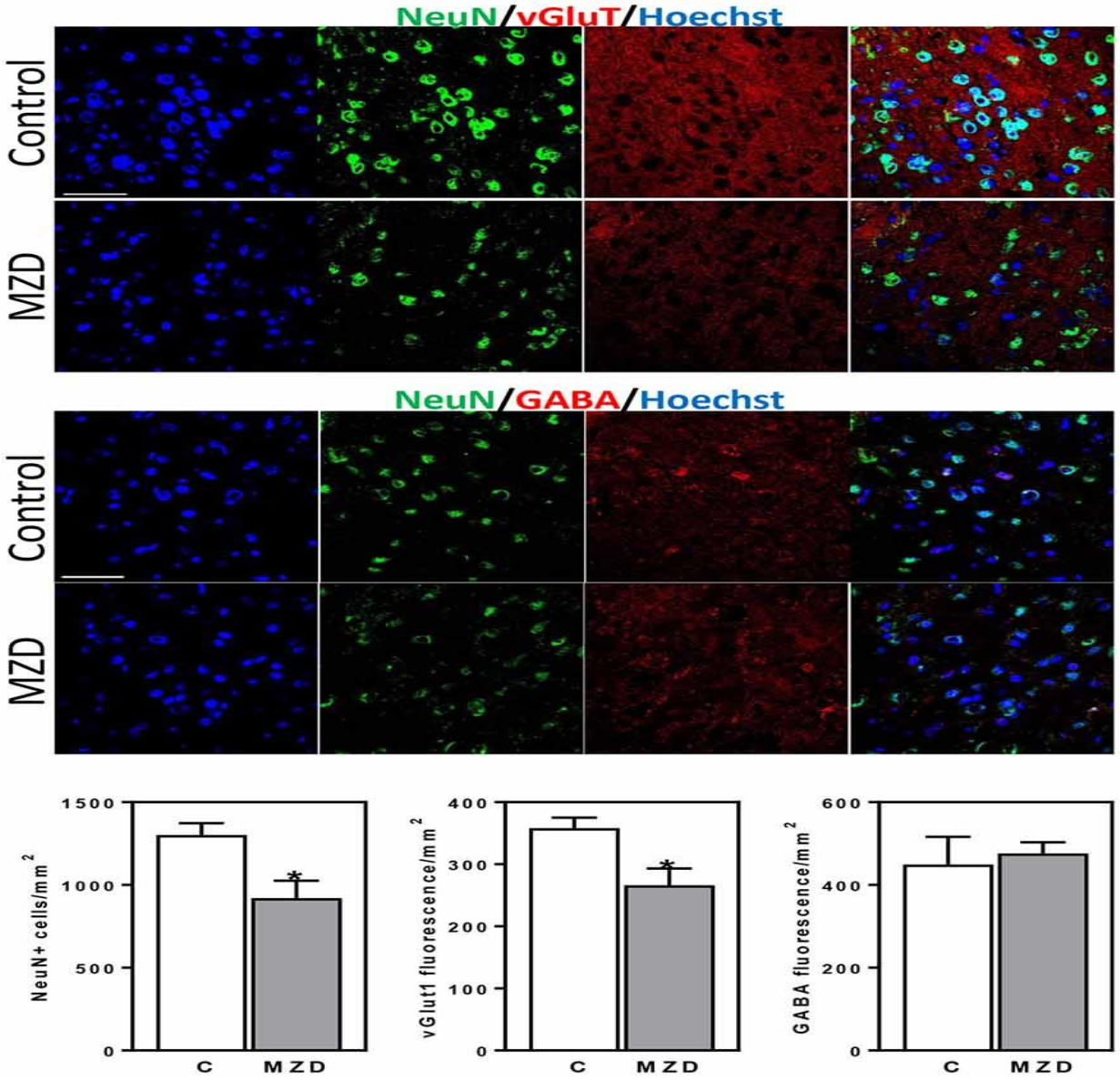

Figure 5. Maternal marginal zinc deficiency from E0 to P2 affects the number and specification of neurons in the young adult brain. NeuN (green fluorescence), VGLUT1 (red fluorescence) and GABA (red fluorescence) were measured by immunofluorescence in the frontal cortex at P56. Nuclei were visualized with Hoechst staining (blue fluorescence; Scale bar, 100 µm). Quantifications were done as described in methods. Results are shown as mean ± SEM of offspring brain from 4 litters/group. *Significantly different from the control group ($p<0.05$, Student's t-test).

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Chapter two

Di-2-ethylhexyl Phthalate Affects Zinc Metabolism and Neurogenesis in the Developing Rat Brain

In preparation

Di-2-ethylhexyl Phthalate Affects Zinc Metabolism and Neurogenesis in the Developing Rat Brain

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Running Title: DEHP and zinc in neurogenesis

Keywords: DEHP, MEHP, zinc, brain development, ERK1/2, SOX2, TBR2, zinc deficiency

Abbreviations: DEHP, di-2-ethylhexyl phthalate; EMX1, empty spiracles homeobox 1; ERK1/2, Extracellular signal-regulated kinase 1 and 2; ELK1, E26 transformation-specific (ETS) Like- 1; GAD65, glutamic acid decarboxylase 65; MEHP, monoethylhexyl phthalate; MEK1/2, Extracellular Signal-regulated Kinase Kinase; NeuN, neuronal nuclei protein; PAX6, Paired box protein 6; PP2A, protein phosphatase 2A; SOX 2, SRY (sex determining region Y)-box 2; TBR1 and TBR2, T-box brain 1 and 2; VGLUT1, vesicular glutamate transporter 1.

Abstract

We previously observed that developmental Marginal zinc deficiency affects neurogenesis. Phthalates are proposed to disrupt zinc homeostasis secondary to the triggering of an acute phase response. In the current study, we investigated if gestational exposure to di-2-ethylhexyl phthalate (DEHP) in rats alters neurogenesis as mediated through decreasing zinc availability to the fetus. Dams were fed an adequate (25 µg zinc/g diet) (C) or a marginal zinc deficient (MZD) (10 µg zinc/g diet) diet, without or with DEHP (300 mg/kg BW) (C+DEHP, MZD+DEHP) from embryonic day (E) 0 to E19. Neurogenesis was evaluated in the E19 fetal brain by measuring parameters of neural progenitor cells (NPC) proliferation and differentiation. Zinc concentrations were lower in brain cytosolic fractions of MZD, C+DEHP and MZD+DEHP groups than C. Protein markers of NPC proliferation (PAX6, SOX2, EMX1), differentiation (TBR2, TBR1) and mature neuron (NeuN) were reduced in MZD, MZD+DEHP and C+DEHP than in C in E19 brain, being the lowest in the MZD+DEHP group. VGLUT1 levels, a marker of glutamatergic neurons, were lower in C+DEHP, MZD and MZD+DEHP than in C E19 brain, while those of GABAergic neurons, GAD65, were similar among groups. The ERK1/2 phosphorylation was reduced by both MZD and DEHP, being the MZD+DEHP group the most affected. Results from human neuroblastoma IMR-32 cells exposed to MEHP and from E19 DEHP exposed brains in point to the zinc-regulated phosphatase PP2A to be in part responsible for DEHP-mediated ERK1/2 downregulation and impaired neurogenesis. Overall, gestational exposure to DEHP caused secondary zinc deficiency and impaired neurogenesis in the E19 fetal rat brain, in part through PP2A activation and ERK1/2 downregulation.

1. Introduction

Di(2-ethylhexyl) phthalate (DEHP) is one of the phthalate plasticizers commonly used in the production of polyvinyl chloride (PVC). DEHP is present in many products, including plastics containers, medical devices, household products and cosmetics [1]. DEHP is lipophilic and is non-covalently bound to plastic polymers. It can be easily released into air, water, food, and into human body fluids if used in medical settings. After intestinal absorption, DEHP is rapidly metabolized into monoethylhexyl phthalate (MEHP), which is the primary active metabolite and major mediator of the adverse effects of DEHP on human health [2]. Among the main adverse effects of DEHP, gestational exposure to this toxicant represents a risk for both pregnancy complications and for the normal development of the offspring [3, 4].

Gestation and the early postnatal period are particular windows of vulnerability to DEHP toxicity. During pregnancy, MEHP is readily transported across the placenta from maternal blood to the fetus [5], negatively affecting pregnancy and birth outcomes [6-8]. Perinatal exposure of mice to DEHP affects neurodevelopment by reducing neural progenitor cells (NPCs) proliferation, increasing cell death [9] and decreasing the number of neurons and synapses in the prefrontal cortex, which ultimately results in cognitive flexibility in adults [10]. A large number of epidemiological studies have evaluated the relationship between maternal DEHP exposure and children's neurocognitive development. These studies indicate that prenatal and neonatal DEHP exposure is associated with impaired children's intelligence quotient scores [11, 12] and abnormal neurobehavior in infants [13], including autism spectrum disorders [14]. Based on the above, exposure to DEHP during gestation and early infancy may impose a long-term

threat to brain development, ultimately leading to behavioral/neurological alterations later in life.

Zinc is an essential trace element for normal development [15, 16]. Besides nutritional deficiency, during gestation zinc bioavailability to the fetus can be compromised by various conditions that affect zinc absorption and/or excretion and that triggers an acute phase response [16]. A large body of evidence showed that DEHP exposure affects zinc metabolism. Zinc concentration decreases after 5 d of DEHP exposure in adult rats, independently of the expression of zinc transporters [17]. On the other hand, DEHP exposure can cause functional zinc deficiency [18] and alter the expression of genes involved in the regulation of zinc homeostasis [19]. We previously showed that gestational DEHP exposure increases the liver expression of the zinc-binding and acute phase protein metallothionein in the dam liver, resulting in zinc retention in maternal liver [20]. This leads to a decrease in maternal plasma zinc concentration which exposes the fetus to a lower zinc bioavailability [20].

Nutritional marginal zinc deficiency (MZD) throughout gestation decreased zinc concentration in the cortex of fetal rat bran in the embryonic day 19 (E19) brain cortex. This was associated with a low number of NPCs in the brain cortex ventricular zone (VZ), and in alterations in neuronal differentiation, maturation, and specification in the developmental brain. Mechanistically, MZD affected ERK1/2 signal pathway that is essential for neurodevelopment [21, 22]. ERK1/2 promotes neurogenesis via increasing NPC proliferation and preventing apoptosis of neural stem cells [23]. Moreover, ERK1/2 is important for neuronal differentiation of embryonic stem cell [24]. Gestational MZD had long-term effects on the mature brain cortex, which showed a lower number of neurons

and altered neuronal specification, i.e. lower number of glutamatergic neurons, but not of GABAergic neurons [21]. Developmental zinc deficiency can also lead to impaired brain function in the offspring, that can manifest in altered behavior, cognitive and motor performance [25-31], attentive symptoms, e.g. ADHD [27, 32, 33], depression [25], and altered child psychomotor development [34-36].

Taking all the above into consideration, this work investigated the impact of prenatal exposure to marginal zinc intake and/or the environmental toxicant DEHP in rats on neurogenesis of fetal rat brain. We hypothesize that prenatal exposure to DEHP impairs neurogenesis in part through decreasing zinc bioavailability to the fetus. The underlying mechanisms were characterized.

2. Materials and Methods

2.1. Materials

IMR-32 cells were obtained from the American Type Culture Collection (Rockville, MA). Cell culture media components were obtained from Invitrogen Life Technologies (Carlbad, CA). Primary antibodies for β-actin (#12620), phospho (Ser217/221)-MEK1/2 (#9154) and MEK1/2 (#9126), phospho (Thr202/Tyr204)-ERK (#4370), and ERK (#9102), phospho (Ser383) -Elk-1 (#9181) and Elk-1 (#9182) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies for glutamic acid decarboxylase 65 (GAD65; SC-377154), Empty Spiracles Homeobox 1(EMX1; SC-398115), NEUROD (sc-46684) and Tbr1 (SC-376258; Western blot) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for Neu-N (MAB377) and Tbr2 (AB15894) were from Millipore (Burlington, MA, USA). The antibody for Ki67 (550609) was obtained from BD

Pharmingen (San José, CA, USA). Polyvinylidene difluoride (PVDF), membranes and molecular weight standards for Western blot were obtained from BIO-RAD (Hercules, CA, USA). The enhanced chemiluminescence (ECL) Western blotting system was from Thermo Fisher Scientific Inc. (Piscataway, NJ, USA). DEHP (#117091000) was from Fisher Scientific (Waltham, MA, USA). MEHP (# 796832), the PP2A Immunoprecipitation Phosphatase Assay Kit (#17-313) and all other reagents were of 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 NIH Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Institutional animal care and use committee 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 (Wilmington, MA, USA). Female rats (200–225 g) were housed individually in stainless steel cages in a temperature (22–23 °C)- and photoperiod (12 h light/dark)-controlled room. Distilled water was provided through a daily flushed automatic watering system. An egg-white protein-based diet with adequate zinc (25 µg zinc/g) was the control diet [37]. Animals were fed the control diet for 5 days before breeding. Males and females were caged together overnight and the following morning, embryonic day (E) 0, after the presence of a sperm plug confirmed successful breeding; female rats (6-9 animals/group)

were randomly divided into four groups and fed *ad libitum* the control or one of the other three experimental diets. The control diet (C group) contained 25 μg zinc/g diet. The marginally zinc deficient diet (MZD group) contained 10 μg zinc/g diet. The remaining two diets were the C and marginal zinc diets supplemented with 300 mg DEHP/kg body weight (BW) (C+DEHP and M+DEHP groups, respectively). DEHP were administrated in the mixture of food in the diet based on BW of the dams weekly. Food intake was recorded daily, and animals body weight was measured at 5-day intervals. At gestation day 19, dams were anesthetized with isoflurane (2 mg/ kg BW), and laparotomies were performed. The gravid uterus was removed, and fetuses were weighed. Whole brains or brain cortices were excised, weighed, and processed for immunohistochemistry or frozen in liquid nitrogen, and stored at -80 °C. All fetal brains from same dam were pooled as one litter, and litter is considered as statistical units.

2.3. IMR-32 cell cultures

IMR-32 cells were cultured at 37 °C in high glucose DMEM medium supplemented with 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin. For the experiments, cells at 70% confluency were incubated with or without 0.1-5 μM MEHP for the corresponding time periods. For the zinc supplementation experiments, cells were incubated without or with 0.5 μM MEHP and in the absence or the presence of 0.5-10 μM $ZnCl_2$.

2.4. Cell Viability

Cell viability was evaluated using the thiazolyl blue tetrazolium bromide (MTT) test. IMR-32 cells were seeded in 96-well plates (2 x10^4 cells/well). After 24 h, the medium was removed and replaced with fresh medium containing various concentration of MEHP (0-50 µM) and further incubated for 24 h. After medium removal, cells were added with 100 µl PBS and 10 µl (5 mg/ml) MTT and incubated at 37°C for another 4 h. The medium was removed, and cells were added with 200 µl DMSO and incubated at room temperature until dissolution of the purple formazan crystals. Absorbance (λ_{570}, λ_{690} nm) was measured using a BioTek Synergy H1 plate reader (BioTek Instruments, Winooski, VT) and expressed as percentage of untreated (control) cell values.

2.5. Determination of mineral concentrations

The concentration of zinc in the diets and of zinc, iron and copper in brain cortex and IMR-32 cell 100,000 x g supernatants was measured by inductively coupled plasma atomic emission spectroscopy as described by Clegg et al. [38]. Brain cortices were weighed, homogenized in ice-cold PBS (1:10), and centrifuged for 60 min at 100,000 x g at 4°C. IMR-32 cells (2 x 10^7 cells) were homogenized in 1 ml of ice-cold PBS and centrifuged for 60 min at 100,000 x g at 4°C. Protein concentration was measured in brain supernatants using the Bradford assay [39]. Supernatant, brain cortex and diet samples were added with 3 ml of 16 N HNO_3 and digested for 72 h at room temperature. Samples were dried and the obtained residue resuspended in ultrapure water. Zinc, iron, and copper concentrations were determined by ICP-AES (Trace Scan; Thermo Elemental, Franklin, MA, USA). Certified reference solutions (QC 21; Spec CentriPrep, Metuchen, NJ, USA) were used to generate standard curves. A sample of a National Bureau of

Standards bovine liver (SRM1577; U.S. Department of Commerce, National Bureau of Standards, Washington, DC, USA) was included with the samples to ensure accuracy and reproducibility. Final minerals concentration in cortex is normalized by total protein in brain cortex tissue, and final minerals concentration in supernatant is normalized by total protein in the cytosol fraction of brain cortex.

2.6. Western blot analysis

Total proteins from brain cortex and cells were isolated as previously described [40]. Protein concentration was measured [39], and aliquots containing 25–50 μg of protein were separated by reducing 10% (w/v) polyacrylamide 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 TBST (Tris-buffered saline pH 7.4, 0.1% Tween 20) at 4^{o}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 (Amersham, Piscataway, NJ).

2.7. PP2A Activity Assay

E19 brain cortex or IMR-32 cells were homogenized on ice in 20 mM imidazole-HCl buffer, pH: 7.0, containing 1 mM PMSF and 1X 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

immunoprecipitate was measured using the PP2A Immunoprecipitation Phosphatase kit following the manufacturer's protocol. PP2A activity was expressed as pmoles of phosphate released in 30 min and normalized to total PP2A C subunit recovered in the immunoprecipitate as measured by Western blot.

2.8. Statistical Analysis

Data were analyzed by one-way and two-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC) 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.

3. Results

3.1. Animal outcomes

Pregnant dams were fed a control or marginal zinc diet with and without DEHP (300 mg/kg diet) from E0 until E19 (**Fig 1A**). As previously described for this set animals, marginal zinc deficient diet, DEHP and M+DEHP did not affect overall maternal and fetal outcomes. While there were no significantly difference in fetal body weight and brain weight, maternal body weight gain was reduced in MZD, C+DEHP and M+DEHP dams [20, 22]. Zinc concentration was measured in both brain cortex and cytosolic fraction (100,000 x g supernatants of tissue lysate) of brain cortex (**Fig 1B).** Compared to the C group, zinc concentration was significantly lower in C+DEHP, MZD and M+DEHP, in both E19 brain cortex and cytosolic fractions (21, 19, 24% reduction and 58, 51, 61% reduction, respectively). The greater reduction in cytosolic zinc content compared to that in the

whole cortex tissue points to profound and particular effects of both zinc deficiency and DEHP exposure on labile, the rapidly accessible zinc pools. Tissue iron and copper concentrations in brain cortex were similar among the groups (**Fig. 1C**)

3.2. Maternal exposure to MZD and DEHP affects markers of neuronal progenitor cells proliferation in E19 brain cortex

We evaluated the brain expression of EMX1, Sox2 and Pax6 in E19 brain cortex by Western blot (**Fig. 2**). These three proteins are transcription factors that are involved in the regulation of NPC proliferation and self-renewal. While MZD and DEHP exposure caused a marked decrease in brain cortex EMX1, Sox2 and Pax6 protein levels, the combination of both treatments (M+DEHP group) caused an even more pronounced decrease of the three proteins compared to the control group (65, 58 and 78%, for MZD, DEHP and M+DEHP groups respectively).

3.3. Maternal exposure to MZD and DEHP affects markers of neuronal differentiation, migration, and neurite expansion in the E19 brain cortex

To evaluate neuronal differentiation, we next measured the protein levels of TBR1, TBR2 and NeuroD in E19 brain cortex (**Fig. 3 A**). Gestational MZD led to lower levels of TBR1, TBR2 and Neuro D (43, 37 and 51% reduction, respectively) compared to controls. DEHP exposure caused *per se* a significant decrease in TBR1 and TBR2 (39 and 48 %, respectively), but not in NeuroD. In the M+DEHP group, TBR1, TBR2 and NeuroD protein levels were significantly lower than in the other three groups (50, 79 and 65% lower than the C group, respectively). As indicators of neuronal migration and neurite outgrowth we measured the protein levels of doublecortin (DC) and vimentin in E19 CT. The content of

both proteins was lower in the MZD, C+DEHP and M+DEHP than in the C group, but no significant differences were observed among these three treatment groups.

3.4. Early developmental MZD and DEHP affect markers of neuronal number and altered neuronal specification in the E19 brain cortex

To evaluate neuronal number and specification we measured by Western blot the protein levels of NeuN, a marker of post mitotic mature neurons, VGLUT1 (vesicular glutamate transporter 1), a marker of glutamatergic neurons and GAD65 (glutamic acid decarboxylase), a marker of GABAergic neurons, in E19 brain cortices (**Fig. 4**). While both gestational MZD and DEHP exposure had a major impact on NeuN protein levels (43 and 60% lower in MZD and DEHP compared to C, respectively), the combination of both caused an 88% decrease in E19 brain cortex NeuN content. Lower levels of VGLUT1 were observed in MZD, C+DEHP and M+DEHP cortices compared to C, while GAD65 protein levels were similar among the four experimental groups.

3.5. Early developmental MZD and DEHP affects the ERK1/2 pathway in the E19 brain cortex.

We previously observed that gestational MZD inhibits the ERK1/2 pathway, at least in part through the activation of the serine phosphatase PP2A [22]. Given the critical role of ERK1/2 in NPCs proliferation, we evaluated the effects of MZD and DEHP maternal exposure on the activation of this cascade (**Fig. 5A**). As measured by Western blot, levels of phosphorylated MEK1/2 (Ser 217/221), ERK 1/2 (Thr 202/Tyr 204) and ELK1 (Ser383) were lower in the MZD and C+DEHP groups than in the control group. The combination of both, MZD and DEHP exposure caused an additional decrease in MEK1/2 and ERK1/2

activation (75 and 86% lower than controls, respectively). PP2A dephosphorylates and inactivates ERK1/2. Zinc is a physiological inhibitor of this enzyme, and we previously found that zinc deficiency causes PP2A activation [22]. PP2A protein levels were significantly lower in the MZD, C+DEHP and M+DEHP E19 brain cortex compared to the C group (**Fig. 5B, left panel**). However, PP2A activity, in embryos from dams fed control or marginal zinc diets, DEHP exposure was associated to a 320% increase in cortical PP2A activity (**Fig. 5B, right panel**).

3.6. MEHP inhibits ERK1/2 phosphorylation in human neuroblastoma IMR-32 cells

MEHP is the primary and highly toxic mammalian metabolite of DEHP. We used human neuroblastoma IMR-32 cells to investigate the mechanisms underlying the impaired activation of ERK1/2 in fetal brain caused by maternal DEHP exposure. In cells exposed for 24h to 0.5 to 50 µM MEHP, cell viability measured using the MTT method, was only affected at the highest MEHP concentrations tested (15-50 µM MEHP) (**Fig. 6A**). MEHP caused a time- and dose-dependent inhibition of ERK1/2 phosphorylation in IMR-32 cells (**Fig. 6B, C**). At 0.5 µM concentration, and between 1 to 24 h incubation, MEHP caused between 36 to 85% decrease in Thr 202/Tyr 204 ERK 1/2 phosphorylation.

3.7. MEHP inhibits ERK1/2 phosphorylation in IMR-32 cells in part through alterations in zinc homeostasis

To assess if MEHP could act disrupting neuronal cell zinc homeostasis, we next measured cellular zinc in IMR-32 cells after 24 h incubation with 0.1-10 µM MEHP (**Fig. 7**). Cellular zinc was significantly lower at 0.1 µM MEHP and no additional decreases

were observed up to 10 µM MEHP (**Fig. 7A**). On the other hand, after 24 h incubation, MEHP (0.5 µM) did not affect copper and iron cellular concentrations (**Fig. 7B**). Supporting a role of zinc in MEHP-mediated alterations in ERK1/2 regulation, zinc supplementation (0.5-10 µM) caused a dose-dependent increase in MEK1/2 and ERK1/2 phosphorylation in MEHP (0.5 µM)-treated IMR-32 cells (**Fig. 7 C, D**).

3.8. MEHP-mediated inhibition of ERK1/2 phosphorylation in IMR-32 cells can be in part due to PP2A activation

We next assessed if MEHP could inhibit ERK1/2 phosphorylation by activating PP2A. IMR-32 cells were incubated for 24h in the presence of 0.5 µM MEHP. PP2A protein content was significantly lower in the MEHP-treated cells compared to controls (**Fig. 8A, upper panel**). On the other hand, PP2A specific activity was 120% higher in MEHP-treated than in control cells (**Fig. 8A, lower panel**). We subsequently evaluated the effect of adding zinc (0.5-10 µM) when measuring the enzyme activity in PP2A immunoprecipitated from cells incubated with 0.5 µM MEHP for 24h. Zinc addition causes a dose-dependent inhibition of MEHP-mediated increase in PP2A activity (**Fig. 8B**).

4. Discussion

Our previous study showed that gestational exposure to DEHP and MZD decrease maternal weight gain and disrupt corticoid metabolism in rats [20]. In the same set of animals, we now observed that DEHP exposure affects zinc homeostasis and impairs neurogenesis. Thus, maternal DEHP exposure led to decreased zinc levels in fetal brain and disrupted parameters of NPCs proliferation, differentiation, migration, and specification in the E19 brain. Mechanistically, DEHP exposure decreased brain zinc

content, leading to an increase in the activity of PP2A, which dephosphorylates and deactivates ERK1/2, a signal pathway required for normal brain development [41].

Gestational MZD and DEHP exposure decreased zinc concentrations in total brain cortex and brain cortex cytosolic fractions, particularly affecting the latter. A decrease in cytosolic zinc pools is highly relevant, because they mostly consist of rapidly available zinc which is required for dynamic cellular events including cell signaling regulation, gene expression and enzymatic reactions [42]. Multiple toxicants, including DEHP, can decrease zinc availability due to the promotion of an acute phase response [18, 43]. Accordingly, we observed that DEHP causes interleukin 6- and STAT3-dependent maternal liver zinc accumulation by increasing the expression of the zinc binding protein metallothionein, thus decreasing zinc availability to the fetus [20]. Similar findings showed that DEHP causes liver zinc accumulation and a zinc decrease in other organs, including testes and fetal brain [18, 44, 45]. In addition, maternal DEHP exposure can in part disrupt zinc metabolism by affecting maternal and fetal brain zinc transporters [19]. On the other hand, our results of MEHP-mediated decrease in IMR-32 cell zinc content, indicate the existence of a direct mechanisms of disruption of cell zinc homeostasis. This is a very relevant finding that cannot be mechanistically explained with the current evidence, but that will be the focus of future research.

Numerous studies have shown that DEHP exposure has detrimental effects on neurodevelopment. High levels of urinary DEHP metabolites in infants and children are associated with abnormal physical growth [46], high incidence of asthma and allergy [47] and abnormal genital and pubertal development [48]. In animals model, a high dose (1000 mg DEHP/kg BW) induces teratogenicity including high percentage of resorptions, high

frequency of fetal anencephaly, exencephaly, and neural tube defects [18, 49]. Similar effects have been reported for severe zinc deficiency in animals models [50] and in humans [51]. Prenatal exposure to 100 mg DEHP/kg BW decreased the number of proliferating NPCs and caused abnormal neuronal distribution, while 500 mg/kg BW caused cell death in the neonate mouse brain cortex [9]. Maternal exposure to 200-500 mg DEHP/kg BW decreased the number of hippocampus pyramidal neurons and caused long-term effects on behavior, including on anxiety and recognition memory [52]. Postnatal 100 mg DEHP/kg BW also affects NPCs proliferation and differentiation in the hippocampal dentate gyrus [53]. According to Peters et al., high dose (1000mg DEHP/Kg BW) have severe effects on fetal brain development causing high incidence of brain malformation, and these effects are caused by DEHP induced severe zinc deficiency [18], on the other hand, moderate/mild dose (300 mg DEHP/kg BW) potentially induced MZD, which had no major effects on organ malformation and fetal and maternal outcomes [20]. As a result, the current study provides relevant evidence showing that gestational exposure to 300 mg DEHP/kg BW affects fetal brain zinc homeostasis and that a maternal suboptimal zinc status can increase the susceptibility to DEHP neurodevelopmental toxicity.

Gestational MZD causes the down-regulated of SOX2 and PAX6 expression in SVZ of fetal brain cortex at E14 and E19 when NPCs are actively dividing [21]. Gestational exposure to DEHP, MZD, and DEHP+MZD impaired neurogenesis as evidenced by decreased markers of proliferating NPCs including SOX2, EMX1, and PAX6. SOX2 is a critical transcription factor that expresses in neural radial glia cells (RGCs) and NPCs in VZ and SVZ both in the developing and adult brain, being important for NPCs self-

renewal, maintenance of NPCs population and induction of neurogenesis [54, 55]. Similarly, the homeobox protein EMX1 is a transcription factor that expresses in both proliferating NPCs in the forebrain [56] and postmitotic neurons in the dorsal striatum [57]. Deletion of the EMX1 gene decreases the capacity of embryonic NPCs self-renewal and proliferation but do not affect glial cell differentiation [56]. EMX1 is found in cortical glutamatergic pyramidal neurons suggesting that EMX1 neurogenic actions are specific to the glutamatergic subtype [58], being cortical excitatory neurons specifically derived from EMX1- expressing NPCs [59]. Accordingly, we observed low levels of EMX1 in DEHP, MZD, and MZD+DEHP E19 brain cortex, in association with low levels of glutamatergic neuron marker VGLUT1. Cortical neurons can be derived directly from RGCs in the SVZ [60]. PAX6 regulates RGCs self-renewal, proliferation, survival and differentiation towards a neurogenic lineage [61, 62] and promotes TBR2 and TBR1 expression [63]. In comparison with C group, protein expression of TBR2, TBR1 were lower in brain cortex from DEHP, MZD and MZD+DEHP groups at E19, which is possibly due to the lower PAX6 expression [63, 64]. In the developing cortical plate, PAX6, TBR2 and TBR1 are expressed in VZ, SVZ and intermediate zone, respectively, where proliferation, differentiation, and maturation of glutamatergic neurons take place [64], with TBR2 and TBR1 being regulators of glutamatergic neurons generation [65, 66]. Accordingly, a lower number of mature neurons and glutamatergic neurons were observed in the E19 brain cortex from DEHP, MZD and MZD+DEHP groups. Parameters of neuronal migration and neurite outgrowth (doublecortin and vimentin) were also affected in the DEHP, MZD and MZD+DEHP E19 brain cortex. Doublecortin, is a microtubule-associated protein expressed in NPCs and immature neurons. Doublecortin

is important in maintaining neuronal cell integrity, neuronal migration [67], axon outgrowth [68], NPCs proliferation and differentiation [69, 70]. Vimentin, a cytoskeletal intermediate filament, is required for neurite outgrowth [71], neuronal differentiation [72], and migration [73]. Overall, these results demonstrate that DEHP and MZD disrupt the process of neurogenesis including NPCs self-renewal, proliferation, differentiation, and neuronal migration and neurite outgrowth, eventually leading to abnormal and imbalanced neuronal population in the fetal brain cortex. Supporting a role for zinc on the neurodevelopmental toxicity of DEHP, findings are similar for embryos from dams exposed to DEHP or MZD, while most parameters measured were more severely affected by the combination of MZD and DEHP exposure.

DEHP affects signaling pathways that can contribute to impaired neurogenesis. We previously reported the downregulation of ERK1/2 in zinc deficient IMR-32 cells and in E14 and E19 fetal brain cortex [21, 22]. Similarly, a major decrease in MEK1/2, ERK1/2, and ELK1 phosphorylation was observed in the brain cortex from DEHP group at E19. This may partially explain the associated changes in NPCs proliferation, differentiation, migration, and neuronal specification. ERK1/2 signaling is important for neuronal development. Double knockout of *ERK1* and *ERK2* alters neurogenesis, causing the thinning of the VZ and neuronal cells death [74]. ERK1/2 activates ELK1 [75], which controls the expression of several target genes that are relevant to neurogenesis [76, 77], including early growth factor 1 (EGR1), involved in neuronal cell differentiation [78], c-fos, involved in cell proliferation and differentiation [79], cyclin dependent kinase 7 (CDK7), and B-cell lymphoma 2 (BCL2), necessary for neuronal survival [80], and matrix

metalloproteinases (MMPs), involved in neuronal cell migration and neurite outgrowth [81].

Inactivation of MEK1/2 and ERK1/2 can be in part due to DEHP-mediated disruption of zinc homeostasis and consequent PP2A activation. Given that, PP2A dephosphorylates both kinases [82, 83]. Zinc inhibits PP2A activity both *in vivo* [84] and *in vitro* [85]. Zinc located in synaptic vesicle inhibits PP2A promoting tau hyperphosphorylation and maintaining microtubule stability in the central nervous system [86]. We previously showed that nutritional zinc deficiency increases PP2A activity both in E19 fetal brain cortex and IMR-32 cells [22]. In IMR-32 cells, MEHP decreased the phosphorylation of MEK1/2 and ERK1/2 in parallel with decreased intracellular zinc levels and PP2A activation. Findings show that zinc supplementation inhibited PP2A activity and restored MEK1/2 and ERK1/2 phosphorylation, indicates that MEHP-mediated disruption of the ERK1/2 signaling pathway is due to a decrease in available zinc necessary to inhibit PP2A. Additionally, zinc supplementation protected against MEHP cytotoxicity which further stresses the central relevance of a deregulation of zinc homeostasis on DEHP neurotoxicity.

In conclusion, our findings support the concept that the developmental toxicity of DEHP is in part due to a disruption in zinc homeostasis either secondary to an acute phase response and/or to a direct effect of MEHP on cellular zinc metabolism. This is further supported by the observed alterations in neurogenesis, including altered neuronal specification particularly affecting glutamatergic neurons, which are similar for both gestational DEHP exposure and nutritional MZD. Impaired ERK1/2 signaling secondary to PP2A activation can be a central mechanism in both zinc deficiency- and DEHP-

mediated alterations in neurogenesis. Importantly, a condition of zinc deficiency could increase DEHP neurotoxicity which makes low-income communities more susceptible to the deleterious effects of DEHP. Zinc supplementation during critical developmental periods emerge as a potential preventive strategy to mitigate the adverse consequences of developmental DEHP exposure.

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Conflict of interest

The authors have no conflict of interest to declare.

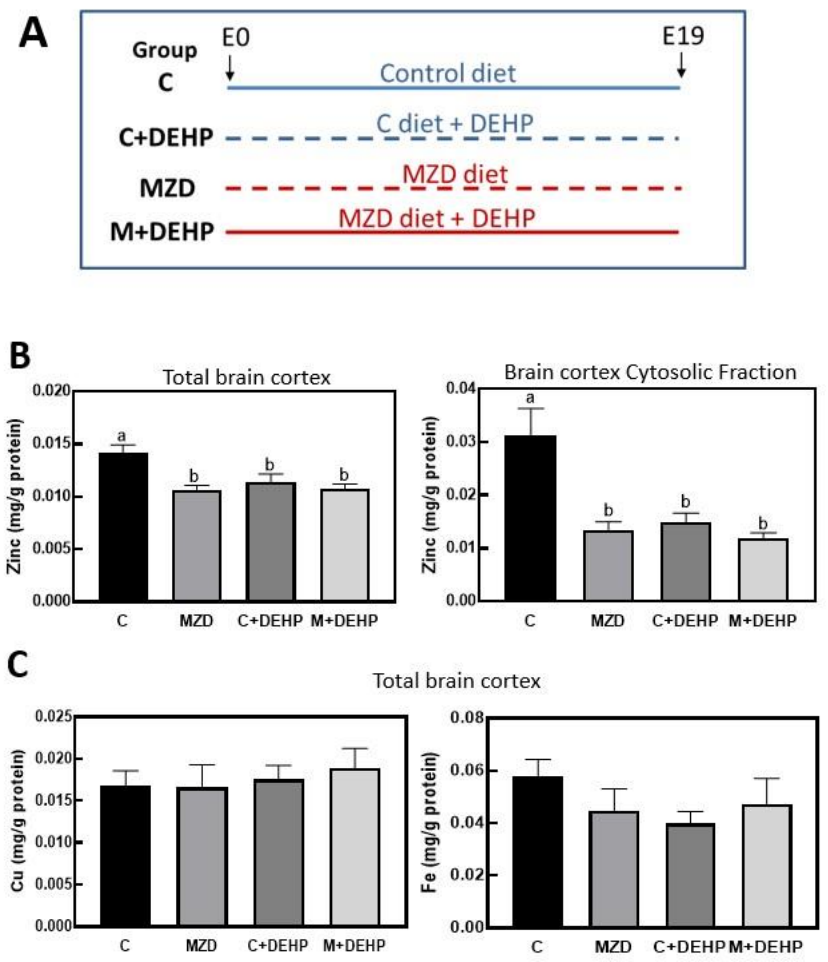

Figure 1. Zinc, copper, and iron concentrations in the E19 brain after maternal exposure to a marginal zinc diet and DEHP. (A) Experimental design; **(B)** Brain zinc concentration in E19 brain cortex and 100,000 x g brain cortex supernatants (cytosolic fraction); **(C)** Brain cortex copper (Cu) and iron (Fe) concentrations in E19 brain. Zinc, copper, and iron concentrations were measured by AES. Final minerals concentration in brain cortex is normalized by total proteins from tissue and final cytosolic minerals concentration is normalized by total protein in cytosol. Data are shown as mean ± SEM and are the average of 4 litters per group. Values having different superscripts are significantly different ($p<0.05$, One Way ANOVA).

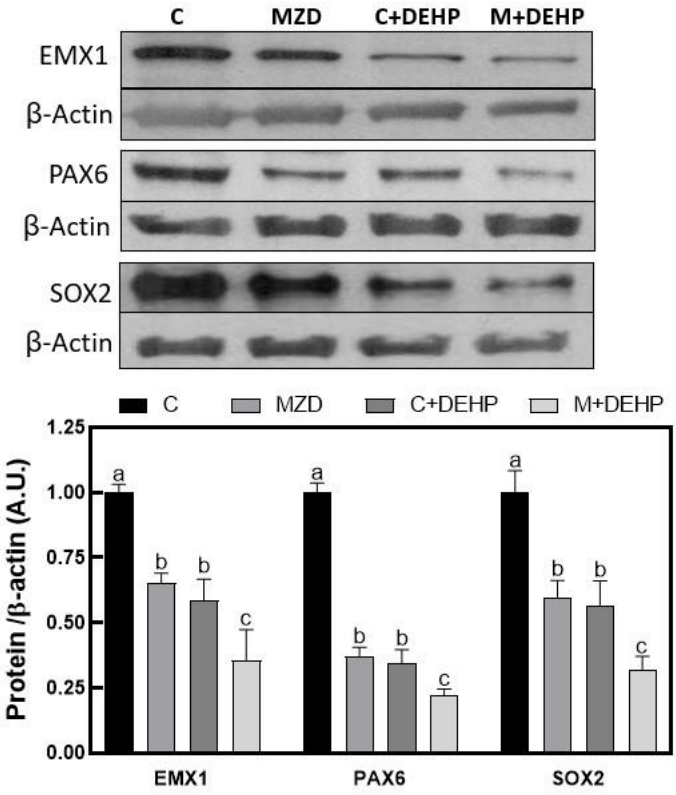

Figure 2. Maternal MZD and DEHP exposure affects the expression of markers (transcription factors) involved in NPC proliferation and self-renewal in the E19 rat brain cortex. EMX1, Pax6 and Sox2 protein levels were measured by Western blot in E19 brain cortex homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (A.U.: arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM of E19 brain cortex from 6 litters/group. Values having different superscripts are significantly different ($p<0.05$, One Way ANOVA).

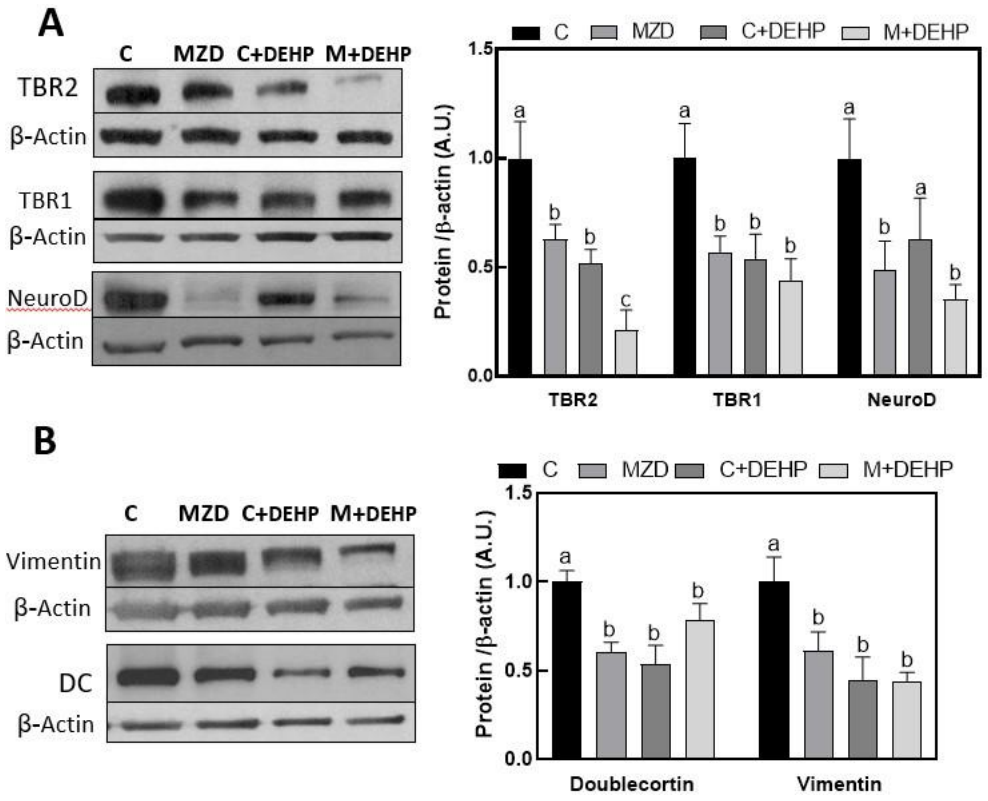

Figure 3. Maternal exposure to MZD and DEHP affects markers of neuronal differentiation, migration, and neurite expansion in the E19 brain cortex. (A) TBR2, TBR1, NeuroD and **(B)** vimentin and doublecortin (DC) protein levels were measured by Western blot in E19 brain cortex homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (A.U.: arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM of E19 brain cortex from 5-6 litters/group. Values having different superscripts are significantly different ($p<0.05$, One Way ANOVA).

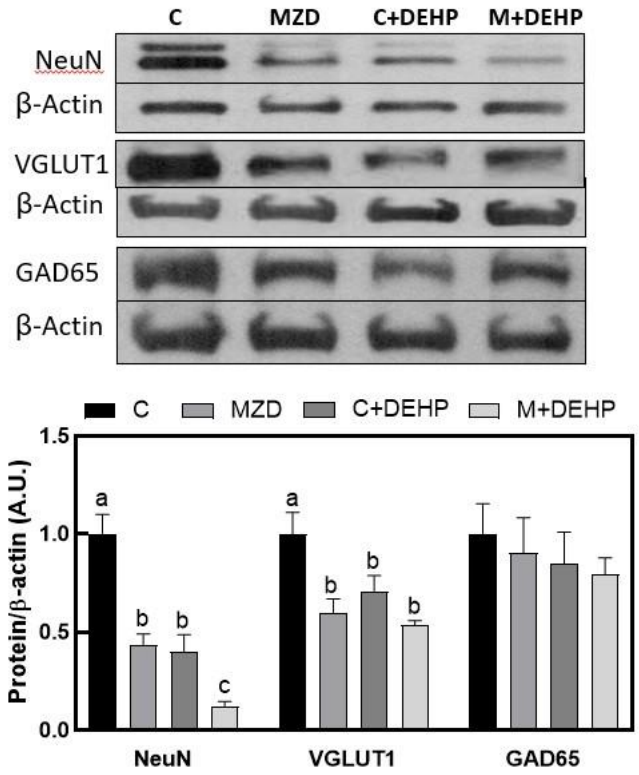

Figure 4. Maternal exposure to MZD and DEHP affects markers of mature neuron number and specification in the E19 brain cortex. NeuN, VGLUT1 and GAD65 protein levels were measured by Western blot in E19 brain cortex homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (A.U.: arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM of E19 brain cortex from 6 litters/group. Values having different superscripts are significantly different ($p<0.05$, One Way ANOVA).

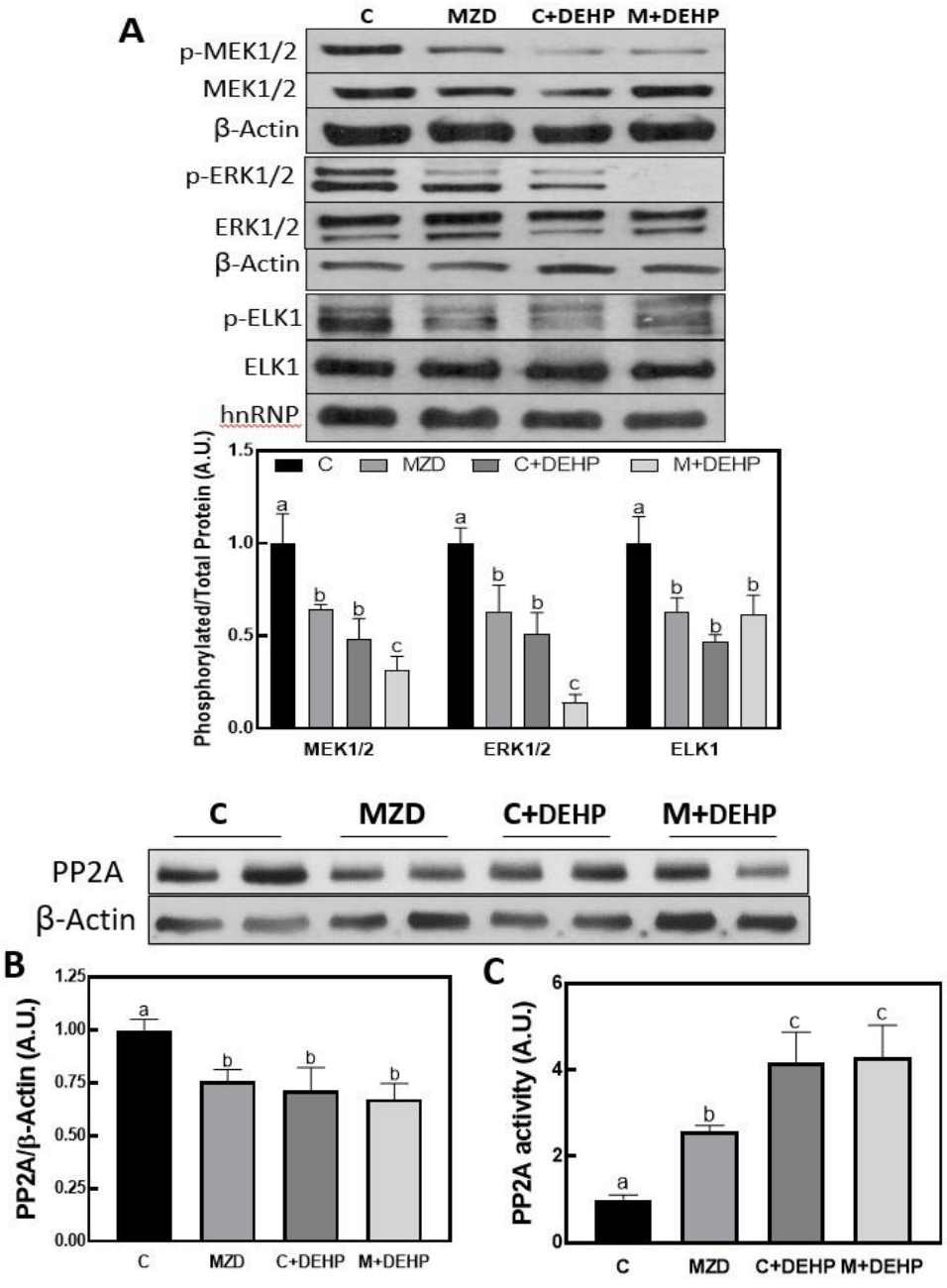

Figure 5. Maternal exposure to MZD and DEHP affects the ERK1/2 signaling pathway in the E19 brain cortex. (A) Phosphorylated and total MEK1/2, ERK1/2 and ELK1 protein levels were measured by Western blot in E19 brain cortex homogenates. After quantification of bands, phosphorylated protein levels were referred to the corresponding total protein content. Values (A.U.: arbitrary units) were normalized to

those of the control group. **(B)** PP2A protein levels were measured by Western blot in E19 brain cortex and **(C)** PP2A specific activity was measured as described in methods. Values were normalized to those of the control group. Results are shown as mean ± SEM of E19 brain cortex from 5-6 litters/group. Values having different superscripts are significantly different ($p<0.05$, One Way ANOVA).

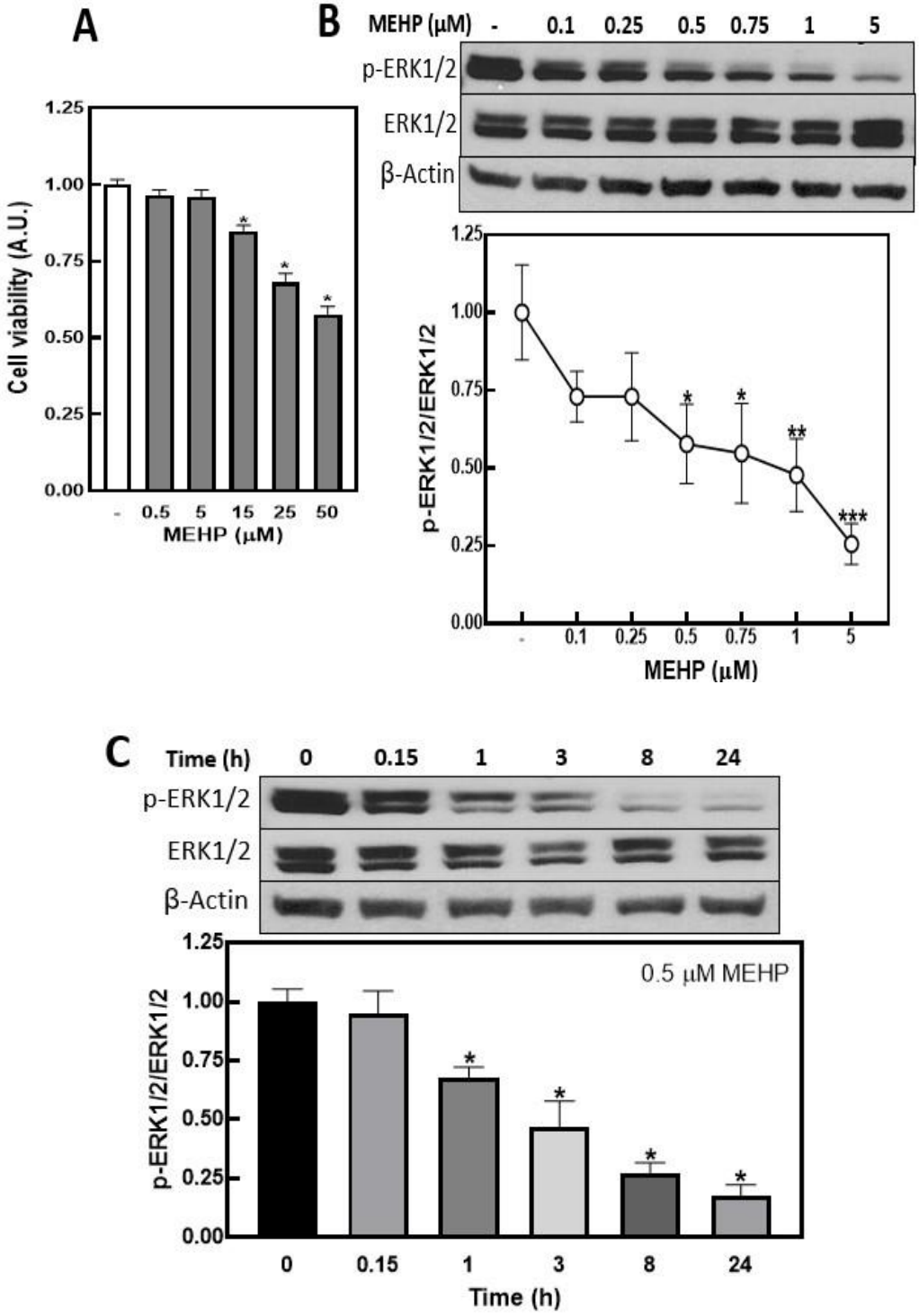

Figure 6. MEHP inhibits ERK1/2 phosphorylation in human neuroblastoma IMR-32 cells. (A) IMR-32 cells were incubated for 24 h in the presence of MEHP (0.5-50 μM),

and cell viability was measured using the MTT assay. Data were referred to control values. **(B)** IMR-32 cells were incubated for 24 h in the presence of MEHP (0.1-50 µM) and **(C)** for 0-24 h in the presence of 0.5 µM DEHP. **(B, C)** Phosphorylated and total ERK1/2 protein levels were measured by Western blot in cell homogenates. After quantification of bands, phosphorylated ERK1/2 levels were referred to total ERK1/2 protein content. Values (A.U.: arbitrary units) were normalized to those of the control group. **(A-C)** Results are shown as mean ± SEM of at least 4 independent experiments. *Significantly different from values for untreated cells ($p<0.05$, One Way ANOVA).

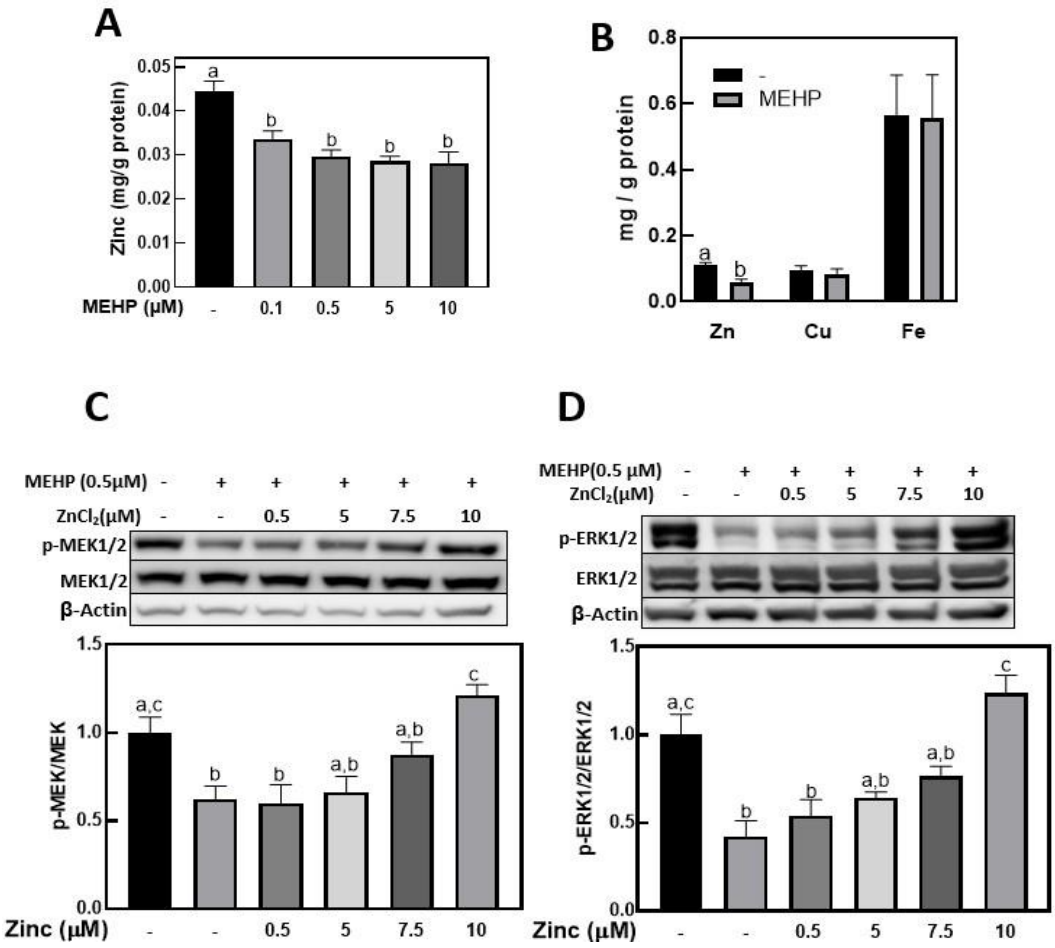

Figure 7. MEHP affects zinc homeostasis in human neuroblastoma IMR-32 cells. **(A, B)** IMR-32 cells were incubated for 24 h in the presence of **(A)** (0.1-10 µM) MEHP or **(B)** 0.5 µM MEHP, and zinc (Zn), copper (Cu), and iron (Fe) concentration in cells was measured by AES. **(C, D)** IMR-32 cells were incubated for 24 h in the presence of 0.5 µM MEHP and 0-10 µM $ZnCl_2$. Phosphorylated and total **(C)** MEK1/2 and (**D)** ERK1/2 protein levels were measured by Western blot in cell homogenates. After quantification of bands, phosphorylated MEK1/2 and ERK1/2 levels were referred to the respective total protein content. Values (A.U.: arbitrary units) were normalized to those of untreated cells. **(A-D)** Results are shown as mean ± SEM of at least 4 independent experiments. Values having different superscripts are significantly different ($p<0.05$, One Way ANOVA).

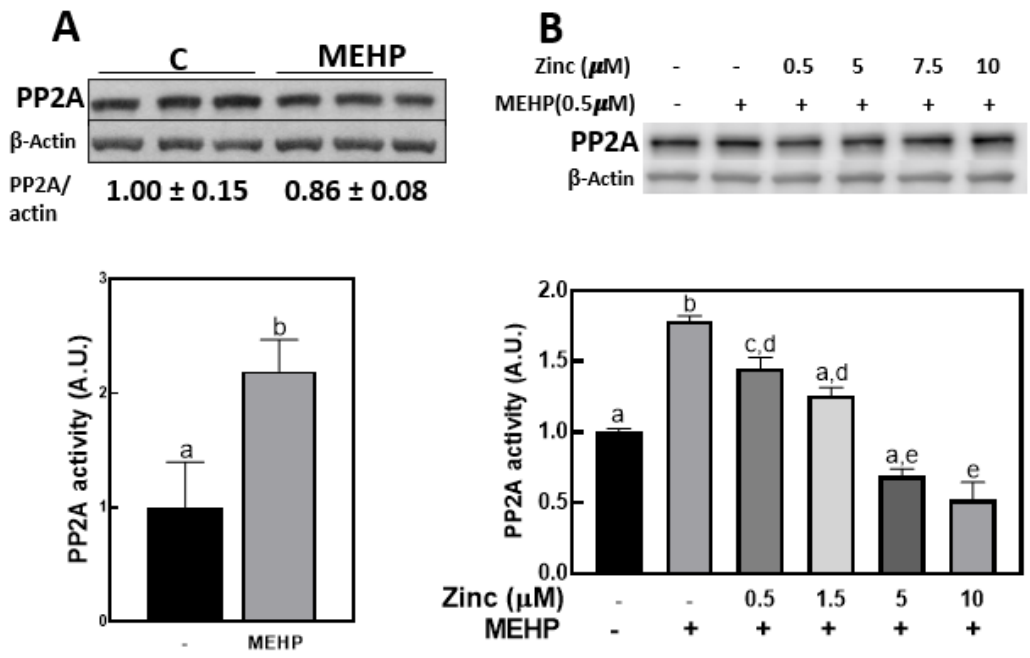

Figure 8. MEHP activates PP2A in human neuroblastoma IMR-32 cells. (A, B) IMR-32 cells were incubated for 24 h in the absence or the presence of 0.5 μM MEHP, and PP2A in cell homogenates was immunoprecipitated. **(A, upper panel)** Western blot for immunoprecipitated PP2A. PP2A content was referred to β-actin levels. **(A, lower panel)** PP2A specific activity was measured as described in methods. **(B)** $ZnCl_2$ 0.5-10 μM were added to the enzyme activity assay medium. Values (A.U.: arbitrary units) were normalized to those of untreated cells. **(A, B)** Results are shown as mean ± SEM of at least 4 independent experiments. Values having different superscripts are significantly different ($p<0.05$, One Way ANOVA).

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Chapter three

Gestational and Lactational Marginal Zinc Deficiency in Rats Affects Oligodendrogenesis, Motor Performance and Behavior in the Offspring

In preparation

Gestational and Lactational Marginal zinc deficiency in Rats Affects Oligodendrogenesis, Motor Performance and Behavior in the Offspring

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Abbreviations: CNS, central nervous system; CNPase, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase; ERK1/2, Extracellular signal-regulated kinase 1 and 2; GLI, glioma-associated oncogene homolog; MAG, myelin associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; NG2, neuron-glial antigen 2; Olig2: oligodendrocytes transcription 2; PDGFRα, platelet-derived growth factor receptor alpha; PLP, Proteolipid protein; SOX10, SRY (sex determining region Y)-box 10; SHH, sonic hedgehog; SMO, smoothened;

Abstract

Oligodendrocytes are responsible for myelin production in the central nervous system (CNS), and hypomyelination may slow saltatory nerve signal conduction and affect motor performance and behavior in the adult. Gestational marginal zinc deficiency (MZD) in rats significantly decreases proliferation of neural Stem cells (NSCs), leading to a lower number of neurons and altered specification. Given that NSCs also generate oligodendrocytes, this study investigated if MZD during early development in rats affects oligodendrogenesis in the offspring CNS. 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 pup's weaning at postnatal day (P) 20, and subsequently all offspring were fed the control diet until P60. Oligodendrogenesis was evaluated in the offspring at P2, P5, P10, P20 and P60, by measuring parameters of oligodendrocyte precursors cells (OPC) proliferation, differentiation, maturation, and of myelin formation. The expression of signaling proteins that regulate OPC proliferation (SHH, SOX10, Olig2) was lower in the brain from MZD than C offspring. This was associated with a low expression of OPC markers (NG2, PDGFRα), of myelin proteins (MBP, MAG, MOG, PLP) at various stages of development and a lower amount of myelin content at P20 and P60. Accordingly, parameters of motor performance and behavior (grip strength, rotarod, elevated T-maze, and open-field tests) were impaired in the MZD offspring at P60. 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.

1. Introduction

Pregnant women and children under 10 years 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 causes teratogenicity, including neural tube defects, skeletal malformations, growth retardation and defects of the heart, lung and brain [2]. In this regard, low serum zinc levels in pregnant mothers and neonates are correlated with pre-term delivery and neural tube defects [3]. On the other hand, a condition of MZD that does not cause teratogenicity and/or major adverse impact on pregnancy outcome, is more common in pregnant women and young children [4, 5]. However, developmental MZD 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 (ASD) [7] and attention-deficit/hyperactivity disorder (ADHD) symptoms in a middle-class American populations [8]. Additionally, MZD is correlated with behavioral disorders including delayed psycho-motor development and social interactions in infants [9], impaired cognitive performance, increase 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 MZD in rats impairs neurogenesis by decreasing the number of

Ki67 positive cells (proliferating NSCs) cells in the fetal brain ventricular zone (VZ) at embryonic day 19 (E19) [16]. Maternal 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]. While NSCs are also precursors of oligodendrocytes, there is limited information on the relevance of zinc for oligodendrogenesis and myelination.

NSCs also generate radial glial cells (RGCs), which can differentiate into intermediate progenitors cells (IPCs). IPCs can further differentiate into oligodendrocyte progenitor cells (OPCs) in the embryonic brain subventricular zone (SVZ) [19]. Oligodendrocytes are a type of neuroglial cells that generate the myelin sheath which maintains the integrity of the axon and provides axons with electrical insulation which enable a faster speed of action potential transmission [20]. Zinc is important to regulate and maintain the oligodendrocyte population and myelin formation [21, 22]. Three weeks old weanling rat treated with severe zinc deficiency (0.007ug/g) diet for 4-7 weeks decreased the number of myelinated axons and myelin sheath thickness in the optical nerve, suggesting that zinc is important for maintaining myelin integrity in the CNS [23]. Similarly, decreased myelin thickness and axonal degeneration are present in the sciatic nerve from 21 days old weanling rats fed a severe zinc deficient diet for 4 weeks, indicated that zinc is also important for maintaining myelin structure in the peripheral nervous system [24]. Additionally, zinc is a critical structural component of the myelin basic protein (MBP) [25], which has an important role in optimizing interactions between the myelin lipid bilayer and intercellular proteins [26]. Furthermore, zinc also play important role in maintaining functional myelin sheath by binding to the cytoplasmic domain of myelin

associated glycoprotein (MAG) and induce its dimerization [27]. Maternal MZD also causes alterations in myelin protein profiles in the offspring rat brain, although the individual proteins were not characterized [28].

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

2. Material and Methods

2.1. Materials

Primary antibodies for β-actin (#12620), phospho-MEK1/2 (Ser217/221) (#9154) and MEK1/2 (#9126), phosphor-ERK1/2 (Thr202/Tyr204) (#4370), and ERK1/2 (#9102), MAG (#9043), MBP (#78896) and GLi (#2643) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies for 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase; SC-166558) and myelin oligodendrocyte glycoprotein (MOG; SC-376138) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for Olig2 (AB35128) and NG2 (AB15894) were from Millipore (Burlington, MA, USA). Antibodies for PLP (ab28486) and PDGFRα (ab203491) were from Abcam Inc. (Cambridge, MA, USA). The antibody for SOX10 (MBS3201449) was from MyBioSource (San Diego, CA, USA). Secondary antibodies, immunohistochemistry Detection Reagents (HRP, Rabbit #8114) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) Substrate Kit (#8059), and hematoxylin

(#14166) were obtained from Cell Signaling Technology (Danvers, MA, USA). Polyvinylidene difluoride (PVDF), membranes and molecular weight standards for Western blot were obtained from BIO-RAD (Hercules, CA, USA). The enhanced chemiluminescence (ECL) Western blotting system was from Thermo Fisher Scientific Inc. (Piscataway, NJ, USA).

2.2. 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 (Wilmington, MA, USA). Female rats (200–225 g) were housed individually in stainless steel cages in a temperature (22–23 °C)- and photoperiod (12 h light/dark)-controlled room. Distilled water was provided through a daily flushed automatic watering system. An egg-white protein-based diet with adequate zinc (25 μg zinc/g) was the control diet [29]. Animals were fed the control diet for 7 days 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; 22 female rats were randomly divided into 2 groups (11 animals/group) 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 marginal zinc diet or the control diet from gestation day 0 until P20. Subsequently, offspring from both dietary groups were fed the control diet until P60 (**Fig. 1A**). Food intake was recorded daily, and body weight was measured at 5-day intervals. Offspring at P2, 5, 10, 20 and 60 and dams at P20 were anesthetized with isoflurane (2 mg/kg body weight) and blood and whole brain collected immediately after anesthesia. Meninges were removed and 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 3,000 x g for 15 min at 4°C to separate the plasma, which was subsequently stored at -80°C until analysis.

2.3. Determination of zinc concentration

The concentration of zinc in diets, brain tissue, and brain supernatants was measured by inductively coupled plasma atomic emission spectroscopy (AES) as described by Clegg et al. [30]. Brains were weighed, homogenized in ice-cold PBS (1g/10 ml), and centrifuged for 60 min at 100,000 x g at 4°C. Protein concentration was measured in the 100,000 x g supernatants using the Bradford assay [31]. Whole brain (50-100mg), 100,000 x g supernatants (1ml), and diet samples (500 mg) were added with 3 ml of 16 N HNO3 and digested for 72 h. Samples were dried and resuspended in ultrapure water. For plasma zinc concentration analysis, plasma (100µl) was added into 1ml of 0.1 N ultra-pure nitric acid and samples were digested at room temperature for 5 d. Zinc concentration was determined by ICP-AES (Trace Scan; Thermo Elemental, Franklin, MA, USA). Certified reference solutions (QC 21; Spec CentriPrep, Metuchen, NJ, USA) were used to generate standard curves. A sample of a National Bureau of Standards

bovine liver (SRM1577; U.S. Department of Commerce, National Bureau of Standards, Washington, DC, USA) was included with the samples to ensure accuracy and reproducibility.

2.4. Western blot analysis

Total brain proteins were extracted as previously described [32]. Protein concentration was measured [31], and aliquots containing 25–50µg of protein were separated by reducing 10% (w/v) polyacrylamide 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 TBST (Tris-buffered saline pH 7.4, 0.1% Tween 20) at 4^{o}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 (Amersham, Piscataway, NJ).

2.5. Assessment of brain myelin volume

Luxol Fast Blue (LFB) (Fisher, #212170250) was dissolved (1 mg LFB/ml) in 95% (v/v) alcohol and 5µl 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 a solution of 0.5 g/l lithium carbonate in distilled water. Finally, slides were counterstained with Cresyl Echt violet (Sigma, #C1791) (1 mg/ml in water) for 6 min and dehydrated in 95% (v/v) alcohol, 100% (v/v) alcohol, and xylene, sequentially, 10 drops

each for 3 times, and finally mounted with mounting medium (Cell signaling, #14177). Images were captured in a Bio Twin CM120 microscope (Philips/FEI, Hillsboro, OR, USA). Micrographs were taken with a Bioscan digital camera (Model 792; Gatan, Pleasanton, CA, USA). The volume of corpus callosum (CC) stained with LFB stain was measured with Image J (Image J, National Institutes of Health, Bethesda, Maryland, USA). Image analysis was performed according to previously described protocols [33]. In brief, blue images were selected by using the Color Deconvolution plugin of image J software with the Vector FastRed Fastblue DAB. The threshold intensity was manually set to subtract the staining background and LFB optical density was determined as previously reported [34, 35]. Myelin volume in CC was calculated by multiplying tissue thickness and area of CC stained with LFB.

2.6. 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 one hour in 5% (w/v) goat serum in TBST at room temperature then incubated overnight at 4 °C with the monoclonal 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-anti-rabbit). After three 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, Hillsboro, OR, USA).

Micrographs were taken with a Bioscan digital camera (Model 792; Gatan, Pleasanton, CA, USA). Image J software was used to analyze the resulting micrographs. Image analysis were performed according to described protocols [33]. In brief, the brown images were selected by using the Color Deconvolution plugin of image J software with the Vector H-DAB. The threshold intensity was manually set to subtract the background of staining and DAB optical density was determined as described in [34, 36].

2.7. Rotarod

Rotarod (Rotamex 5, Columbus Instruments (Ohio, USA)) is a widely used test to assess motor performance and neuromuscular coordination in rodents. The apparatus consists of four 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. Adult rat offspring (20 females and 20 males from each group) were tested in the rotarod. Animals were pre-trained on the rotating rod with constant speed of 4 revolutions per minute (rmp) before testing. At the testing session, animals were placed on the rods at an increasing speed (4-40rpm). The latency to fall and the speed at which they fell were recorded automatically on the display. Each animal was subjected to the trial three times and the results were averaged.

2.8. Grip strength test

The grip strength apparatus (San Diego Instrument, San Diego, CA) was used to measure animals' neuromuscular strength. The apparatus consists of an acrylic base, a force gauge and an adjustable grip. To avoid personal variability and to produce reliable data, the same experimenter performed all the grip force measurements. 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 degrees 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 to 10 seconds, and the time interval between each trial was 10-30 min to allow animal to rest. Five trials were performed with each animal and the three best force readings (in grams) were averaged. The final mean force readings were normalized by the weight of the animal (force/weight ratio). All procedures are performed by the same operator to avoid interindividual variability.

2.9. 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 x 56 cm) with a wall height of 60 cm. The center zoom was set as 30 cm x 30 cm to evaluate the anxiety levels of the animals. 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 auto-record and analyze the rat's behavior in the open field. Each rat was placed in the same corner of the open field arenas facing the center. The total distance traveled in the open field was measured to assess locomotor activity. Frequency to enter the center

zoom and duration to stay in the center zoom were measured to assess animal's anxiety levels.

2.10. 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. [37]. The ETM consists of one enclosed and two open arms, being the closed arm perpendicular to the open arms. The maze arm dimensions are 50 cm x 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 four paws into the open arms. The entire experiment was recorded with a video camera above the ETM 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 ETM 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 analysis were performed by a single examiner who was blinded to the sample group.

2.11. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC) and Graphpad Prism (San Diego, CA, USA). 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.

3. Results

3.1. Animal outcome

A marginal zinc nutrition throughout gestation did not affect overall maternal and fetal outcome. However, maternal weight gain and litter size were significantly lower in the MZD than in the C group (**Table 1**). Maternal plasma zinc concentration at lactational day 20 was 21% lower in MZD dams compared to controls, whereas plasma copper and iron concentrations were similar between groups (**Fig. 1D**). Plasma zinc concentration was significantly lower at P2, P5, P10, and P20 (34%, 25%, 37%, and 40%, respectively) in MZD pups compared to the control group (**Table 1**). Offspring brain weight was similar between groups at all developmental stage (**Table 1**). Whole brain zinc concentration in the P2, P5, P10, P20, and P60 offspring was similar between groups **(Fig. 1B**). Zinc concentration in brain 100,000 x g supernatants isolated from P2, P5, P10, and P20 offspring was significantly lower (79%, 79%, 71% and 64%, respectively) in the MZD offspring compared to controls (**Fig. 1C**). Iron and copper concentrations in the whole brain and in 100,000 x g supernatants were similar between groups at all developmental stages (**Supplemental Fig. 1**).

3.2. Maternal MZD affected markers of oligodendrogenesis in the P2 offspring rat brain.

Our previous work showed that zinc is important in NSCs proliferation, self-renewal, and differentiation [17]. We now investigated the effects of maternal MZD on the

proliferation and differentiation of OPCs 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 (**Fig. 2**). NG2 and PDGFRα are protein markers that specifically express in brain OPCs. Maternal MZD caused a 32% decrease in NG2 and 38% decrease in PDGFRα expression in the P2 offspring brain (**Fig. 2**). Olig2 and SOX10 are transcription factors that control OPCs proliferation and differentiation. Maternal MZD caused 35% and 50% reduction of Olig2 and SOX10 protein expression, respectively, in the P2 offspring rat brain compared to controls. (**Fig. 2**). CNPase is a myelin-associated enzyme involved in OPCs differentiation, MAG is a marker of mature oligodendrocytes and PLP is a major structural protein component of myelin. MZD caused 17% and 49% reduction in CNPase and PLP protein levels in the P2 brain compared to controls. MAG protein levels were similar between groups (**Fig. 2**).

3.3. Maternal MZD affected sonic hedgehog (SHH) and extracellular regulated protein kinase 1 and 2 (ERK1/2) signaling pathways in the P2 offspring rat brain

We next examined the effects of early developmental zinc deficiency on signaling pathways that regulate brain OPCs, proliferation, and differentiation. Key proteins involved in the SHH pathways were measured by Western blot including cleaved SHH, smoothened (SMO) and glioma-associated oncogene homolog (GLI1). At P2, cleaved SHH, SMO and GLI1 protein levels were 61, 17 and 54% lower in the MZD offspring brain compared to controls (**Fig. 3A**). Our previous work showed that brain ERK1/2 activation was affected by maternal MZD at E19 [16]. In the P2 offspring rat brain, ERK1/2 phosphorylation was 34% lower in the MZD group compared to controls, while the

activation (phosphorylation) of upstream Extracellular Signal-regulated Kinase Kinase (MEK1/2) and a downstream target cAMP response element-bind protein (CREB) was similar between groups (**Fig. 3B**).

3.4. Maternal MZD affected markers of oligodendrogenesis in the P5 and P10 rat offspring brain

At P5, a similar pattern as that of 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 to controls (**Fig. 4**). Transcription factors Olig2 and SOX10 protein levels were 41 and 38% lower, respectively, in the brain of MZD rat pups at P5 compared to controls (**Fig. 4**). CNPase and PLP protein levels were 30 and 21% lower, respectively in MZD brains compared to controls, whereas MAG protein levels were similar between groups (**Fig. 4**).

At P10, while brain NG2, PDGFRα, and CNPase protein levels were similar between groups, 68 and 67% lower levels of Olig2 and SOX10, respectively, were observed in MZD compared to controls (**Fig. 5**). At this stage, oligodendrocytes reach a mature state and actively produce myelin. At P10, PLP, MAG, MBP protein levels were significantly lower (31%, 51%, 49%, respectively) in the MZD offspring brain than in controls (**Fig. 5**).

3.5. Maternal MZD affected markers of oligodendrogenesis 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 to the control group (**Fig. 6A**). Brain NG2, PDGFRα, and CNPase protein

levels were similar between groups (**Fig. 6A**). MBP protein level, measured by Western blot, was 64% lower in the P20 MZD offspring brain compared to controls (**Fig. 6A**). Immunohistochemistry characterization of MBP intensity stain by DAB showed 48% and 31% lower levels in both the CC (**Fig. 6C, D and G**) and the hippocampus (**Fig. 6C, D and H**), respectively, in the MZD offspring compared to controls. Myelin content in CC was evaluated by LFB staining. A lower (18%) myelin volume was measured in the CC of MZD compared to control offspring (**Fig. 6B and E**). Myelin staining intensity of LFB in CC was 31% lower in MZD than in controls (**Fig. 6B and F**). These results indicate that early developmental MZD affected myelin formation in the P20 offspring rat brain.

3.6. Maternal MZD 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 MZD on oligodendrogenesis and myelination. At P60, no differences were observed in brain NG2 and PDGFRα protein levels in males (**Fig. 7A, C**) and females (**Fig. 7B, D**) offspring of both groups. Moreover, CNPase expression were similar between groups in both male (**Fig. 7A, C**) and female (**Fig. 7B, D**) offspring. Even after 40 d in the control diet, lower levels of Olig2 (31% in males (**Fig. 7A, C**) and 33% in females (**Fig. 7B, D**)) and SOX10 (32% in males (**Fig. 7A, C**) and 36% in females (**Fig. 7B, D**)) were observed in the MZD offspring than in controls. Levels of the myelin proteins MAG, MBP, MOG, and PLP were lower in both male (35, 72, 40 and 31%, respectively, (**Fig. 7A, C**)) and female (27, 34, 25 and 26%, respectively, (**Fig. 7B, D**)) offspring of the MZD group compared to the control group.

Immunohistochemistry analysis also showed lower levels of brain myelination in the MZD group compared to controls in both P60 males and females 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 to controls (**Fig. 8A, D, E**). In the female MZD P60 offspring brain, lower myelin volume and LFB intensity (25% and 44%, respectively) were observed compared to controls in CC (**Fig. 8H, K, L**). Immunohistochemistry analysis showed lower MBP intensity stained by DAB in both CC and hippocampus (75% and 53%, respectively) in P60 males MZD offspring rat brain (**Fig. 8B, C, F, G**) compared to controls. Similar effects were seen in MZD P60 females, that showed 68% and 38% lower MBP intensity in CC and hippocampus, respectively, compared to controls (**Fig. 8I, J, M, N**). Results indicated that early developmental MZD affected the oligodendrogenesis and had long-term effects on myelination in the mature adult rat brain.

3.7. Early developmental MZD 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 which evaluates 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 in controls (**Fig. 9A**), being 29 and 25% lower in MZD males and females, respectively (**Fig. 9B**). In the rotarod test, the latency to fall from the rotating rod was 21% shorter for the P60 MZD group compared to the control group (**Fig. 9C**). The latency was

26 and 34% shorter, in MZD males and females, respectively, compared to controls. (**Fig. 9D**).

Locomotor activity was evaluated using an open field test in MZD and control offspring pups at P60. Within 10 min MZD rats travelled 13% less distance compared to controls (**Fig. 10A, G**). This overall difference was due to 71% lower total distance traveled by females MZD compared to controls, while for males it was similar for both groups (**Fig. 10B, G**). Anxiety levels were assessed by measuring the frequency to enter and duration to stay in the center zone of the open field. No significant differences were observed between MZD and control groups (**Fig. 10C, D, E, F**). However, in the ETM test, anxiety levels were 67% higher in MZD rats compared to controls. This was observed when combining both females and males data (**Fig. 9E**), while differences were not significant when analyzing each sex separately (**Fig. 9F**).

4. Discussion

Zinc plays a central role in brain development [11]. This work investigated the effects of a maternal marginal zinc nutrition on OPCs proliferation and progression of OPCs to pre-oligodendrocytes and immature oligodendrocytes and eventually myelin-producing oligodendrocytes. This study presented evidence that a decreased proliferation of OPCs resulting from maternal marginal zinc deficient diet leads to a disruption of oligodendrogenesis in the rat offspring brain. Lower expression of OPCs protein marker during early development is associated with a decreased expression of protein marker of mature oligodendrocytes and hypomyelination in the MZD offspring rat brain that extends

into adulthood and results in neuromuscular strength, locomotor and behavioral alterations.

Critical periods of susceptibility to zinc deficiency during brain development have been studied in rodents. Gestational severe zinc deficiency is associated with teratogenic effects affecting multiple organs including neural tube defects, decreased brain size, malformations of eyes and olfactory bulbs [38-40]. In contrast, maternal mild/marginal zinc deprivation does not cause major birth defects in rodents but causes behavioral alterations [6]. Maternal MZD is associated with deficits in learning and memory [41], impaired social interaction, increase emotionality including anxiety and depression, and aggressiveness [42, 43], and decreased locomotor function [44] in the offspring. Additionally, lower serum zinc levels have been reported in children suffering from neurological disorders such as ASD [7], ADHD [8], mood disorder (depression, anxiety, and aggressiveness) [45], schizophrenia [46, 47], and cognitive and motor function [48]. Even though behavioral testing was conducted later in adulthood, these behavioral problems could be in part explained by exposure to developmental zinc deficiency that could have long lasting effects on the neurological function through the early disruption of neurogenesis and/or oligodendrogenesis.

Similarly to what we previously observed [17], we currently observed that maternal MZD did not affect maternal and fetal outcomes. The observed lower maternal weight gain at gestational day 19, is associated with a lower litter size in the MZD compared to the control group. Maternal marginal zinc nutrition affected offspring plasma zinc concentration at P2, P5, P10 and P20, suggesting that maternal milk also had a lower zinc content. While maternal MZD did not affect whole brain zinc concentration in the

offspring, it caused a significant decrease in cytosolic zinc concentration. This finding suggests that the most rapidly accessible zinc pool present in the cytosol is adversely impacted by MZD. Thus, there would be less of this available zinc pool for increased requirements associated with active processes during early development, including cell proliferation and cell signaling regulation. In this regard, we showed that zinc is required to inhibit protein phosphatase 2A, which activation due to decreased bioavailable zinc caused ERK1/2 de-phosphorylation and inactivation of downstream pathways involved in NSCs proliferation [16].

Zinc deficiency-associated disruption of the ERK1/2 signaling pathway affects NSCs proliferation, differentiation, and specification in various stages of neurogenesis [17, 49, 50]. ERK1/2 is important in regulating the cell cycle [51], neuronal differentiation [52], and survival [53]. 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 involved in regulating OPCs proliferation. In this regard, maternal MZD also decreased SHH activation and the levels of the SHH-regulated OPCs proteins NG2, PDGFRα and Olig2. SHH is a morphogenic protein present throughout the brain during development, being involved in embryonic tissue induction and patterning [54]. SHH is also important for OPCs proliferation and migration [55, 56], differentiation [57], maturation and myelination [58]. SHH signaling is key regulator in NG2 and PDGFRα positive OPCs proliferation in the ventral forebrain and late in the dorsal cortex [59]. SHH also regulates the transcription factor Olig2 which controls OPCs proliferation [60]. Additionally, the differentiation of OPCs is under the modulation of SHH. Thus, inhibition of SHH with cyclopamine, a potent inhibitor of the SHH signaling activator SMO, decreases MBP and

MAG expression in differentiating oligodendrocytes [57]. In postnatal oligodendrogenesis, SHH regulates mature oligodendrocyte production and contributes to myelination and remyelination in the adult brain [58, 61]. Our results suggest that ERK1/2 and SHH could crosstalk during oligodendrogenesis. In fact, SHH agonists activate ERK1/2 increasing cell proliferation *in vitro* [62]. Overall, SHH emerges as key altered signaling cascade, which in combination with ERK1/2, can in part explain the altered offspring brain oligodendrogenesis caused by maternal MZD.

Postnatally, OPCs are originated from the ventricular zone and then migrate to distant sites, while continuously dividing and differentiating throughout development, which persists into adulthood [63, 64]. Maternal MZD also disrupted offspring OPCs differentiation into pre-oligodendrocytes as evidenced by decreased expression of CNPase, Olig2 and SOX10 at P2 and P5. CNPase is an enzyme presents in pre-oligodendrocytes [65], and is important for oligodendrocyte differentiation and myelination [66, 67]. Olig2 and SOX10 are both largely expressed in differentiating OPCs, pre-oligodendrocytes and mature oligodendrocytes throughout development [68, 69]. SOX10 is thought to be necessary for the development of myelin-forming oligodendrocytes [70] and the presence of Olig2 is critical for maintaining the population of mature oligodendrocytes [71]. We observed that oligodendrocyte maturation and subsequent myelination are both affected in the marginal zinc deficient offspring rat brain at P10 and P20, as evidenced by low levels of the mature oligodendrocyte proteins MAG, MOG, MBP, and PLP and decreased myelin amounts at P20. Overall, results showed that maternal MZD leaded to decreased expression of protein markers of OPCs in the

offspring's early stages of development (P2, P5) and was affecting OPCs proliferation and differentiation into mature oligodendrocytes.

Early developmental MZD altered oligodendrogenesis which had long lasting adverse effects in the young adult brain. Consumption of a zinc sufficient diet from weaning to P60 did not reverse or alleviate the negative neuromuscular and neurocognitive outcome despite the restoration of zinc status. Hypomyelination was evidenced by lower brain myelin proteins and a decreased myelin mass in the adult rat CC and hippocampus in MZD. This was observed in both the male and female offspring, indicating that maternal MZD equally affects oligodendrogenesis in both genders. These alterations were reflected in an impaired motor performance as evidenced by the rotarod and grip strength tests. The rotarod test is commonly used to evaluate de-myelination diseases in rodent models and evaluates neuromuscular, sensorimotor, ability to maintain balance, and integrative neurological function [72-74]. Studies show that the number of falls from the rotating rod is increased in hypomyelinated [75, 76] and in multiple sclerosis mouse models [77]. The grip strength test is used to access muscle strength, neuromuscular function, and ability to react to the pulling force [78, 79]. Alterations in the grip strength test are observed in mouse models of white matter injury [80] and cuprizone-induced demyelination [76]. Motor activity assessed in the open field test suggested that maternal MZD affects more females that males. The open field test is commonly used to measure locomotor activity in a novel environment [81, 82]. Mixed results are observed in animals models of demyelination which showed no alterations [74, 83], increased [74, 75] or decreased [76] locomotor activity measured by total distance travelled in the open field test. The T-maze test is a modification of the elevated plus maze designed to

evaluate anxiety level in animals [84, 85]. Higher [76] and lower [86] anxiety levels were reported using this test in mouse models of demyelination. Higher levels of anxiety were observed in the T-maze test for the adult MZD offspring. However, how the latter can relate to hypomyelination is not clear. The previously observed alterations in neurogenesis and imbalance between excitatory and inhibitory cortical neurons with maternal MZD can in part contribute to the higher anxiety levels. Relevant to our current results, demyelination in mouse model exhibits schizophrenia type of behavior [87]. Lower number of OPCs and mature oligodendrocytes, and consequently of hypomyelination, have been observed in a mouse model of ASD [88, 89]. Patients with multiple sclerosis, characterized by demyelination, have co-existing mental disorders such as schizophrenia [90]. 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.

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 effects of maternal MZD on NSCs proliferation that affect 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 a 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.

Table 1. Maternal and offspring outcome

Groups	C	MZD	C	MZD	C	MZD	C	MZD	C	MZD
Gestational day	GD0		GD5		GD10		GD15		GD19	
Maternal body wt gain	--	--	22 ± 1.78	19.6 ± 1.55	42.5 ± 3.03	38.1 ± 2.33	70 ± 3.28	60.9 ± 3.54	116.4 ± 5.12	101.2 ± 5.87 **
Maternal Food intake	14 ± 0.76	14.44 ± 0.68	18.06 ± 0.43	18.22 ± 0.83	19.27 ± 0.54	18.02 ± 0.80	22.98 ± 0.91	21.48 ± 1.16	20.85 ± 0.57	20.49 ± 0.64
Postnatal days	P2		P5		P10		P20		P60	
Litter size	13.71 ± 0.43	11.1 ± 3.16*	--	--	--	--	--	--	--	--
Maternal/pup food intake	27.59 ± 1.82	24.28 ± 1.66	33.16 ± 1.79	28.58 ± 2.09	32.90 ± 1.77	33.01 ± 1.69	48.31 ± 2.37	47.93 ± 1.88	46.43 ± 3.24	48.38 ± 3.79
Pup body wt (mg)	7.06 ± 0.25	7.88 ± 0.18	11.78 ± 0.43	12.15 ± 0.42	20.75 ± 1.08	22.08 ± 1.03	37.2 ± 2.15	41.43 ± 2.04	233.71 ± 11.27	233.36 ± 10.64
Pup brain wt (mg)	0.31 ± 0.01	0.33 ± 0.01	0.51 ± 0.14	0.54 ± 0.01	0.91 ± 0.04	0.98 ± 0.02	1.26 ± 0.03	1.35 ± 0.02	1.79 ± 0.03	1.75 ± 0.03
Pup brain wt/body wt	0.045 ± 0.001	0.042 ± 0.001	0.043 ± 0.012	0.045 ± 0.001	0.045 ± 0.001	0.045 ± 0.002	0.035 ± 0.001	0.033 ± 0.001	0.008 ± 0.001	0.008 ± 0.001
Pup Plasma [Zn] µM	45.80 ±1.45	30.20 ±1.71*	41.96 ± 0.42	31.30 ± 0.81*	33.10 ± 1.95	20.80 ± 0.97*	15.30 ± 0.42	9.20 ± 0.88*	20.30 ± 1.42	19.80 ± 1.03
Pup Plasma [Cu] µM	13.30 ±0.42	13.89 ±0.50	16.33 ± 0.35	16.79 ± 0.49	17.94 ± 0.40	18.75 ± 0.36	19.15 ± 0.48	19.81 ± 0.48	26.93 ± 0.80	25.78 ± 0.67
Pup Plasma [Fe] µM	58.73 ± 4.45	59.04 ± 2.45	54.32 ± 5.02	57.10 ± 5.30	34.69 ± 2.41	35.65 ± 1.53	41.42 ± 1.81	42.57 ± 1.38	41 ± 1.04	42 ± 1.28

Rat dams were fed control (25 µg zinc/g) (C) or marginal zinc (10 µg zinc/g) (MZD) diets from gestation days 0 to postnatal day (P) 20. All pups were fed the control diet from P20 to P60. Results for the different parameters measures are shown as means ± SEM, n=10, analyzed by one-way ANOVA with Fisher's LSD (*$p < 0.05$ **$p < 0.01$ relative to control). Wt: weight

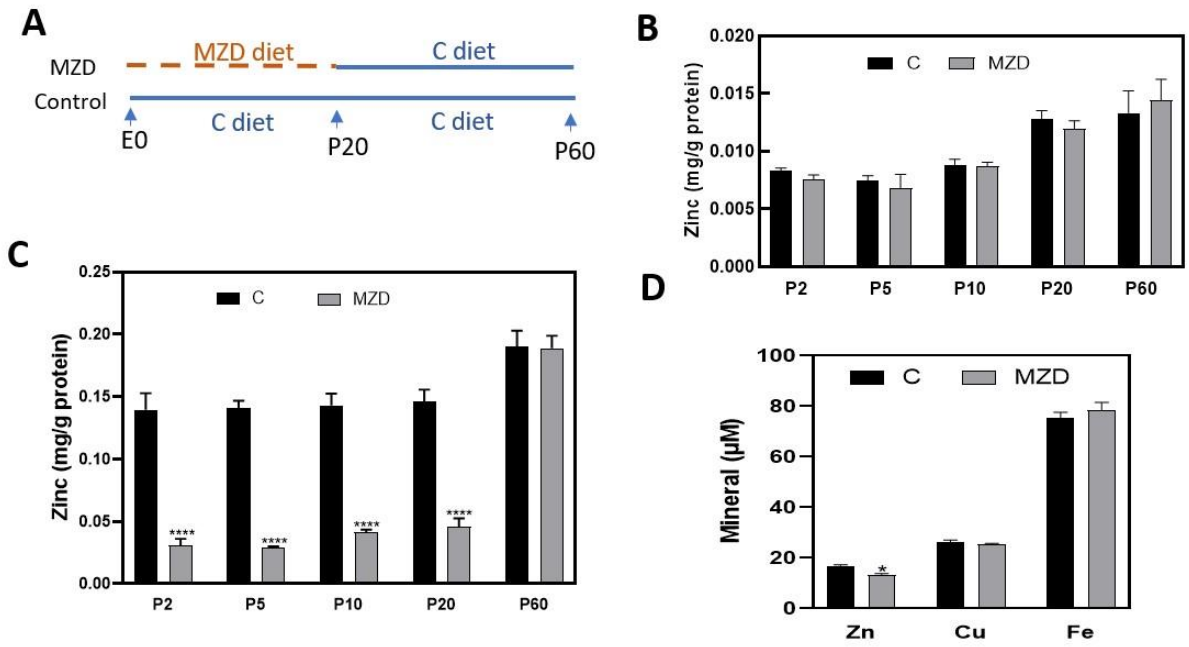


Figure 1. Maternal and offspring minerals concentration in plasma and/or brain. (A) Experimental design. **(B)** Zinc concentration in the offspring (P2-P60) whole brain and **(C)** in brain 100,000 × g supernatants. **(D)** Maternal plasma zinc, copper, and iron concentrations at lactational day 20. Minerals were measured by AES as described in methods. Data are shown as mean ± SEM and are the average of 9 litters per group. *, ****Significantly different from the control group at the same developmental stage (t-test, *P<0.05, ****P<0.0001).

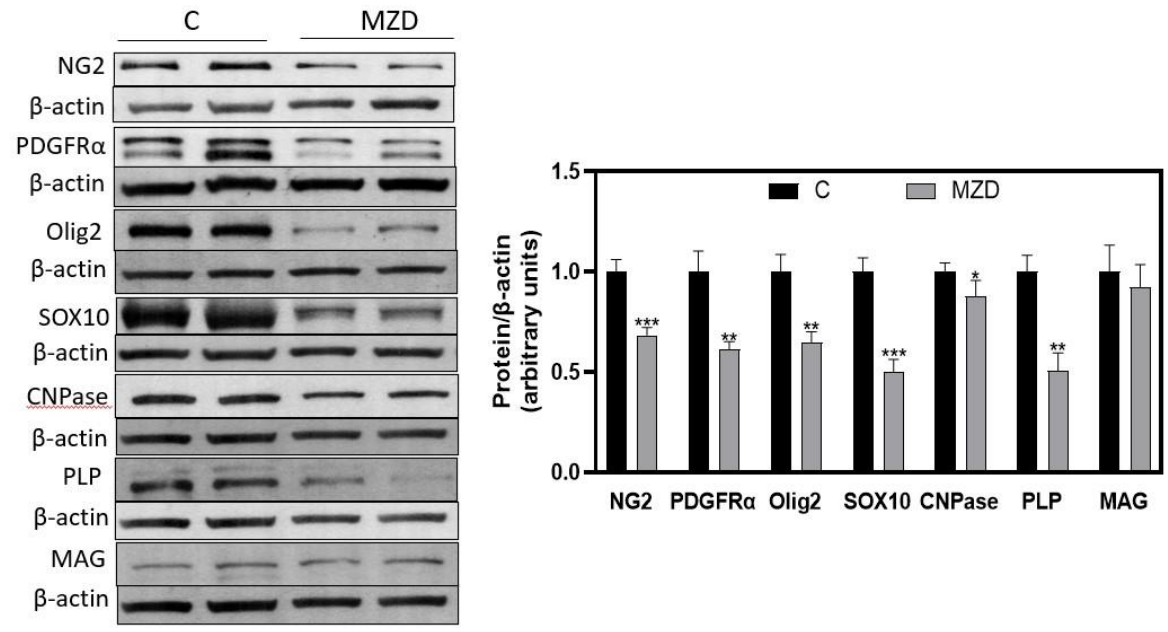


Figure 2. Maternal MZD affects parameters of oligodendrogenesis in the P2 offspring rat brain. NG2, PDGFRα, Olig2, SOX10, CNPase, PLP and MAG protein levels were measured by Western blot in P2 offspring rat brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM from 9 litters/group. *, **, ***Significantly different from the control group (*p < 0.05, **P<0.01, ***P<0.001, Student's t-test).

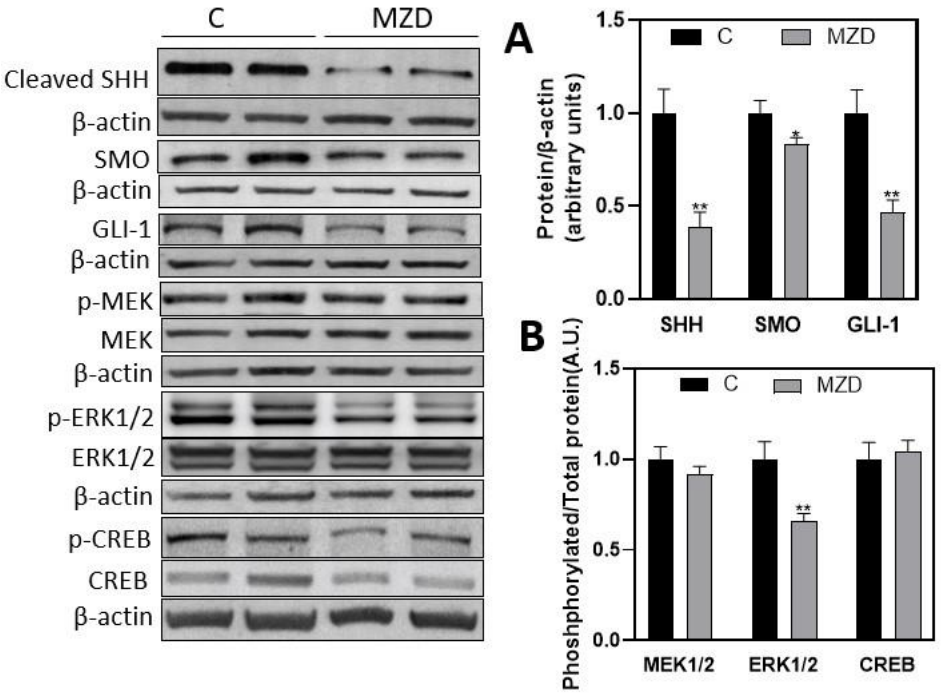


Figure 3. Maternal MZD affects SHH and ERK1/2 signaling pathway in the P2 offspring rat brain. (A) Cleaved SHH, SMO and GLI-1 proteins levels and **(B)** phosphorylated and total MEK1/2, ERK1/2 and CREB protein levels were measured by Western blot in P2 rat brain homogenates. After quantification of bands, proteins were referred to **(A)** β-actin levels, and **(B)** 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/group. *, ** Significantly different from the control group (*p<0.05, **P<0.01, Student's t-test).

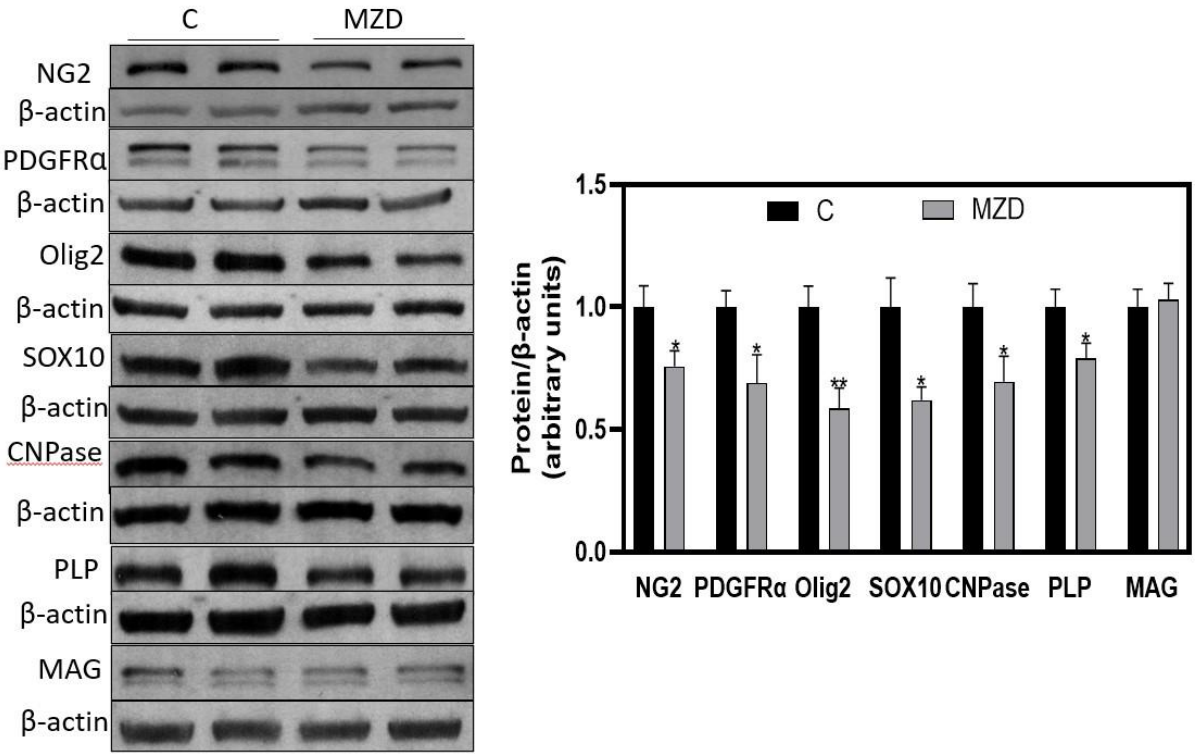


Figure 4. Maternal MZD affects parameters of oligodendrogenesis in the P5 offspring rat brain. NG2, PDGFRα, Olig2, SOX10, CNPase, PLP, and MAG protein levels were measured by Western blot in P5 offspring rat brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM from 9 litters/group. *, **Significantly different from the control group (*p < 0.05, **P<0.01, Student's t-test).

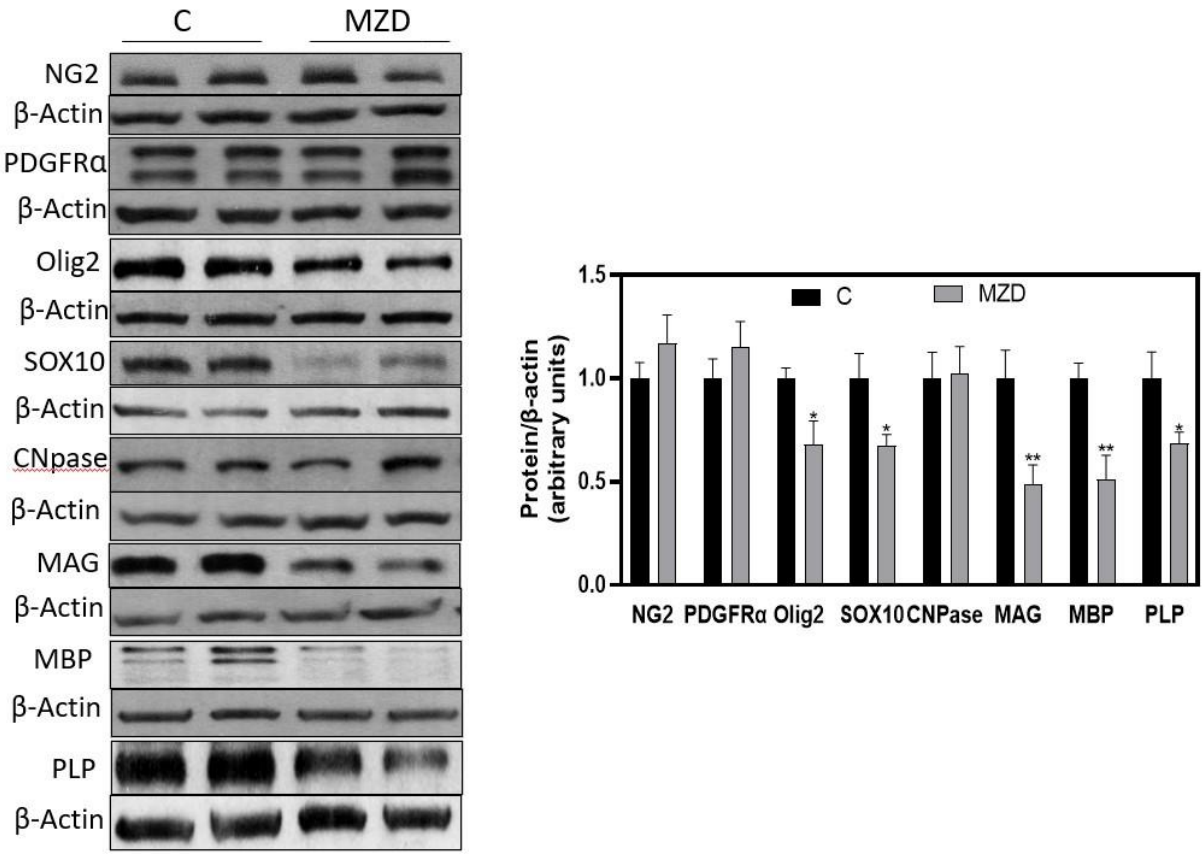


Figure 5. Maternal MZD affects parameters of oligodendrogenesis in the P10 offspring rat brain. NG2, PDGFRα, Olig2, SOX10, CNPase, MAG, MBP, and PLP protein levels were measured by Western blot in P10 offspring rat brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown as mean ± SEM from 9 litters/group. *, **Significantly different from the control group (*p < 0.05, **P<0.01, Student's t-test).

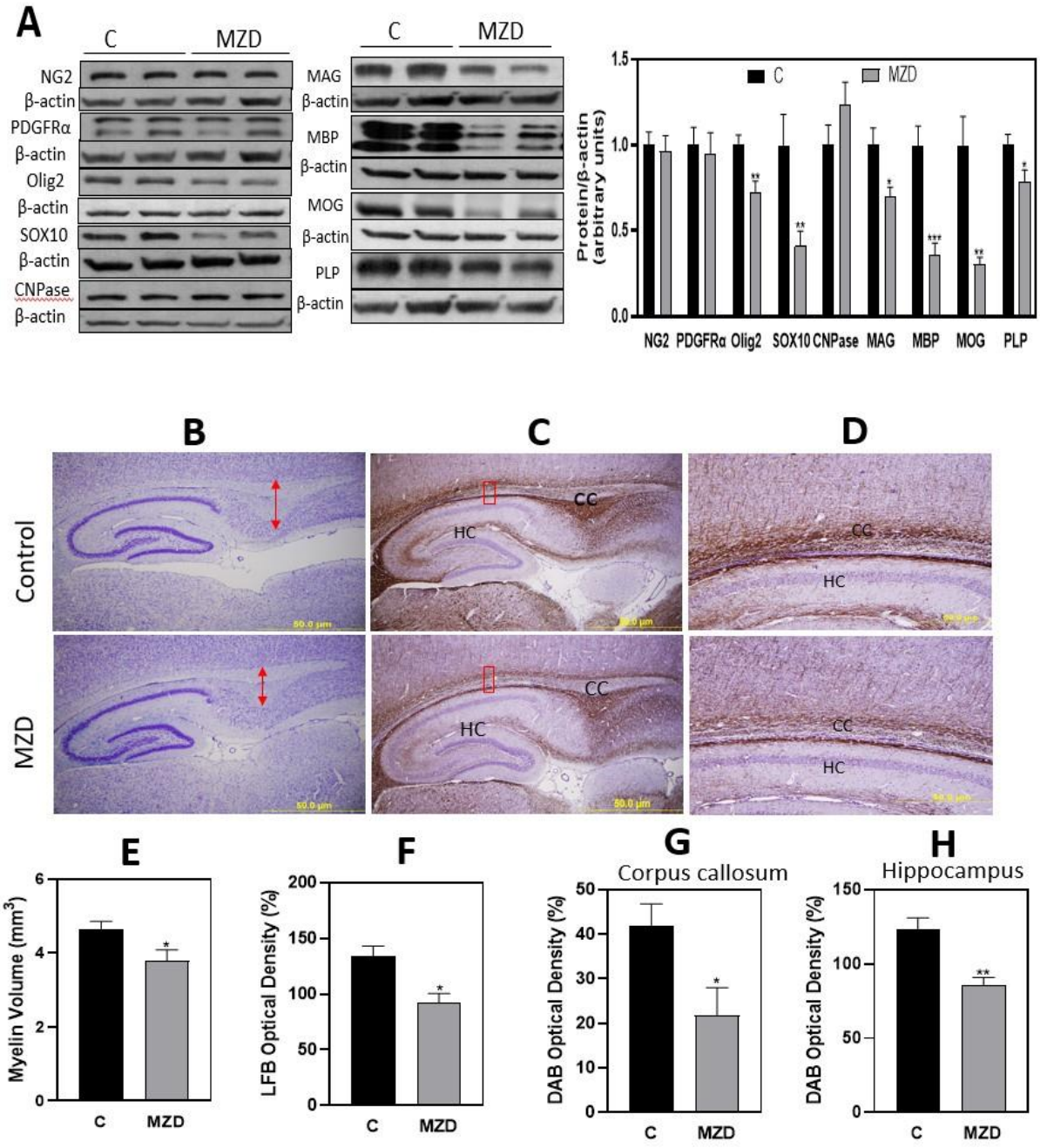


Figure 6. Maternal MZD affects parameters of oligodendrogenesis in the P20 offspring rat brain. (A) NG2, PDGFRα, Olig2, SOX10, CNPase, MAG, MBP, MOG and PLP protein levels were measured by Western blot in P20 offspring rat brain homogenates. After quantification of bands, proteins were referred to β-actin levels. Values (arbitrary units) were normalized to those of the control group. Results are shown

as mean ± SEM from 9 litters/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 combination of (**B**) LFB and anti-MBP immunohistochemistry staining at (**C**) 4x and (**D**) 10x magnification (Scale bar, 50 μm). **(E)** Quantification of myelin volume by measuring same area of CC (using Image J), multiplied by thickness of the tissue mounted on the slides. **(F)** Quantification of LFB in % of optical density. **(G)** Quantification of anti-MBP immunohistochemistry stained with DAB in CC. **(H)** Quantification of anti-MBP immunohistochemistry stained with DAB in hippocampus. Quantifications were done as described in methods. Results are shown as mean ± SEM of P20 brains from 5-9 litters/group. *, **, ***Significantly different from the control group (*p < 0.05, **P<0.01, ***P<0.001, Student's t-test).

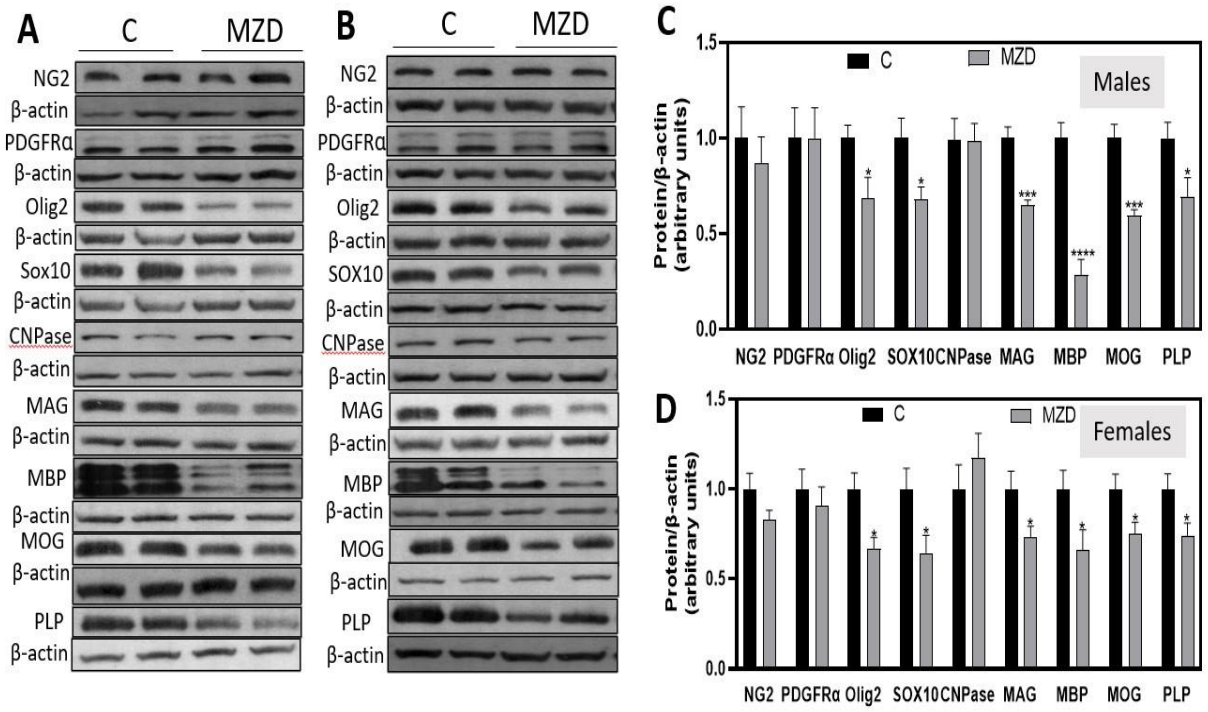


Figure 7. Maternal MZD affects parameters of oligodendrogenesis in the P60 offspring rat brain. (A-D) NG2, PDGFRα, Olig2, SOX10, CNPase, MAG, MBP, MOG and PLP protein levels were measured by Western blot in P60 (**A, C**) males and (**B, D**) female offspring brain homogenates. After quantification of 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 of 9 litters/group. *, ***, ****Significantly different from the control group (*p < 0.05, ***P<0.001, ****P<0.0001, Student's t-test)

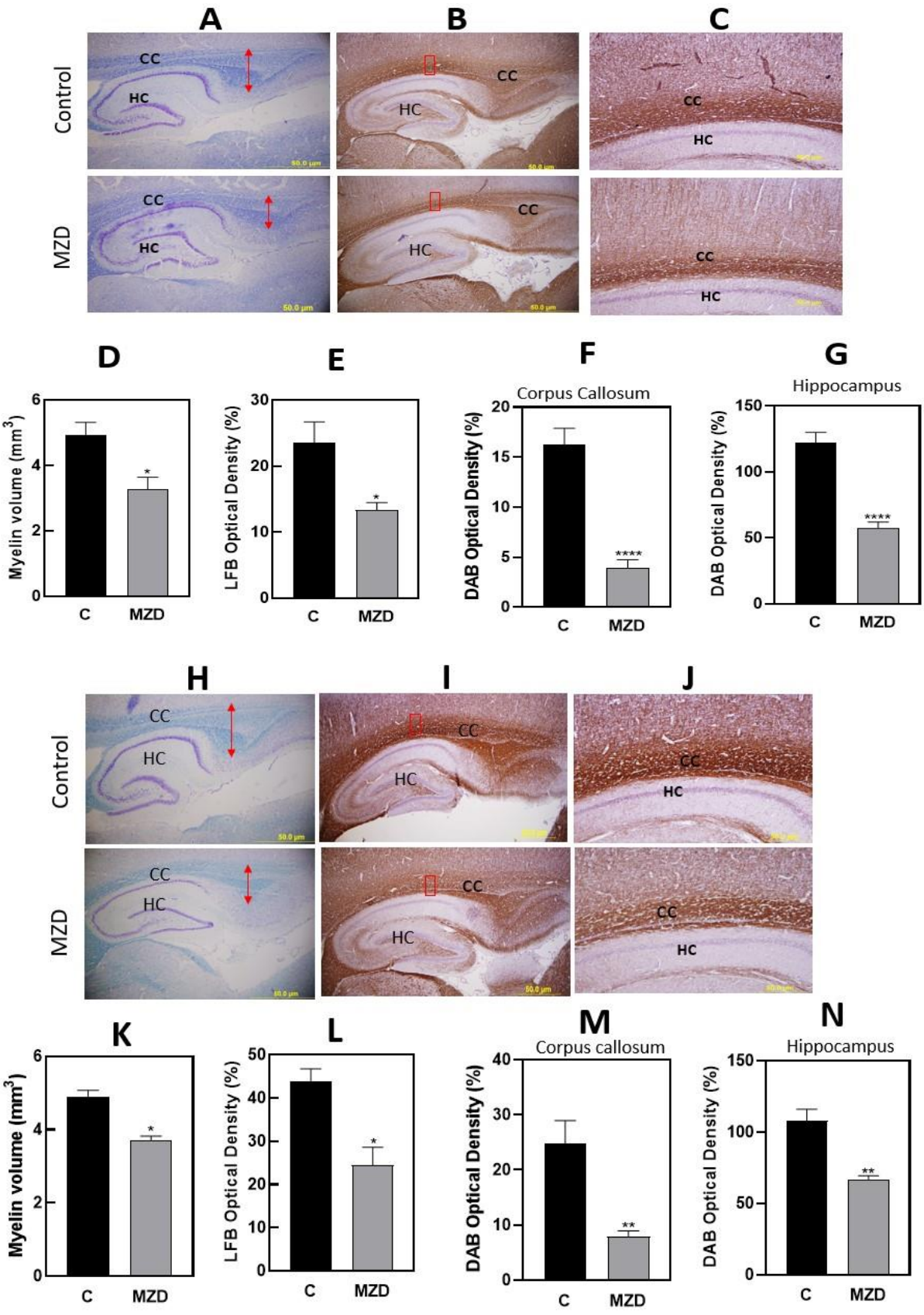

A
B
C
Control
MZD
CC
HC
D
E
F
G
Myelin volume (mm3)
LFB Optical Density (%)
DAB Optical Density (%)
Corpus Callosum
Hippocampus
C
MZD
H
I
J
K
L
M
N
Corpus callosum

Figure 8. Maternal MZD 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 at (**B, I**) 4X and (**C, J**) 10X magnification (Scale bar, 50 μm). **(D, K)** Myelin volume was evaluated by measuring area of CC (Using Image J) stained with LFB multiplied by the thickness of the tissue mounted on the slides. **(E, L)** Quantification of LFB staining, **(F, M)** Quantification of anti-MBP stained with DAB in the CC. **(G, N)** Quantification of anti-MBP stained with DAB in the hippocampus. Quantifications were done as described in methods. Results are shown as mean ± SEM of from 5-9 litters/group. *, **, ***Significantly different from the control group (*$p < 0.05$, **$P<0.01$, ***$P<0.001$, Student's t-test).

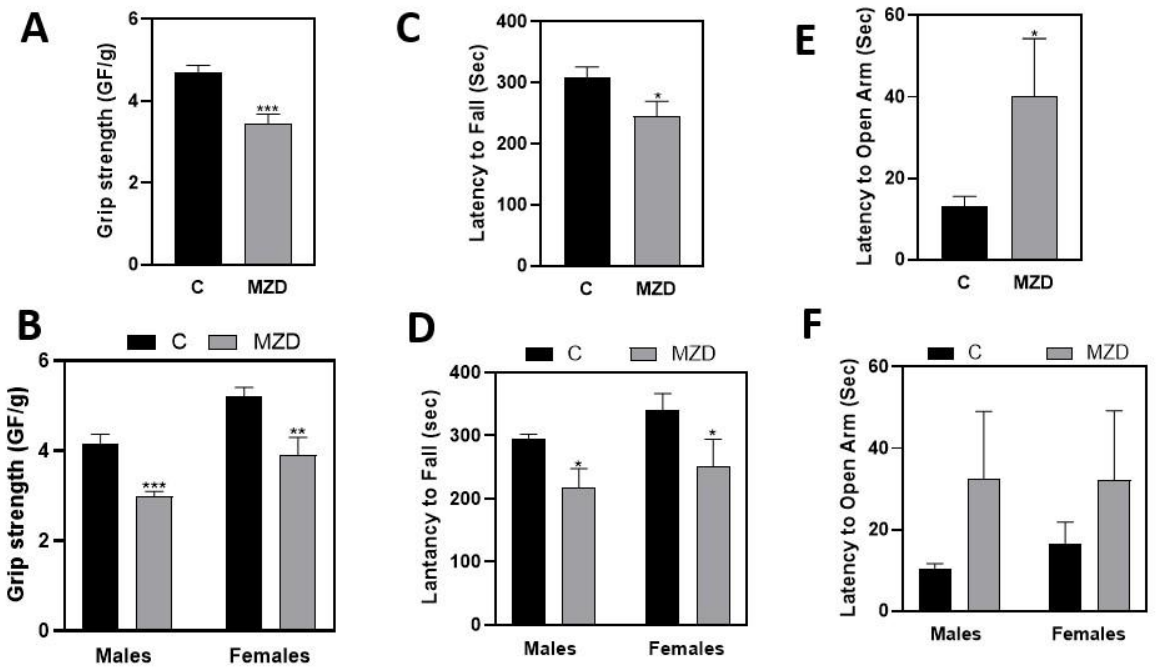


Figure 9. Maternal MZD causes behavioral changes in the P60 offspring. (A, B) Grip strength in **(A)** all offspring and **(B)** males and females separately. Grip strength was measured in gram of force and normalized per gram of body weight. Results are shown as mean ± SEM of 5-9 litters/group. **(C, 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 a T-maze in **(C)** all offspring and **(D)** males and females separately. Results are shown as mean ± SEM of P60 pups from 5-9 litters/group. **, ***Significantly different from the control group (**P<0.01, ***P<0.001, Student's t-test).

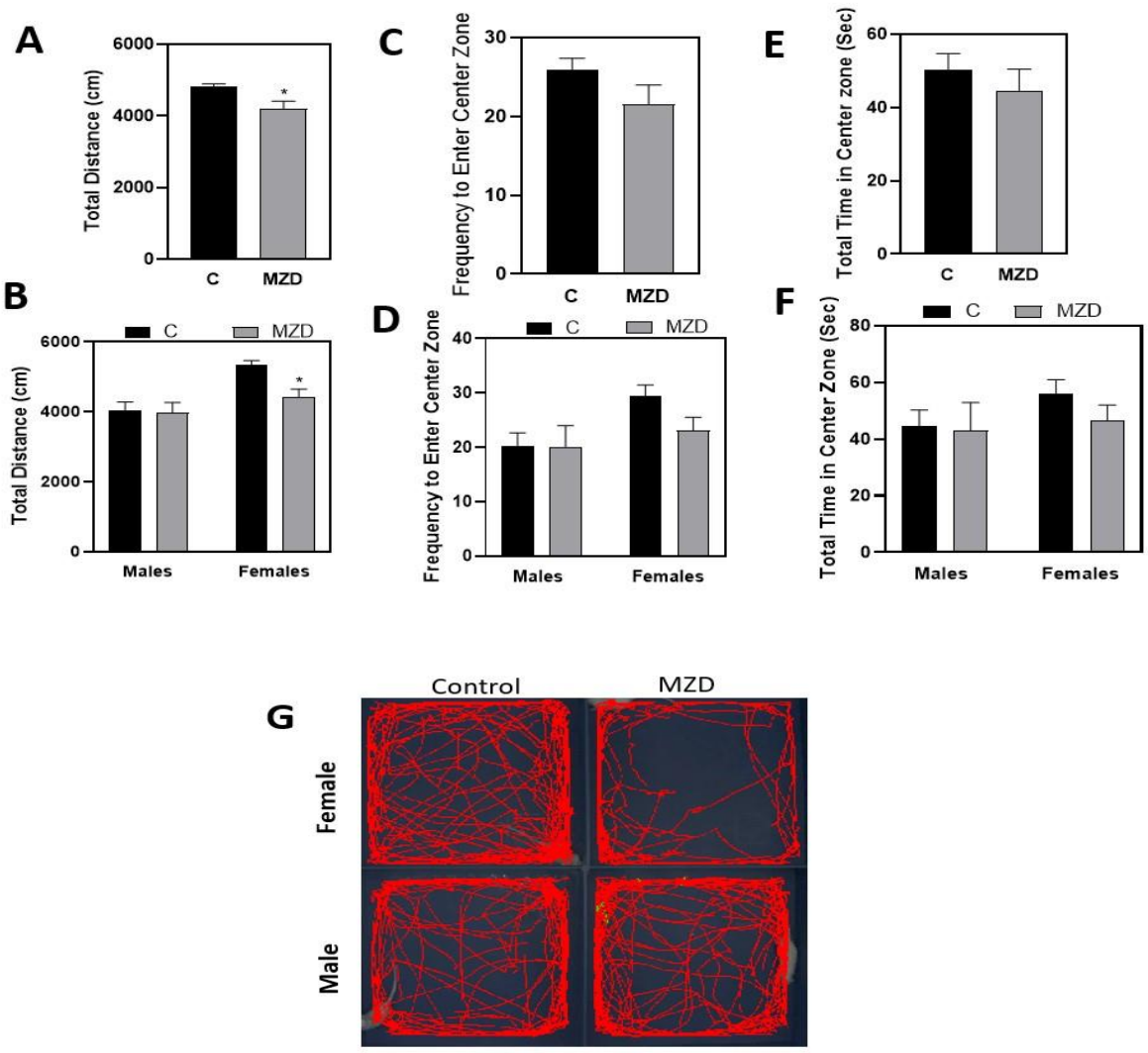
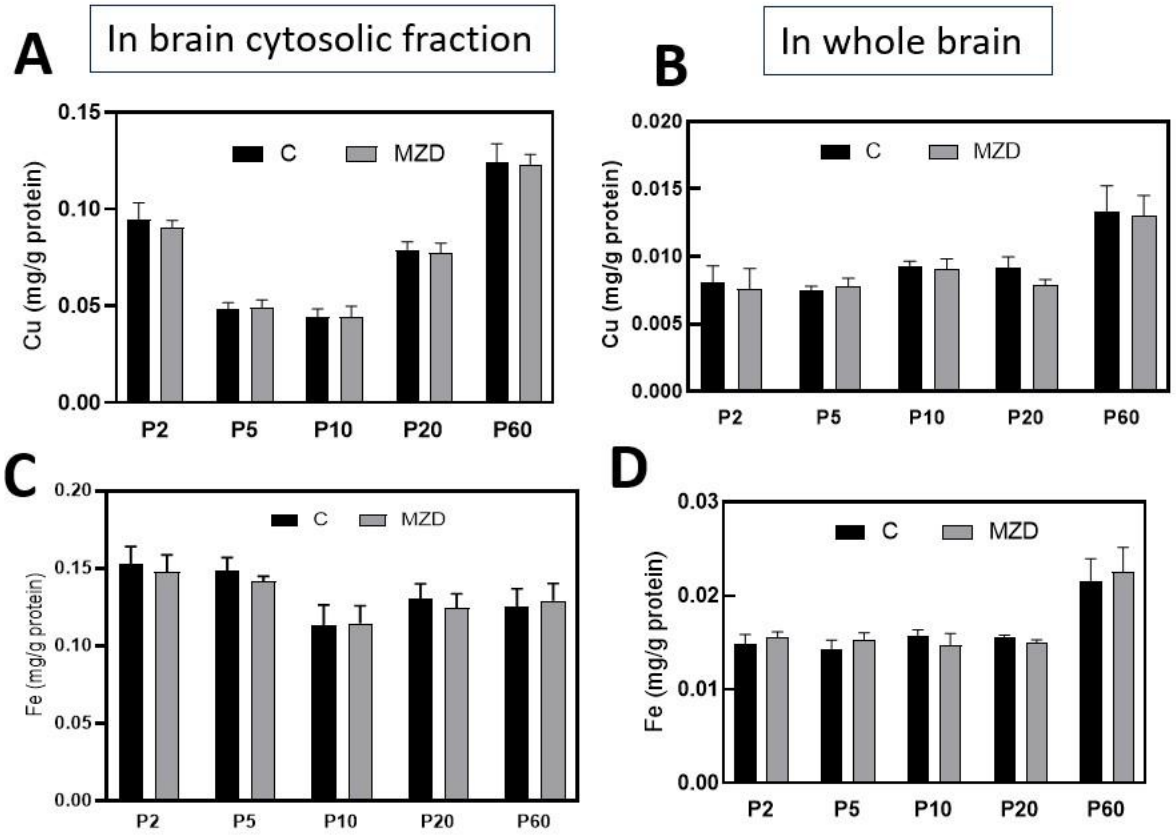


Figure 10. Maternal MZD causes behavioral changes in the P60 offspring rat brain. (**A, B**) Locomotor activity was measured as total distance travelled in an open field test. (**A)** all offspring and **(B)** males and females separately. Offspring anxiety level was measured in the open field test as (**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 ± SEM of P60 pups from 9 litters/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.



Supplemental Figure 1. Copper and iron concentrations in the P2, P5, P10, P20 offspring brain after maternal exposure to a control and marginal zinc diet. (A, B) Copper concentration in P2, P5, P10, P20 and P60 **(A)** 100,000 x g brain supernatants and (**B**) whole brain (**C, D)** Iron concentration in P2, P5, P10, P20 and P60 in **(C)** 100,000 x g brain supernatants and (**D**) whole brain. Copper and iron concentrations were measured by AES. Data are shown as mean ± SEM and are the average of 4 litters per group. (One Way ANOVA).

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Conclusion

This dissertation work evaluated the effects of maternal MZD on neurodevelopment in rats, specifically on neurogenesis and oligodendrogenesis. Zinc deficiency is a worldwide health concern particularly for pregnant women and children under 10 years of age [1]. Zinc deficiency during pregnancy and early growth can be caused by inadequate intake or secondary to decreased absorption at the gastrointestinal tract, increased urinary loss and limited availability in plasma due, among others, to infections and exposure to toxicants [2]. Gestational exposure to the environmental toxicant DEHP can cause, by triggering an acute phase response, retention of zinc in the maternal liver, limiting zinc availability to the fetus [3]. Zinc is a regulatory element in multiple cellular events including signal transduction pathways, the antioxidant defense response and regulation of gene expression. During brain development, zinc plays an essential role in NSCs self-renewal, proliferation, differentiation, and apoptosis [4, 5]. My thesis research provides evidence that maternal suboptimal zinc nutrition has adverse impacts on neurogenesis and oligodendrogenesis involving a complex cellular and signaling regulation.

Results from chapter one showed that zinc deficits affect neurogenesis at different stages in development and at different cellular processes. Maternal marginal zinc nutrition during gestation and until postnatal day (P) 2 caused a lower expression of NSCs proliferating marker (SOX2, PAX6). Maternal MZD also affected NSCs differentiation, resulting in decreased levels of intermediate progenitor cells marker TBR2 and TBR1, which are the transcription factors that regulate the neurogenic cell lineage towards neurons. Importantly, the number of mature neurons (NeuN) was decreased, and

neuronal specification was affected in the young adult brain. Specifically, a lower number of glutamatergic neurons were observed, while GABAergic neurons were not affected, leading to unbalanced excitatory and inhibitory neuronal populations in the adult brain cortex. Mechanistically, the ERK1/2 signaling pathway, which is central to neurodevelopment, was downregulated in MZD offspring during the process of neuronal cell proliferation and differentiation. Accordingly, our previously study showed that gestational MZD caused a decreased number of Ki67 positive NSCs in the E19 SVZ in parallel with inhibition of ERK1/2 [6]. Overall, this part of the thesis showed that maternal consumption of a marginal zinc diet during pregnancy and until P2 affected NSCs proliferation and differentiation into neurons. Of major relevance, even after restoring normal zinc intake to the dam and to the offspring up to adulthood, that early zinc deficiency had long-lasting adverse in the number and specification of cortical neurons.

A condition of decreased zinc availability to the fetus can be also secondary due to the maternal exposure of the environmental toxicant DEHP. Previously study showed that maternal exposure of DEHP causes an acute phase response, leading to increased zinc accumulation in the maternal liver and decreased zinc availability to the fetus [3]. Thus, in the second chapter of my thesis I investigated the effects of maternal exposure to DEHP on zinc homeostasis and neurogenesis. In the DEHP-treated group, DEHP caused a decrease in fetal brain cytosolic zinc concentration similarly to that caused by a marginal zinc nutrition, suggesting that under the studies conditions DEHP disrupts zinc metabolism and decreases zinc availability to the fetus. Zinc concentration was also lower in MEHP-treated human neuroblastoma IMR-32 cells. This indicates that MEHP can also have a direct effect on neuronal zinc homeostasis. Similar effects on neurogenesis were

found in MZD and DEHP groups, with lower expression of protein markers of NSCs proliferation (SOX2, PAX6) and differentiation (TBR2, TBR1), combined treatment with MZD+DEHP further decreased these markers. The number of mature neurons in the offspring's brain cortex was decreased in the MZD and DEHP groups, and this was even more pronounced in the combined MZD+DEHP-treated group. Mechanistic studies showed that MZD and DEHP increased PP2A activity, which dephosphorylated and inactivated the ERK1/2 signal pathway. Overall, this study showed that gestational exposure to DEHP can disrupt zinc homeostasis in the offspring through direct and indirect mechanisms affecting neurogenesis in the E19 fetal brain.

Maternal marginal zinc nutrition could also affect non-neural lineage development in the CNS. The third chapter of this thesis showed that gestational and early postnatal MZD disrupted oligodendrogenesis, which persisted into adulthood, leading to hypomyelination and consequent altered motor performance and caused behavioral abnormalities. The expression of protein markers of OPCs proliferation (NG2, PDGFRα), differentiation (CNPase), mature oligodendrocytes (MBP, MAG, PLP, MOG) and transcription factor (Olig2, SOX10) were significantly lower in the MZD offspring. Hypomyelination was observed at P20, when the dam/offspring were consuming the zinc deficient diet and at P60 when all offspring had been fed the zinc adequate diet after weaning (P20 to P60). Hypomyelination was associated with impaired motor function, muscle strength and anxiety disorder. The above finding indicated that early developmental marginal zinc deficiency affects oligodendrogenesis causing long-lasting adverse effects on myelination and behaviors.

In summary, finding from this dissertation provided critical evidence that insufficient zinc intake or disruption of zinc homeostasis during pregnancy and the early postnatal period can have a detrimental impact on brain development. Maternal marginal zinc deficiency and exposure to DEHP affects neurogenesis and/or oligodendrogenesis that cause altered brain cellularity and behavior in the adult offspring which could be irreversible. Furthermore, the effects of early developmental decreased zinc availability on region-specific (e.g., hippocampus, cerebellum, spinal cord) neurogenesis and oligodendrogenesis and other neuronal processes such as axon guidance and synapses interaction during development are important aspects for future investigations.

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Additional publication

Zinc and the Modulation of Nrf2 in Human Neuroblastoma Cells

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