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*Original Article*

# Maternal Vit D supplementation in AMA mice and the role of Vit D/VDR signaling in the offspring's cognition

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**Abstract:** Objective: To explore the molecular mechanism underlying the effect of maternal vitamin D (Vit D) supplementation before pregnancy in advanced maternal age (AMA) mice on the offspring's cognitive function. Methods: Thirty-two-week-old female mice either received 10 IU/g body weight vitamin $D_3$ dissolved in 200 μl corn oil (32W+VD group), or 200 μl corn oil (32W group) per day for one week. Another group of eight-week-old female mice received the same amount of corn oil as 32W group was set as normal reproductive age control (8W group). Then the three groups of female mice were mating with ten-week-old male mice at 2:1 ratio, the offspring were weaned at the age of 3 weeks and housed until the age of 6 weeks. Vit D metabolites and enzymes involved in Vit D metabolism were measured in both mothers and their offspring. Vit D receptor (VDR) and synaptic markers were determined in the offspring hippocampus. Vit D response elements in HIF-1α promoter were predicted, and VDR transcriptional target genes and related signaling molecules were also detected. Results: Vit D intervention markedly improved the serum 1,25 dihydroxy vitamin $D_3$ ($1,25(OH)_2D_3$) concentration in early pregnancy, middle pregnancy and late pregnancy stages in AMA mice. The hippocampal $1,25(OH)_2D_3$ levels in the offspring showed the similar pattern. Subsequently, the expression of Cyp27b1, the gene encoding enzyme that converts $25(OH)D_3$ to $1,25(OH)_2D_3$, in the hippocampus of the offspring from AMA mice was significantly lower than that of the offspring from normal female mice, and was restored by Vit D supplementation. VDR (Vit D receptor), which mediates the cellular actions of active $1,25(OH)_2D_3$, was also rescued by Vit D supplementation, especially in dentate gyrus (DG) region of hippocampus. Concurrently, the synaptic markers NR1, NR2A, and PSD-93 in the hippocampus were reversed in 32W+VD group. Finally, we found that Vit D supplementation may affect PI3K-AKT, PLC-ERK1/2, and p38-MAPK signaling molecules by mediating HIF1α expression via VDR. Conclusion: Our findings highlight the biological significance of maternal Vit D supplementation before pregnancy on Vit D metabolism, and signaling molecules in the offspring, underlying the potential mechanism of the cognitive impairment in the offspring born to AMA mice.

**Keywords:** Vitamin D supplementation, advance maternal age, synaptic biomarkers, VDR, HIF-1α/VEGF

## Introduction

Vitamin D (Vit D) is of great concern for public health since Vit D deficiency is common and associated with various diseases [1-3]. In addition, maternal Vit D insufficiency is proved to have a negative impact on the offspring brain development [4-6]. Meanwhile, maternal Vit D supplementation is associated with a reduced risk of small for gestational age (SGA) and improved infant growth, while the effects on offspring brain development and cognitive function are less discussed [7].

Studies have shown that childbearing age over 35 years in women has an important impact on the development of offspring's cognition and mental behavior [8-11]. Compared with adults, pregnant and lactating women have elevated prevalence of Vit D deficiency [12]. In our previous work, we found that offspring born to mice with advanced maternal age (AMA) had impaired

spatial learning and memory when compared with offspring born to mice with normal reproductive age, and maternal Vit D supplementation before pregnancy rescued the impairment. Nevertheless, the molecular mechanism is still unclear yet.

Vit D has to be activated through enzymatic hydroxylation to $25(OH)D_3$ by Cyp27a1 in the liver and subsequently to $1,25(OH)_2D_3$ (bioactive form of VitD) by Cyp27b1 in the kidney [13, 14]. $1,25(OH)_2D_3$, acting as a fat-soluble hormone with a variety of biological activities, now is considered as a neurosteroid involved in neurogenesis, behavioral function, and neuroprotection [15].

Only when $1,25(OH)_2D_3$ and VDR bound together are various transcription factors attached to this complex, resulting in changes in expression of various genes [16, 17]. Mice with VDR knockout showed severe impairments in the behavior function [18]. Important polymorphisms in the VDR gene are proved to be associated with risk of mild cognitive impairment and longitudinal cognitive changes [19-21]. The typical effect of Vit D/VDR signaling is known as bone homeostasis. Previous studies reported that Vit D/VDR inhibits oral epithelia inflammation by regulating HIF-1α signaling [22], promotes re-endothelialization and restores impaired angiogenesis in the femoral artery ligation model by increased HIF-1α [23]. Maintaining the HIF-1α level is effective to attenuate the nerve damage [24]. In particular, HIF-α expression changes can activate or inhibit PI3K/Akt/mTOR pathway which is related to cognitive function [25-27].

With this study, we aim to investigate the role of Vit D/VDR signaling in genes expression related to behavioral and brain functions in the offspring born by AMA mice.

## Materials and methods

### *Animal model*

SPF (specific pathogen free) grade C57BL/6J mice were purchased from Hubei Provincial Center for Disease Control and Prevention, (license#: SCXK (E) 2015-0018, animal quality certificate#: 4200006000031051, 4200-0600030394). All animals were housed at 25±2°C, humidity of 60±2% and 12-hour light-dark cycle, and mice were adapted to the experimental environment for one week before Vit D supplementation. Animals had free access to standard laboratory chow diet and water. The thirty-two-week-old virgin female mice were randomly allocated into two groups: mice in 32W+VD group received 10 IU/g body weight vitamin $D_3$ dissolved in 200 μl corn oil per day for consecutive 7 days by gavage, while mice in 32W group received 200 μl corn oil. The eight-week-old virgin female mice received the same treatment as the 32W group. All female mice were mated with ten-week-old male mice at 2:1 ratio after 7 days treatment. Successful mating was confirmed by the presence of a vaginal plug and the day was recorded as pregnancy (P0.5). At least five mice at each stage of pregnancy (early-before P6, middle-P6-12, and late-P13-18) were sacrificed by cervical dislocation, and blood was collected for serum preparation. The offspring were weaned at 3 weeks old and continued to feed on normal chew for further 5 weeks. Three of young adult offspring in each group were first perfused with cold PBS and then with 4% paraformaldehyde (PFA) before brain dissection. The brains were removed and immersed in formalin for 24 h, paraffin embedded, and sectioned. The rest of young adult mice were anesthetized by diethyl ether, the eyeballs were removed, and blood samples were collected for serum preparation. The mice were sacrificed, and the hippocampi were dissected, immediately frozen and stored at -80°C for further analysis. All experimental protocols involving the use of animals were approved by the committee of the Ethics of Animal Experiments of the Wuhan University School of Medicine in accordance with the regulation of Guide for the Care and Use of Laboratory Animals published by the US National Institution of Health.

### *Q-PCR analysis*

Trizol (Life, USA) was adopted to extract total RNA of hippocampal tissue at 8 weeks of offspring in 3 groups. qPCR was conducted in the StepOne™ Real-Time PCR System (ABI, Singapore) using SYBR® Premix Ex Taq II (TaKaRa, Japan) and the gene-specific primers. β-actin was used as an endogenous control. The relative expression of RNA was calculated using

**Table 1.** The primer sequences used in this experiment

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *β-actin* | 5'CTGTCGAGTCGCGT3' | 5'GATACCTCTCTTGCTCTGGGC3' |
| *AKT* | 5'AAGGACGGTGCCACTATGAA3' | 5'TCCTGGTTGTAGAAGGGCAG3' |
| *BDNF* | 5'GACAGCCTGTATCCGACCCTC3' | 5'ATCAGTTTGTTCGGCTCCACT3' |
| *Cyp27a1* | 5'AAGGGCCTCACCTATGGGAT3' | 5'CACCTGGTCCCCTGATTCAC3' |
| *Cyp27b1* | 5'GCCGAGACTGGGATCAGATG3' | 5'TGATGCCCAGACGGCATATC3' |
| *ERK1* | 5'GGACCAGCTCAACCACATTC3' | 5'ATGCGCTTGTTTGGGTTGAA3' |
| *ERK2* | 5'TCCTTTTTGAGCACCAGACCT3' | 5'AAAGGTCCGTCTCCATGAGG3' |
| *FGF* | 5'CGGTTGTGTACGAAGTCCCA3' | 5'CTTCAACACAAAGCAGGGGC3' |
| *HIF1α* | 5'CTTGACAAGCTAGCCGGAGG3' | 5'TCGACGTTCAGAACTCATCCT3' |
| *IL-2* | 5'CCAAGGGCTCAAAAATG3' | 5'GCGCTTACTTTGTGCTGTCC3' |
| *NR1* | 5'CCCCAGTGCTGTTATGGCTT3' | 5'TGTTTACCCGCTCCTGTGTG3' |
| *NR2A* | 5'TTCTGAAACCTCAAGCCGGG3' | 5'TCCCTGGGAGAACTTGCTTT3' |
| *p38* | 5'ACAACATCGTGAAGTGCCAG3' | 5'TCTCATCATCAGTGTGCCGA3' |
| *PI3K* | 5'AATGCACGGCGATTACACTC3' | 5'GGACACTGGGTAAGAGCAACT3' |
| *PSD-93* | 5'GTTACAAGTCGCCCAGCTCT3' | 5'TCTAGTTTAATCGCCCGGTCA3' |
| *VDR* | 5'GTGCAGCGTAAGCGAGAGAT3' | 5'GGATGGCGATAATGTGCTGTTG3' |
| *VEGF* | 5'GAGAACTGGGCTCTGTG3' | 5'ATGGAGAAAATCGCCAGGCA3' |

AKT, thymoma viral proto-oncogene 1; BDNF, brain derived neurotrophic factor; Cyp27a1, cytochrome P450 family 27 subfamily A member; Cyp27b1, cytochrome P450 family 27 subfamily B member; ERK1, mitogen-activated protein kinase 3; ERK2, mitogen-activated protein kinase 1; FGF, fibroblast growth factor 1; HIF1α, hypoxia-inducible factor 1 alpha; IL-2, interleukin 2; NR1, glutamate receptor, NMDA1 (zeta 1); NR2A, glutamate receptor, NMDA2A (epsilon 1); p38, mitogen-activated protein kinase 14; PI3K, phosphoinositide-3-kinase regulatory subunit 1; PSD-93, Post Synaptic Density 93; VDR, vitamin D receptor; VEGF, vascular endothelial growth factor A.

$2^{-\Delta\Delta Ct}$ method. All primers used in q-PCR analyses were described in **Table 1**.

*Western blotting analysis*

Whole hippocampal lysates of offspring in each group were prepared by RIPA buffer, and then the protein concentration was detected by BCA Protein Assay kit (Beyotime, China). 30 μg of protein was separated by SDS-PAGE and then transferred to a pre-activated Polyvinylidene Fluoride membrane (PVDF). The membrane was blocked for 1 h in TBST buffer (TBS containing 0.1% Tween 20) containing 5% non-fat dry milk followed by an overnight incubation with primary antibody. β-actin (GB11001, 1:2000) was purchased from Servicebio Company. VDR (ab3508, 1:500), Cyp27a1 (ab126-785, 1:500) and Cyp27b1 (ab206655, 1:500) were purchased from Abcam Company. PSD-93 (BM5488, 1:200), NR1 (A01808, 1:200) and NR2A (BA0613, 1:500) were purchased from Boster Company. After extensive washing, the blot was incubated with secondary antibody overnight, and finally, the membranes were washed thrice with TBST and visualized by BeyoECL Moon kit (Beyotime, China). Imaging was then performed using an Alpha Imager HP (NatureGene Corp, USA) and Quantity One software was used to quantify the bands' grayscale.

*ELISA assay*

The serum from maternal and offspring, and the hippocampal lysates from offspring were used for ELISA assay. Vitamin $D_3$ (vitamin $D_3$) pre-coated ELISA kit (CEA920Ge), 25-Hydroxyvitamin $D_3$(25(OH)$D_3$) pre-coated ELISA kit (CEA915Ge) and 1,25-Dihydroxyvitamin $D_3$(1,25$(OH)_2D_3$) pre-coated ELISA kit (CEA467Ge) were used to detect the concentration of Vitamin D, 25(OH)$D_3$ and 1,25$(OH)_2D_3$ respectively, following the manufacturer's protocol.

*Immunohistochemistry*

Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene, rehydrated in alcohol, and subsequently, these sections were subjected to antigen retrieval. Then, the sections were incubated overnight in a humid chamber at 4°C with antibody against VDR (ab3508, 1:500) followed by secondary antibody for 30 min. Immunocomplexes of horseradish peroxidase were visualized by DAB reac-

tion, and sections were counterstained with hematoxylin before mounting.

*VDR binding sites prediction in HIF-1α promoter*

Nuclear receptor VDR regulates transcription of target genes by binding to specific DNA sequences (response elements), and $1,25(OH)_2D_3$ response elements are composed of repeats of Pug (G/T) TCA motifs. Therefore, the promotor of HIF-1α was identified by NCBI (http://www.ncbi.nlm.nih.gov/gene/) and the potential binding sites of VDR were predicted by the online database JASPAR (http://jaspar.genereg.net/).

*Statistical analysis*

Continuous variables were expressed as mean ± standardize deviation, and two-sample t test was applied for comparison between two groups. The difference between 32W group and 8W group was evaluated to measure the effect of maternal age, while the difference between 32W+VD group and 32W group was evaluated to explore the effects of vitamin D intervention before pregnancy for mice with AMA. Values of $P<0.05$ were considered significant. All analyses were performed in SPSS 19.0.

## Results

*Maternal Vit D supplementation rescued the decreased expression of synaptic markers in the offspring's hippocampus*

NR1 and NR2A are critical subunits of NMDA receptors, responsible for synaptic plasticity and memory consolidation [28]. Postsynaptic density 93 (PSD-93) controls synaptic transmission as a scaffolding protein [29, 30]. In our study, the offspring born to AMA mice had lower NR1 (**Figure 1A**, **1D**), NR2A (**Figure 1B**, **1E**) and PSD-93 (**Figure 1C**, **1F**) expression in their hippocampi, but the expression patterns were reversed when the AMA mother received Vit D intervention before pregnancy, consistent with our previous findings in behavioral tests [31].

*Maternal Vit D supplementation before pregnancy increased $1,25(OH)_2D_3$ concentration in the offspring born to AMA mice*

Previous studies showed that maternal Vit D depletion impaired the offspring's brain development [32]. In our study, maternal serum $1,25(OH)_2D_3$ concentration in 32W group markedly decreased during early, middle and late stages of pregnancy (**Figure 2C**), while the serum vitamin $D_3$ (**Figure 2A**) and $25(OH)D_3$ (**Figure 2B**) concentration showed no significant difference, compared with 8W group. Maternal Vit D supplementation before pregnancy retrieved serum $1,25(OH)_2D_3$ levels during the entire pregnancy (**Figure 2C**), though it did not exert significant effect on serum vitamin $D_3$ (**Figure 2A**) and $25(OH)D_3$ (**Figure 2B**) levels, when compared with 32W group. The similar patterns of serum $1,25(OH)_2D_3$, vitamin $D_3$ and $25(OH)D_3$ concentration in the offspring were also observed (data not shown).

To investigate the close link between vitamin D and the ability of learning and memory, we further analyzed vitamin D metabolites in hippocampus of the young adult mouse, and found that the offspring born to AMA mice had lower $1,25(OH)_2D_3$ level, but the $1,25(OH)_2D_3$ concentration (**Figure 2D**) became higher when AMA mice received Vit D intervention, while vitamin $D_3$ and $25(OH)D_3$ remained unchanged among three groups.

With the data above, we speculate that increased hippocampal $1,25(OH)_2D_3$ concentration by maternal Vit D supplementation before pregnancy may contribute to the synaptic connection which leads to the offspring's cognitive improvement.

*Maternal Vit D supplementation elevated Cyp27b1 expression in the offspring's hippocampus*

Vitamin D is hydroxylated to $25(OH)D_3$ by Cyp27a1 (25-hydroxylates vitamin D) in the liver and subsequently to $1,25(OH)_2D_3$ by Cyp27b1 (vitamin D 1α-hydroxylase) in the kidney [13, 33]. Although renal 1α-hydroxylase is a key enzyme, and rate limiting step of circulating $1,25(OH)_2D_3$ levels, many other organs, such as brain, are also able to express 1α-hydroxylase and convert $25(OH)D_3$ to its active form [34]. Substantial elevation of $1,25(OH)_2D_3$ in the offspring's hippocampus prompted us to speculate the enzymatic hydroxylation for local production and breakdown of $1,25(OH)_2D_3$. We found that both mRNA and protein expressionss of hippocampal Cyp27b1, not Cyp27a1,

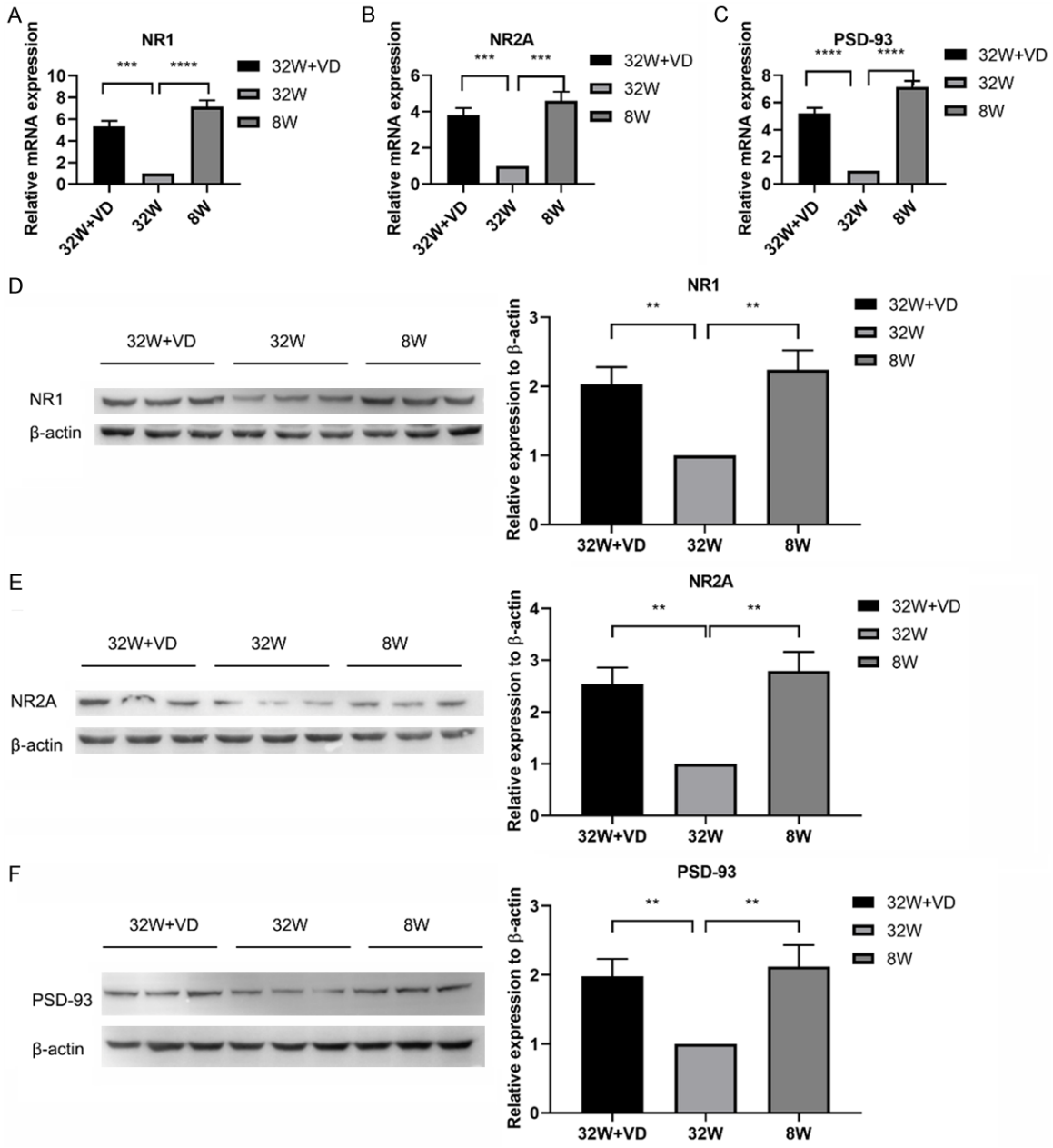


**Figure 1.** Vitamin D supplementation rescued the decreased learning and memory related genes expression in the hippocampus of offspring born to AMA mice. A. Offspring born to 32W+VD and 8W group had elevated hippocampus NR1 mRNA expression than that born to 32W group. B. Offspring born to 32W+VD and 8W group had elevated hippocampus NR2a mRNA expression than that of offspring born to 32W group. C. Offspring born to 32W+VD and 8W group had elevated hippocampus PSD-93 mRNA expression than that of offspring born to 32W group. D. Offspring born to 32W+VD and 8W group had elevated hippocampus NR1 protein expression than that of offspring born to 32W group. E. Offspring born to 32W+VD and 8W group had elevated hippocampus NR2a protein expression than that of offspring born to 32W group. F. Offspring born to 32W+VD and 8W group had elevated hippocampus PSD-93 protein expression than that of offspring born to 32W group. All data are shown as mean ± SD, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

were significantly reduced in the offspring born to AMA mice (**Figure 3B**, **3D**), while maternal Vit D supplementation rescued the reduced Cyp27b1 level when comparing with their counterparts. Therefore, Cyp27b1 may play a vital role in $1,25(OH)_2D_3$ production, resulting in significant elevation of hippocampal $1,25(OH)_2D_3$ in the offspring.

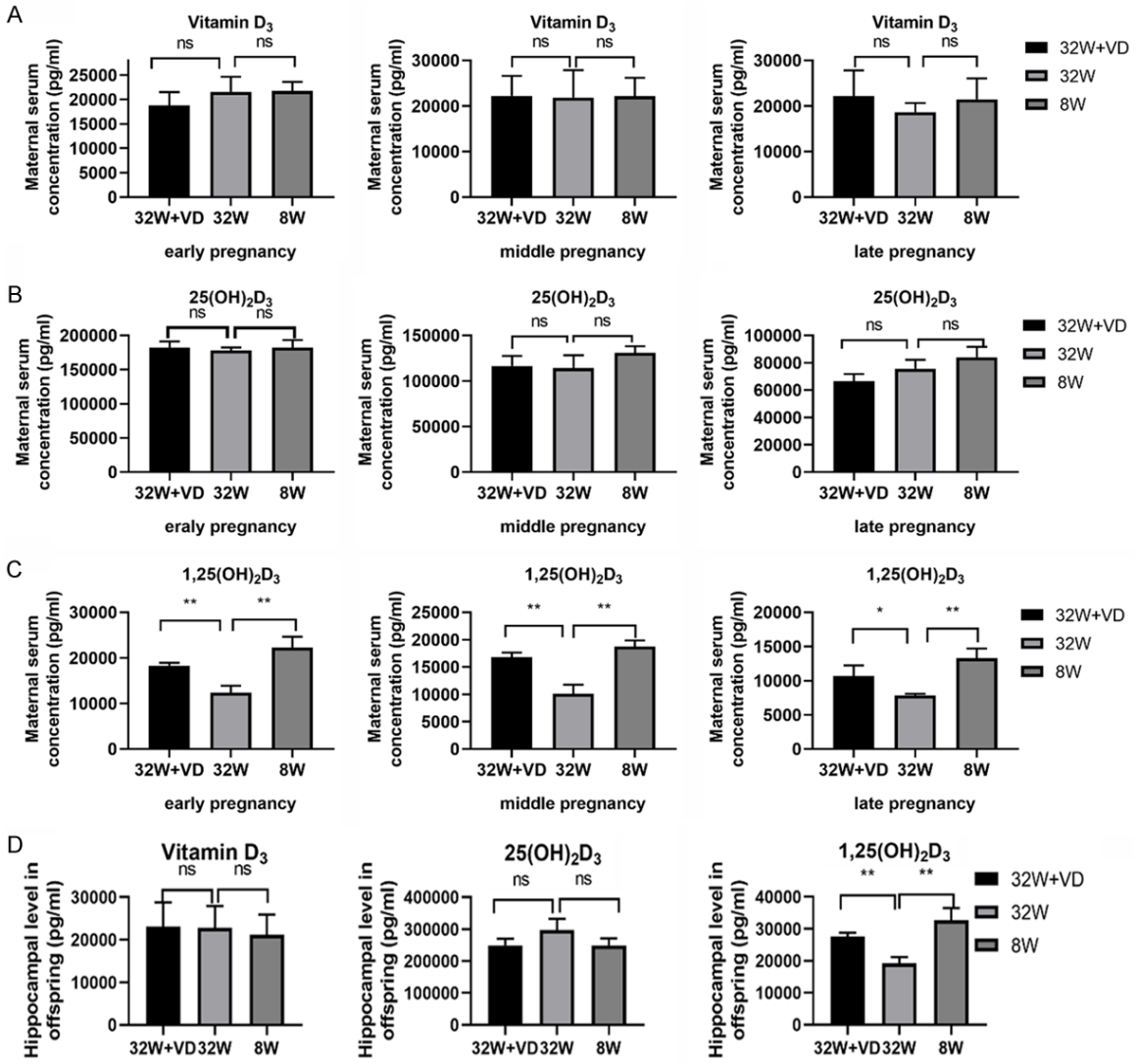


**Figure 2.** Advanced maternal age decreased maternal serum $1,25(OH)_2D_3$ concentration and offspring hippocampal $1,25(OH)_2D_3$ concentration while vitamin D supplementation before pregnancy rescued the effect. A. No significant difference in vitamin $D_3$ was found between 32W+VD and 32W group, so was that between 8W and 32W group. B. No significant difference in $25(OH)D_3$ was found between 32W+VD and 32W group, so was that between 8W and 32W group. C. Serum $1,25(OH)_2D_3$ concentration in the 32W+VD and 8W group was significantly higher than that in the 32W group. D. Offspring hippocampal $1,25(OH)_2D_3$ concentration in the 32W+VD and 8W group was significantly higher than that in the 32W group, but no significant differences in vitamin $D_3$ and $25(OH)D_3$ were found. All data are shown as mean ± SD, ns $P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$.

*Maternal Vit D supplementation rescued the decreased expression of VDR in the offspring's hippocampus*

$1,25(OH)_2D_3$ exerts the various biological functions through binding to VDR [16, 17]. Therefore, we conducted q-PCR, western blotting and immunohistochemistry to measure VDR expression in the offspring's hippocampi. We observed VDR immunoactivity throughout the hippocampus, and the overall VDR expression decreased due to AMA (**Figure 4A**, **4B**), especially in the dentate gyrus area (**Figure 4C**). After AMA mice received Vit D intervention before pregnancy, the hippocampal VDR expression in their offspring was elevated significantly, indicating the existence of rescued effect.

*Vit D/VDR may regulate neural signaling molecules by HIF-1α signaling*

Experimental studies indicated that Vit D/VDR has protective effect on brain function by regu-

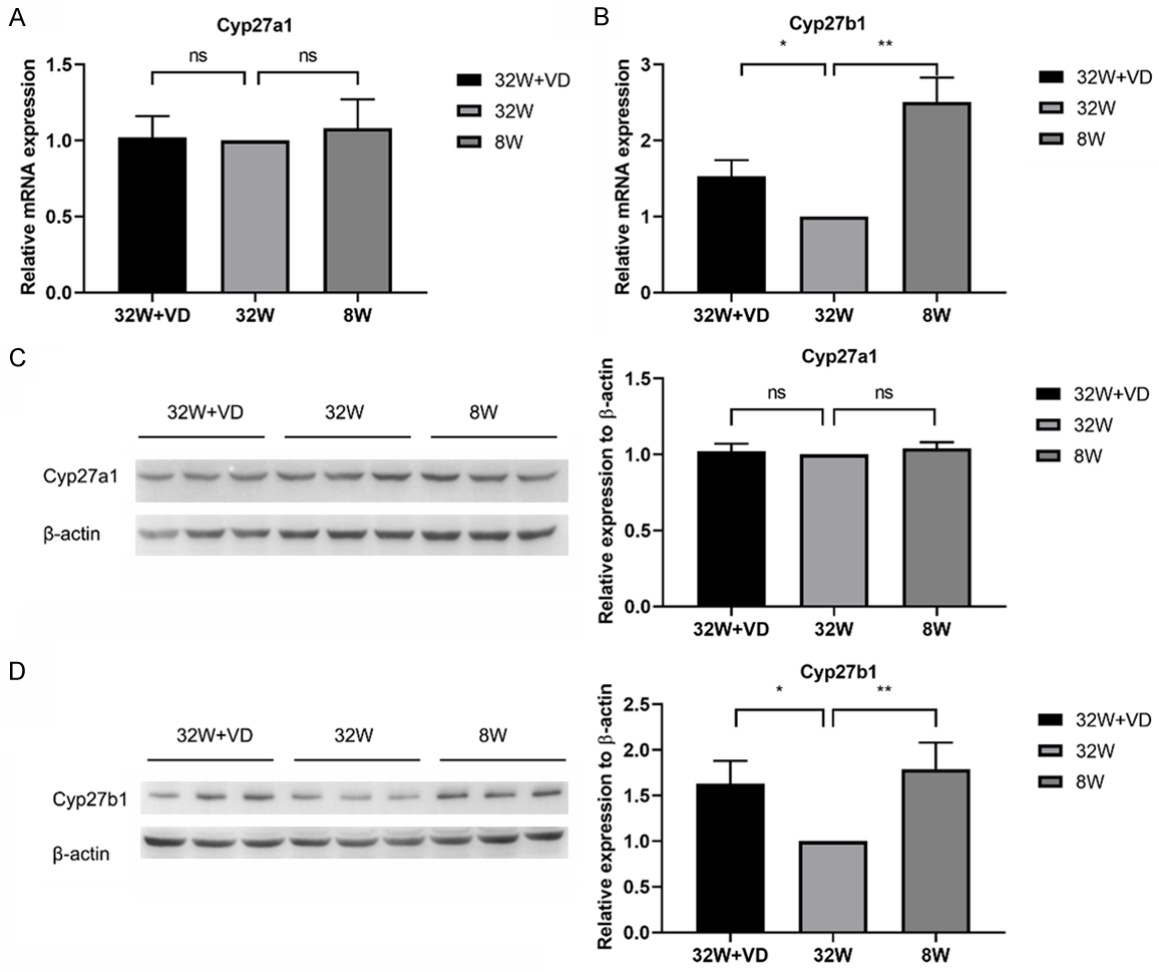


**Figure 3.** Cyp27b1 expression in the offspring's hippocampus led to significant hippocampus $1,25(OH)_2D_3$ expression difference. A. No significant difference in the offspring's hippocampus Cyp27a1 mRNA expression was found between offspring born to 32W+VD and 32W group, so was that between 8W and 32W group. B. Offspring born to 32W+VD and 8W group had elevated hippocampal Cyp27b1 mRNA expression than that of offspring born to 32W group. C. No significant difference in the offspring's hippocampus Cyp27a1 protein expression was found between offspring born to 32W+VD and 32W group, so was that between 8W and 32W group. D. Offspring born to 32W+VD and 8W group had elevated hippocampal Cyp27b1 protein expression than that of offspring born to 32W group. All data are shown as mean ± SD, ns $P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$.

lating angiogenesis, vascular regeneration and anti-inflammatory [35]. We found that Vit D intervention in AMA mice promoted VEGF (Vascular Endothelial Growth Factor), but not BDNF (Brain-Derived Neurotrophic Factor) and IL-2 expression in the offspring's hippocampus (**Figure 5A**).

VEGF is a key target gene of HIF-1α [36], and knockdown of HIF-1α significantly exacerbates the cognitive impairment in rat model of chronic cerebral hypoperfusion [37]. Furthermore, Vit D/VDR suppresses inflammation in oral epithelia by regulating HIF-1α [38]. The above proofs prompted us to hypothesize that Vit D/VDR may regulate HIF-1α in mouse hippocampus.

We identified two possible VDR binding sites in HIF-1α promoter (**Figure 5B**) by bioinformatics prediction, suggesting that HIF-1α is a transcriptional target of $1,25(OH)_2D_3$. The putative binding sequences are CAGTTCACAAAATTTA and AGGTTCAGTGAGTGTC at +644 and -1501, respectively.

We then found that Vit D supplementation in AMA mice did rescue the offspring's hippocam-

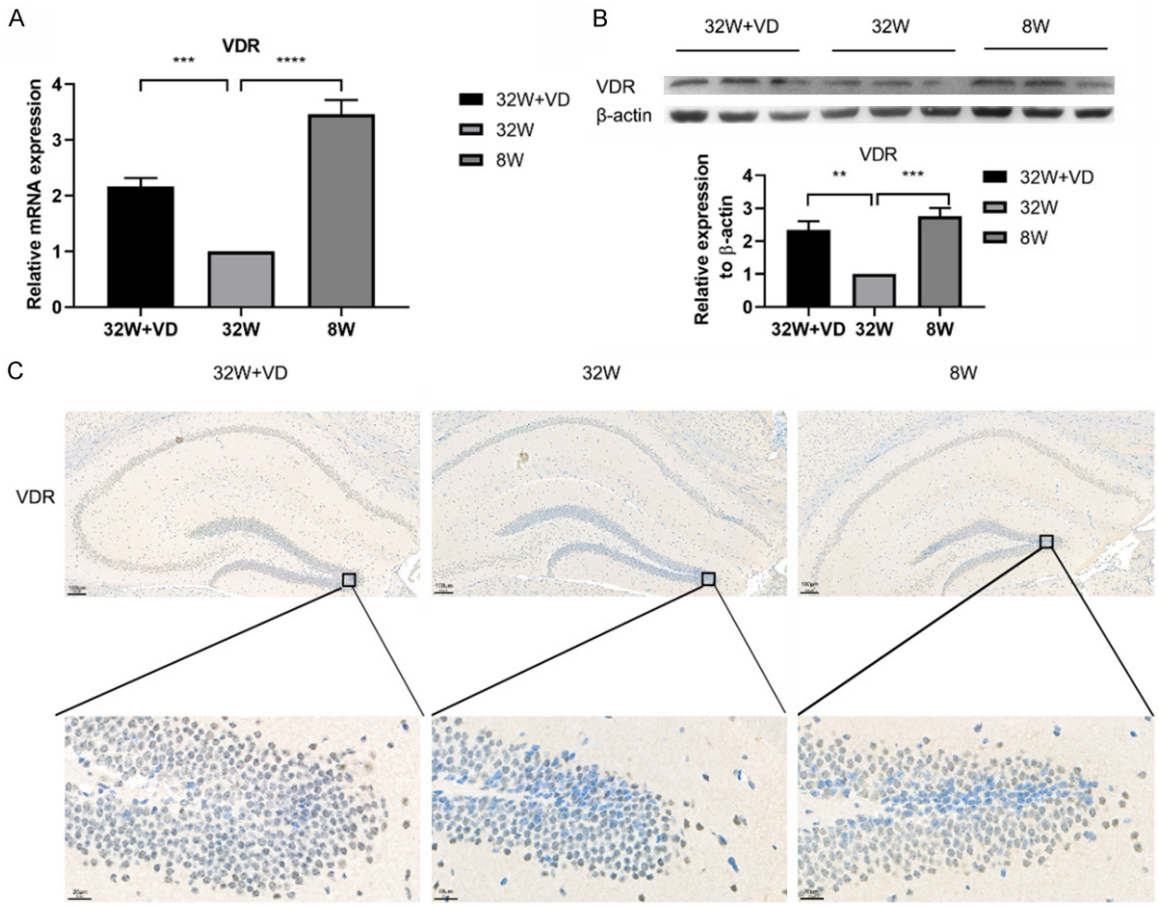


**Figure 4.** Vitamin D supplementation rescued the decreased expression of VDR in the offspring's hippocampus due to AMA. A. Offspring born to 32W+VD and 8W group had elevated hippocampus VDR mRNA expression than that of offspring born to 32W group. B. Offspring born to 32W+VD and 8W group had elevated hippocampus VDR protein expression than that of offspring born to 32W group. C. Immunochemistry showed that VDR expressed in the nucleus, and offspring born to 32W+VD and 8W group had elevated hippocampus VDR protein expression than that of offspring born to 32W group. All data are shown as mean ± SD, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

pal HIF-1α mRNA level, which is similar to VDR expression (**Figure 5C**).

PI3K-AKT, p38-MAPK and PLC-ERK1/2 pathways are related to neural system and proved to be correlated with VEGF [39-41]. Therefore, we measured the expression of these signaling molecules in the offspring hippocampus, and the results showed that the offspring born to AMA mice with Vit D supplementation before pregnancy had higher PI3K, AKT, ERK1, and ERK2 expression, while p38 expression was suppressed (**Figure 5D**).

Based on the above findings, we had a belief that Vit D/VDR may regulate PI3K-AKT and PLC-ERK1/2 pathways by modulating HIF-1α/VEGF expression, resulting in learning and memory function improvement.

## Discussion

Previous studies had shown that pregnant women with AMA tend to have a lower $25(OH)D_3$ concentration and higher percentage of vitamin D deficiency [42, 43]. Our results from animal study also confirmed the above findings [31]. Studies identified the distribution of Vit D metabolic enzymes and VDR in the central nervous system, indicating the role of VDR in the cognitive function [44], and VDR is essential for biological activities of $1,25(OH)_2D_3$. Vit D has great impacts on the proliferation, growth, and differentiation of neurons in fetal hippocampus [45], and protects normal development of cognitive functions [46]. In the present study, we demonstrated that Vit D supplementation before pregnancy not only elevated serum

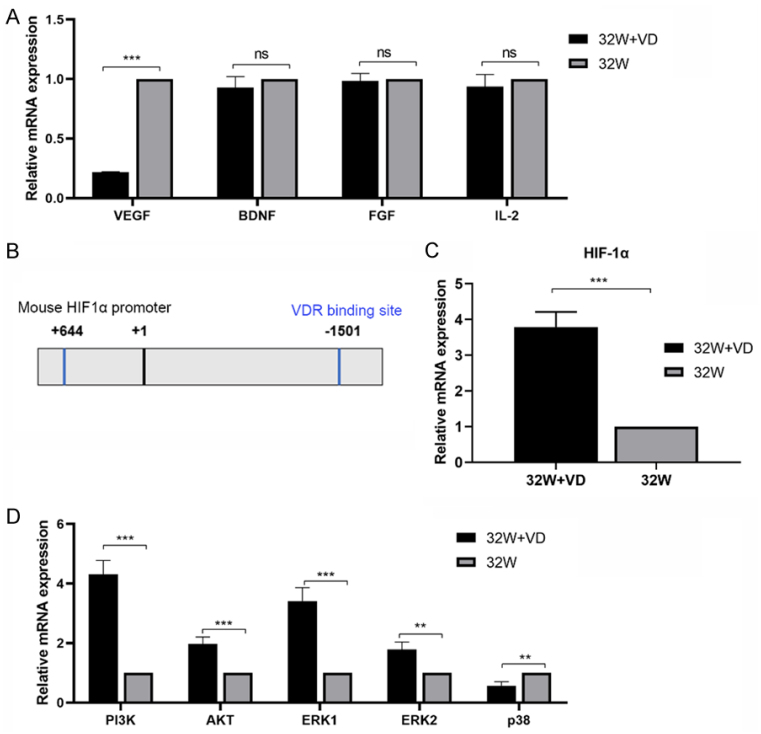


**Figure 5.** Vitamin D supplementation regulated HIF-1α signaling in the offspring's hippocampus. A. Vit D intervention in AMA mice promoted VEGF, but not BDNF, FGF and IL-2 expression in the offspring's hippocampus. B. Two VDR binding sites were identified in the HIF-1α promoter. C. Offspring born to 32W+VD and 8W group had elevated hippocampal HIF-1α mRNA expression than that of offspring born to 32W group. D. Offspring born to AMA mice with Vit D supplementation before pregnancy had higher PI3K, AKT, ERK1, and ERK2 expression, while lower p38 expression.

$1,25(OH)_2D_3$ concentration during the early, middle and late stages of pregnancy in AMA mice, but also $1,25(OH)_2D_3$ concentration in the young adult offspring, especially in their hippocampi. Further investigation found that Cyp27b1, responsible for $1,25(OH)_2D_3$ production, increased in the offspring's hippocampus by maternal Vit D intervention, which may contribute to the significant elevation of hippocampal $1,25(OH)_2D_3$ level. Given the role of Vit D/VDR signaling in the neurodevelopment and cognitive functions, boosted VDR expression and $1,25(OH)_2D_3$ production in the offspring's hippocampus would likely contribute to synaptic connection (NMDA) and transmission (PSD), and subsequently lead to learning and memory improvement.

Among the signaling pathways between VDR and cognitive function, calcitriol is proved to activate the VDR/ERK signaling pathway to reduce cognitive impairments [47]. Yan concluded that Vit D played a protective role by suppressing inflammatory cytokines and subsequently inhibited the progression of apoptosis [48]. Meanwhile, studies found that mutation of VDR in mice led to sensory and cognitive dysfunction [49], which finding linked VDR with cognitive function and is similar to our results.

HIF-1α, the critical regulator of cellular response to hypoxia, was proved to exacerbate the blood-brain barrier disruption, leading to further cognitive impairment [50]. HIF-1α can be suppressed by calcitriol through binding to VDR that involves PI3K/Akt signaling [22, 25, 51]. In addition, activation of VDR promotes VEGF expression in endothelial cells [52], and HIF-1α/VEGF signaling is a pathway to modulate brain dysfunction [53-55]. The current study found that maternal Vit D preventive intervention not only elevated HIF-1α/VEGF expression, but also regulated PI3K-Akt, ERK1/2, and p38-MAPK molecules. Such biological effects are probably through the binding sites of VDR to specific VDR response elements in HIF-1α promoter.

To our knowledge, numerous studies revealed the impacts of maternal Vit D supplementation on maternal health and neonatal outcome. However, few studies focused on the association between maternal Vit D preventive intervention and their offspring's cognitive function, especially the regulation of Vit D/VDR/HIF-1α pathway. Our study showed that the maternal Vit D preventive administration before pregnancy had profound neuroprotective effects on their offspring. Thus, Vit D replenishment may open up new possibilities for therapeutic application.

Nevertheless, the present study has several limitations. First, though we did identify the putative binding sites of VDR in HIF-1α promot-

er, we were unable to verify the direct interaction between VDR and VDR response elements in HIF-1α promoter by luciferase reporter and chromatin immunoprecipitation (ChIP) assay. Second, preventive maternal Vit D intervention did modulate HIF-1α/VEGF and related signaling molecules in the offspring's hippocampus; however, the cause-effects between Vit D/VDR and HIF-1α/VEGF require genetic knockout or pharmaceutic inhibitors of HIF-1α/VEGF (either or both).

In conclusion, our results highlight the biological impacts of maternal Vit D intervention on Vit D metabolism, synaptic connection and transmission, and related signaling molecules in the offspring. Our findings provide experimental evidence which might underlie the potential mechanism of the cognitive improvement in the offspring born to AMA mice with Vit D supplementation before pregnancy.

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## Disclosure of conflict of interest

None.

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