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Technical Note

Effects of vitamin D deficient diet on microtubule-associated protein 2 expression in brains of newborn mice

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Abstract: Functioning as a neuroactive steroid hormone, vitamin D assumes a pivotal role in brain development, function and dynamic equilibrium. Inadequate vitamin D levels can lead to diminished expression of genes related to the growth and apoptosis of mouse brain cells. This deficiency extends to the next generation. Microtubule-associated protein 2 (MAP2), closely linked to neural cell differentiation and migration, acts as a neural cell-specific marker. It is expressed in both the neuronal cytoplasm and dendrites, serving as a substrate for the majority of protein kinases and phosphatases found in neurons. Our study findings unveil a reduction in MAP2 expression in the brains of newborn mice from pregnant mice with a diet deficient in vitamin D. Vitamin D deficiency status thus serves as a potential molecular indicator for diagnosing and prognosticating mental developmental disorders, opening avenues for novel therapeutic strategies.

Keywords: vitamin D deficiency, microtubule-related protein 2, brains of newborn mice

INTRODUCTION

Cumulative evidence underscores the pivotal role of vitamin D, acknowledged as a neuroactive steroid hormone in the human body [1]. In addition to its principal function as a calcium regulator facilitating absorption and metabolism of calcium in the bone, vitamin D can affect cell and tissue development. It contributes to normal bone development, optimal muscle contraction, and cellular function across various body regions [2]. Along with its role in regulating calcium and phosphorus balance and promoting skeletal mineralisation, vitamin D exerts influence on target genes by binding to vitamin D receptor that acts as a nuclear transcription factor after the formation of a vitamin D receptor/retinoid-X receptor/cofactor complex, to participate in diverse biological processes [3]. Vitamin D and vitamin D receptors are widely found in the brain, where vitamin D exists in 25-hydroxyvitamin D_3 and its active hormones [4, 5]. Animal studies have

confirmed the distribution of vitamin D in diverse brain regions, encompassing the prefrontal cortex, middle frontal cortex, middle temporal cortex, cerebellum, corpus callosum, medulla and pons [6]. In the brain vitamin D engages in brain cell differentiation, expression of neurotrophic factors, regulation of intracellular calcium, synthesis of neurotransmitters, antioxidant activity, and the expression of genes related to neuronal structure and metabolism. It plays a role in synaptic plasticity, neuroprotection, neural circuits, and turnover of the dopamine system [5]. The level of vitamin D exhibits a correlation with brain neurodevelopment and function and dynamic balance [7].

Vitamin D deficiency (VDD), defined as serum 25-hydroxyvitamin D concentrations < 25 or < 30 nmol/L, affects almost one billion people today [8]. The escalation of VDD among pregnant mothers in China is evident. Reports indicate notably high rates of VDD, with percentages reaching 90% in Beijing, 69% in Shanghai, and 49% in Nanjing [1]. The awareness of the detrimental effects of VDD on pregnant women and foetuses is widespread globally. In the course of pregnancy, the intricate interplay between vitamin D and calcium metabolism assumes paramount importance. This life stage, marked by its uniqueness and demands, accentuates the foetal developmental requirement for a mineralised structure while concurrently upholding optimal maternal healthm [6]. The 25-hydroxyvitamin D in pregnant women has the propensity to traverse the placenta, constituting the sole source of foetal vitamin D. Inadequate vitamin D status in pregnant women can result in foetal and neonatal VDD [9]. Despite active promotion of vitamin D supplementation for pregnant women in many countries, the prevalence of VDD remains persistently high in these demographics [10]. The development of VDD poses a particular risk to individuals with limited intake, insufficient exposure to sunlight, or inadequate intestinal absorption [2, 11]. .It is now well-recognised that VDD is frequently linked to bone-related disorders such as rickets and osteomalacia, extending its impact to the development of diverse extra-skeletal diseases. These encompass multiple sclerosis, autoimmune conditions, increased susceptibility to infections, respiratory illnesses, cardiovascular disorders, and various forms of cancer [3]. Recent studies have brought to light a link between VDD during brain development and the emergence of neurodevelopmental disorders, notably schizophrenia and autism [12]. Although existing studies propose that VDD influences the differentiation and migration of nerve cells during brain development, detailed mechanisms and specific proteins implicated in these processes still warrant extensive exploration both domestically and internationally [13].

Microtubule-associated protein 2 (MAP2), crucial for neural cell differentiation and migration, stands as a key player in these processes [14]. Serving as a neural cell-specific marker, MAP2 constitutes a component of the cytoskeleton and is predominantly expressed in neurons. Additionally, it acts as a substrate for the majority of protein kinases and phosphatases present in neurons [15]. Studies have validated that MAP2 immunostaining serves as a sensitive method for identifying dendritic lesions associated with various central nervous system conditions in rats, such as schizophrenia and autism [16]. Notably, MAP2 participates in the regulated processes of vitamin D during the brain development, but the impact of VDD on MAP2 expression in the brain remains unexplored in the current literature. This experiment aims to scrutinise potential alterations in MAP2 expression under the influence of a low vitamin D diet. The outcomes could contribute to the scientific groundwork for averting and addressing pathological changes linked to VDD in pregnant mothers.

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METHODS

Animal Culture and Feeding

Six-week-old, specific-pathogen-free, Institute-of-Cancer-Research pregnant mice, weighing between 18-22 g, were obtained from Hunan Silk Jingda Experimental Animal Technology Co. These mice were healthy, disease-free, and acquired with a laboratory animal certificate bearing batch number SCXK (Xiang) 2019-0004. All procedures used in this animal study were carried out in compliance with all applicable regulations and guidelines and approved by Hengyang Medical School Animal Ethics Committee (Hospital Medical Research Ethical Clearance No. EC20220301).

The experimental conditions were upheld at a consistent temperature of 21±2°C with abundant food and unrestricted access to water, and the cages of the experimental group of mice were covered with black cotton cloth to provide a light-shielding environment. The feeding regimen adhered to a 12-hour cycle (7:00-19:00). Both the control group (n = 6) and vitamin-D-deficient group (n = 10) received feed from Jiangsu Nantong Biological Engineering Co. The standard feed in the control group contained vitamin D levels surpassing 1500 IU/kg, whereas the vitamin-D-deficient feed in the experimental group contained less than 25 IU/kg. Postnatal days (PND) of newborn mice were documented, with PND 0 indicating within 24 hr of birth, PND 1 representing the first day of life, and so forth. Brain samples of the newborn mice were randomly collected at both PND 3 and PND 7 irrespective of gender.

Hematoxylin-Eosin (HE) Staining

HE staining, a fundamental and widely used technique in scientific research and pathology, allows for the staining of all components within tissue cells. In the process, hematoxylin, an alkaline dye, imparts a blue colour to the nucleus (which has an acidic structure), while eosin, an acidic stain, causes other cellular components to appear pink (as their structures are alkaline and acidophilic). The histological analysis was performed according to the standard protocol published in the literature.

The brain tissue from newborn mice were rigorously processed and fixed in 4% paraformaldehyde in phosphate-buffered saline for a duration of 24 hr, with careful attention to ensuring thorough infiltration of the paraformaldehyde in the tissue. Subsequently, the brain tissue underwent a repair process, and the repaired brain tissue was then placed in pre-marked dehydration boxes, subjected to a gradient dehydration with ethanol concentrations of 60%, 80% and 95%. After dehydration, the tissue block was successively put into a mixed xylene solution (xylene : absolute ethanol = 1:1) and pure xylene solution for 30 min. for transparent treatment. Subsequently, the treated tissue was placed in a warm box at approximately 56°C and immersed in a mixture of xylene and paraffin (in equal proportions) for 2 hr. Thereafter, the tissue was left in the paraffin for approximately 2 hr until it was thoroughly soaked and enveloped by the paraffin. Then the embedded tissue was positioned carefully in the designated embedded box according to the specified embedding procedure. The embedded tissue was then transferred to a frozen table for cooling, allowing the wax block to solidify completely.

After removing the thoroughly solidified wax block from the embedded box for simple repair treatment, the sample was cut into 5-um thick sections with a paraffin slicing machine. The slices were flattened in warm water and then gently removed and placed on a 37°C baking machine for 1 hr. The dried sections were put in 100% xylene for 10 min., then placed successively in 100%, 95%, 80% and 75% ethanol, each for 1 min., washed with tap water for 2 min., and washed in

phosphate-buffered saline for 5 min. The sections were counterstained in hematoxylin (Haokebio, HK2019, Hangzhou, China) for 5 min. and rinsed with tap water for 5 min. and then stained with 0.5% eosin in 95% ethanol for a while and washed with tap water for a while. The sections were sequentially soaked in 95% ethanol for 5 sec., absolute ethanol for 4 min. and xylene for 3 min. Finally, the sample was desiccated in the oven at 37℃ and sealed with neutral gum and observed under an optical microscope.

Immunohistochemistry Analysis

The location and expression of MAP2 proteins was detected using immunohistochemistry analysis. The brain sample was embedded in paraffin, cut into 3-μm sections, mounted on glass slides and deparaffinised by standard protocols. For antigen retrieval, the tissue was treated with Tris/EDTA solution buffer (10 mM Tris, pH 9; 1 mM EDTA). Incubation with primary rabbit anti-MAP2 antibody (Proteintech, 17490-1-AP, Wuhan, China) was conducted at 4°C overnight followed by phosphate-buffered saline washing. DyLight 488-conjugated anti-rabbit antibody and DyLight 549-conjugated anti-mouse antibody (Jackson, Bar Harbor, ME, USA) were used as secondary antibodies. Then three more washes were performed. The fluorescence of the MAP2 proteins was examined using a confocal microscope (MRC 600; Olympus, Japan) with a krypton argon laser. The fluorescence of the MAP2 proteins was examined using a fluorescence microscope (Eclipse C1, Nikon, Japan). The images collected had an optical thickness of 3 microns for the brain.

Statistical Methods

Statistical analysis was performed using Graphpad Prism 8.0 or SPSS 25.0 software. Data were expressed as mean ± standard deviation (Mean ± SD). One-way analysis of variance (ANOVA) and pairwise comparison with the least significant difference (LSD) were used, and $P < 0.05$ was regarded as statistically significant.

RESULTS

Maternal Weight Changes

The mean body weight on the first day of feeding for the maternal deficiency group was 38.49±2.88 g; for the control group, it was 37.21±2.78 g. There was no statistical difference between the body weights of the maternal deficiency group and the control group. However, the maternal deficiency group showed a significant weight increase at PND1 compared to the normal control group ($P < 0.05$), as illustrated in Figure 1.

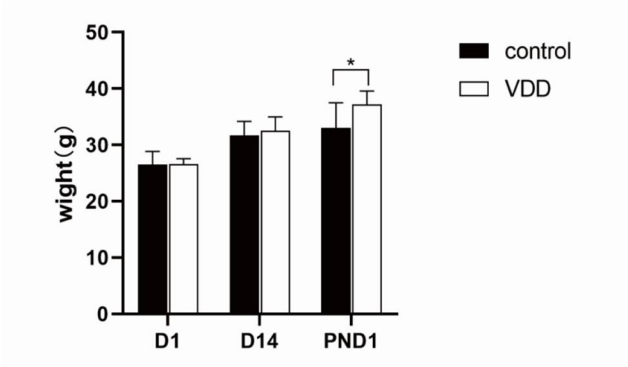

Figure 1. Effect of VDD on pregnant maternal weight (D=day; * $p<0.05$)

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Impact of Maternal Vitamin D on Brain Tissue of Newborn Mice

HE staining results reveal a well-organised arrangement of nerve cells in each layer of the cerebral cortex. In comparison to the control group, a significant reduction in glial cells was observed in the experimental grouip, as depicted in Figure 2

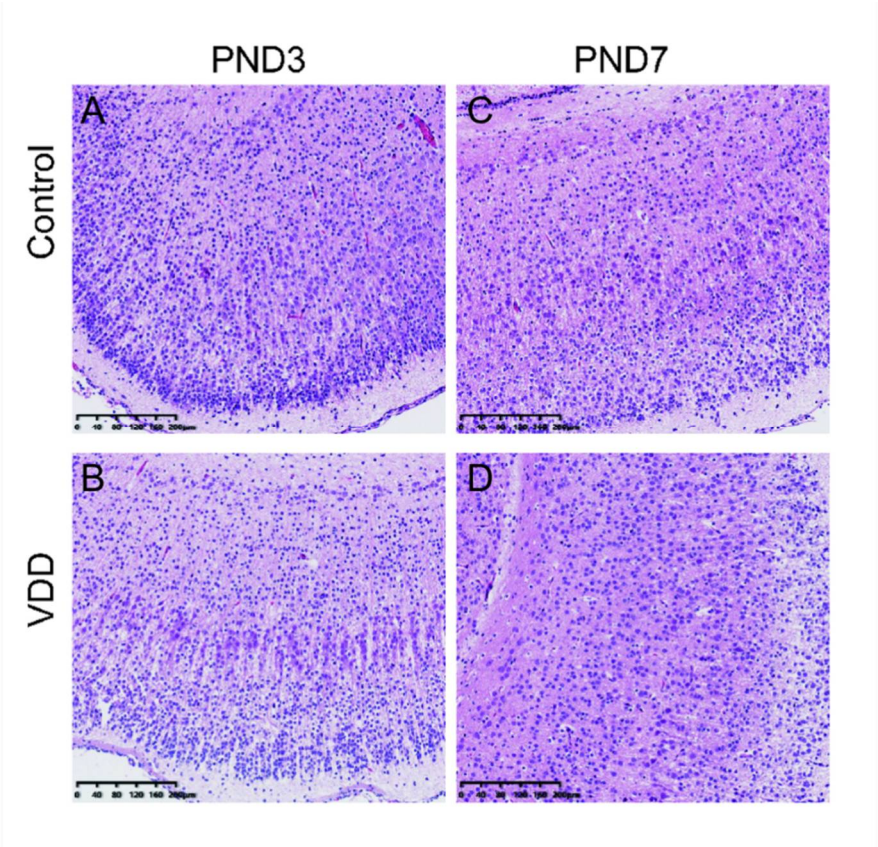

Figure 2. HE staining of cerebral cortex of newborn mice at different periods: (A) control group at PND 3; (B) VDD group at PND 3; (C) control group at PND 7; (D) VDD group at PND 7. (Scale bar = 200 μm)

MAP2 Expression

The expression of MAP2 was assessed at PND 3 and PND7. Positive MAP2 cells, identified by a brownish-yellow colour, were observed in the cerebral cortex. However, a significant decrease in the number of positive cells was noted in the maternal vitamin D deficient group compared to the control group. Under a 400x light microscope, the counting results revealed that on PND 3 the experimental group had 25.50±7.15 positive cells, while the control group had 50.60 ± 8.38 positive cells. On PND 7, the experimental group exhibited 25.60±10.76 positive cells, and the control group exhibited 43.60 ± 6.05 positive cells. The number of positive cells in the offspring of maternal VDD group during the same period was significantly lower than that of the control group ($P < 0.05$). In addition, within the same group at different periods, the control group showed a significantly lower expression of positive cells on PND 7 compared to PND 3 ($P < 0.05$). Interestingly, there was no significant difference in the expression of positive cells between PND 7 and PND 3 in the low diet group ($P > 0$). These results are illustrated in Figure 3.

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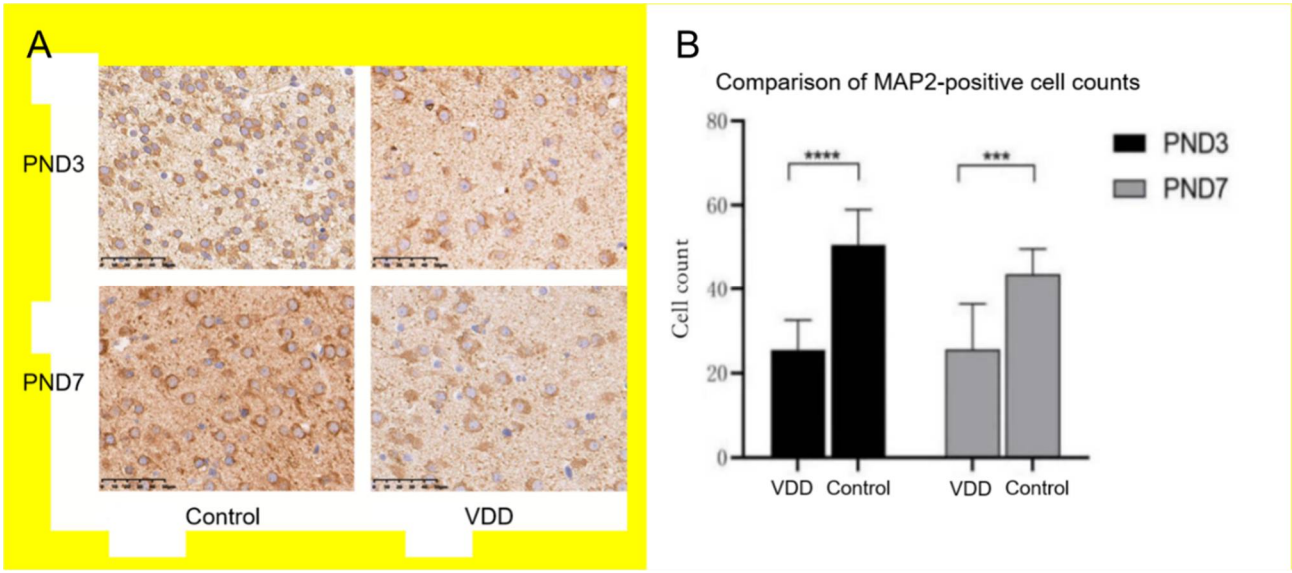

Figure 3. (A) Results of immunohistochemical staining of MAP2 in cerebral cortex; (B) Number of MAP2-positive cells at PND3 and PND7. (Scale bar = 50 μm, **** $p<0.0001$, *** $p<0.001$)

DISCUSSION

VDD presents a hidden and great challenge for the pregnant stage and foetus. Increasing evidence demonstrates that VDD is associated with impairment of brain development and neurological illnesses including Alzheimer's disease, Parkinson's disease, epilepsy and multiple sclerosis [7]. Vitamin D is a steroid molecule and is involved in brain development, but the detailed alternations and underlying mechanisms need to be further explored. In our study maternal VDD in mice leads to neuroanatomical alternations in newborn mice; neural MAP2 is notably decreased in both gender. These fundamental alternations in both the protein expression and neural structure emphasise the essential role of vitamin D in healthy brain development.

The study outcomes highlight significant impairment in offspring born to mothers with a VDD diet on the initial days after birth. HE staining indicates that on PND 3 and PND 7 the VDD group exhibits a less compact arrangement of cortex tissue with a lower count of glial cells compared to the control group. Vitamin D is pivotal in fostering activity in both the embryonic and adult brains, contributing to the connectivity of neural circuits [17].

Our findings of reduced MAP2 protein expression at PND3 and PND7 in the developing cortex of vitamin D deficient foetuses is novel. The MAP2 protein serves as a significant microtubule-associated protein in the vertebrate nervous system, mainly residing in the cytoplasm and dendrites of neuronal cells. In this study the MAP2 expression of the VDD group did not undergo a significant reduction compared to the control group, indicating that the depleted vitamin D changes the expression pattern of the MAP2 during growth.

In conclusion these observations underscore the relationship between VDD and MAP2 expression in brain development during the foetal period. The VDD status emerges as a potential molecular indicator for diagnosing and predicting mental developmental disorders, opening up new avenues for therapeutic approaches.

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