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Effect of marginal vitamin A deficiency during pregnancy on retinoic acid receptors and *N*-methyl-D-aspartate receptor expression in the offspring of rats☆

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Abstract

This study examined whether pregnancy-related marginal vitamin A deficiency (MVAD) influences postnatal development of retinoic acid receptors (RARs) and *N*-methyl-D-aspartate (NMDA) receptor subunit 1 (NR1) in hippocampus of rat pups. Sixteen female rats were randomized equally into control and MVAD groups. Dams and pups were fed with either a normal control diet or one deficient in vitamin A. Eight female pups in each group were killed at 1 day, 2 weeks, 4 weeks and 8 weeks after birth, respectively. Serum retinol levels were monitored. The messenger RNA (mRNA) and protein expressions and subcellular localization of RARα, RARβ and NR1 in postnatal hippocampus were detected. At 1 day, 2 weeks and 8 weeks after birth, serum retinol levels in the MVAD group were significantly lower than those in the control group. Results of Morris water maze test at 7 weeks of age showed that spatial learning and memory in the MVAD group were affected. Vitamin A deficiency resulted in decreased mRNA levels of RARα, RARβ and NR1 ($P<.05$). The protein level of RARα and NR1 in the MVAD group was lower than that of the control group ($P<.05$). There was no significant difference in RARβ between the groups ($P>.05$). A mass of RARα and NR1 colocalized in hippocampal cell cytoplasm on postnatal day 1. Our data suggested that vitamin A deficiency in pregnancy may affect the postnatal expression of RARα and NR1, affecting learning and memory function in the hippocampus and synaptic plasticity of the calcium signaling pathway.

Keywords: Vitamin A deficiency; Retinoic acid receptors; NMDA receptor; Pregnancy

1. Introduction

Vitamin A deficiency (VAD) is a worldwide micronutrient deficiency disease [1]. It is estimated that 125–150 million preschool children have VAD and 20 million pregnant women have marginal VAD (MVAD) or VAD [2]. Although more attention is being given to the issue, VAD and subclinical VAD still pose a threat to the health, especially to pregnant women and children from high-risk groups [3,4]. Vitamin A and its metabolites play a role in the regulation of nerve cell differentiation and the development of axial symmetry in neurological development [5]. In recent years, a close link between vitamin A and cognitive function has been found.

Many studies in rats show that VAD can lead to damage in terms of spatial learning, memory and the somatic sensory system [6,7]. In our previous study, we found that MVAD in pregnancy led to significant impaired learning and memory function in the affected rat pups when they reached adulthood, with depressed long-term potentiation (LTP) in the hippocampal CA1 area, which suggests that synaptic plasticity was affected. In that study, the LTP of the hippocampal slice in the MVAD group increased significantly in response to all-*trans*-retinoic acid (atRA) *in vitro* and the ultrastructure of the synapse that reflected the occurrence of LTP in the MVAD group also changed. After tetanic stimulation, which induces LTP, the calcium ion concentration in the MVAD group was significantly lower than that in the control group [8,9]. These results suggest that MVAD, which has its onset in pregnancy, may affect learning and memory function in the child by regulating the expression of important molecules in the synaptic plasticity of the Ca^{2+}-dependent signaling pathway.

Long-term potentiation is one of the neural electrophysiological bases of synaptic plasticity, as well as the basic mechanism of learning and memory [10]. The influx of Ca^{2+} into the postsynaptic membrane is the initial signal of LTP induction [11,12]. The classical Ca^{2+}-dependent signal transduction pathway shows that *N*-methyl-D-aspartate (NMDA)-type glutamate channels in nerve cells can control Ca^{2+} influx by identifying their ligands [13]. The NMDA receptor (NMDAR) is the main regulator of neuronal differentiation, synaptogenesis, synaptic plasticity, LTP and long-term depression (LTD) and is also the basis for learning and memory [14,15]. The function of the NMDAR is determined by the composition and number of subunits, and NMDAR1 (NR1) plays a key role in this

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Table 1
Primers used in real-time polymerase chain reaction to assess the expressions of RARs and NMDA receptor 1 in the hippocampus

Gene	Primer sequences	Accession number	Product size (bp)	Annealing temperature
RARα	FP:TGGGCAAGTACACTACGAACAAC RP:GGTAATCTGGTCGGCAATGGT	NM-031528	164	60
RARβ	FP:CTTGGGCCTCTGGGACAAAT RP:TGGCGAACTCCACGATCTTAAT	XM_001059523	68	60
NMDAR1 (Grin1)	FP:AAGCTGCACGCCTTTATCTG RP:TTCTCATGGGACTTGAGTATGGA	NM-017010	173	60
β-Actin	FP:GACGTTGACATCCGTAAAGACC RP:TAGGAGCCAGGGCAGTAATCT	NM-031144	112	59

FP, forward primer; RP, reverse primer.

process [16]. A number of *in vitro* studies found that the NR1 subunit is the target gene of RA [17,18].

The hippocampus is closely related to learning and memory, particularly, spatial learning and memory. It was recently found that the memory formation of the hippocampus is also dependent on atRA through its RA receptors (RARs) [19]. However, the exact mechanism is uncertain. It has been confirmed that RA can be synthesized in the hippocampus because of its independent RA anabolic system [20]. Enzymes related to RA synthesis and metabolism, as well as cellular retinol-binding protein and cellular RA binding protein, are present in either the hippocampus or the surrounding meninges [21]. Our previous study found that the changes in RA synthesis caused by catabolic enzymes in the hippocampus contribute to the regulation of hippocampal RA synthesis when exogenous vitamin A supply is insufficient and the level of serum vitamin A is relatively low. The results of that study suggested that the mechanism of MVAD in pregnancy leading to impaired learning and memory in offspring may be related to the downstream effects of the RA signaling pathway; these are the RARs or retinoidXreceptors (RXRs). The expression of nuclear transcription factor RAR and RXR in hippocampus is the most in the brain. RXRs only bind 9-cisRA, while RARs bind both 9-cisRA and atRA. RARs are thought to play a more direct and important role in the RA signaling pathway. In our previous study, LTP decreased significantly when an RARα antagonist was added to hippocampal brain slices, suggesting that RARα may play an important role in synaptic plasticity.

Therefore, in the present study, we investigated the postnatal messenger RNA (mRNA) and protein expression of RARs and NMDAR1 and the impact of pregnancy-onset MVAD on the expression of these genes.

2. Methods and materials

2.1. Animals and diets

Sixteen clean-grade female Wistar rats weighing 220–250 g provided by the Laboratory Animal Center of the Third Military Medical University (Chongqing, China) were randomly divided into two groups: a control group and an MVAD group. After 1 week of acclimatization, the animals were fed with different diets for 3 weeks and then were mated with males. Eight rats in the control group were fed with a standard diet, which contained basic diet (Nutrition Research Center, Chongqing, China) supplemented with 6500 IU retinol/kg. The other eight rats in the MVAD group received the MVAD diet, which consisted of the basic diet plus 400 IU retinol/kg. The basic diet was modified on the basis of AIN765 with the following composition per 100 g dry food: vitamin-free protein, 20%; carbohydrate, 68.005%; soybean oil, 5%; inorganic salt mix, 6.75%, and a vitamin mixture lacking vitamin A, 0.245%.

Female pups born from the 16 female rats were used for the present study. After weaning, the pups of both groups were fed throughout the study with the standard diet or MVAD diet, respectively. Seven weeks after birth, eight pups in each group were randomly selected for the Morris water maze test. At postnatal day 1 (P1d), week 2 (P2w), P4w and P8w, eight female pups from each group were randomly selected and killed.

The rats in both groups were housed in individual stainless steel cages at a constant temperature of 19°C–25°C with 55%–65% relative humidity; artificial daylight was put on between 7 a.m. and 7 p.m., and food and water were available *ad libitum*. The experimental protocol was approved by the Animal Care Committee of Chongqing Medical University. All efforts were made to minimize the number of animals used and alleviate their suffering. All procedures were conducted in strict accordance with international guidelines. No visible clinical manifestations of VAD were detected in any experimental rats.

2.2. Serum retinol detection

The tail blood of female rats was collected for detecting serum retinol level before mating. Heart bloodletting at P1d and femoral bloodletting at P2w, P4w and P8w were performed, and the blood was collected for detecting serum retinol level using high-performance liquid chromatography following a previously described method [22,23] with slight modifications. Briefly, retinol was extracted with hexane after deproteinization with ethanol that contained retinyl acetate as the external standard and evaporated to dryness with nitrogen gas. The residue was dissolved in 0.1 ml methanol. A portion (20 μl) of the sample was injected into the column (Symmetry Shield RP_{18} 3.9×150 mm) installed with the high-performance liquid chromatography apparatus (Waters 1525 Binary HPLC Pump, Waters Breeze, Milford, MA). The mobile phase was a methanol–DH_2O mixture (95:5). The concentration of retinol was determined by spectrophotometry (Waters 2487 Dual λ Absorbance Detector) at 315 nm. All procedures were performed by the same operator in a dark room to protect the serum from light.

2.3. Morris water maze test

At age of 7 weeks, eight female pups of each group were trained to do the Morris water maze test (developed by Richard Morris in 1984) to evaluate spatial learning and memory abilities. The maze is a circular pool (painted black, 150 cm in diameter and 50 cm high) filled with clear tap water at a temperature of 22°C±0.5°C to a depth of 30 cm. A black platform, 10 cm in diameter, is located 2 cm below the surface of the water approximately in the middle of one of the four quadrants. A digital camera is suspended above the pool and connects to a video recorder and tracking device, which permits automated tracking of the path taken by the rats. Each experiment was carried out in a soundproof room, with the light source and surrounding environment remaining unchanged.

There were four kinds of training in the Morris water maze test, which lasted for 10 days [24]. During the first 5 days, rats were given four swimming trails a day and designated a space acquisition test. Each trail was limited to 120 s to record the time to reach the platform, swimming distance and speed. If the platform was not found within the set time, the computer stopped tracking and recorded the time as 120 s. If the rat found the platform within 120 s, it was allowed to stay on it for 30 s; otherwise, it would be guided to the platform to stay for 30 s. Then, a probe trial test was conducted at day 6. The platform was removed on day 6 for a 30-s exploration test, which was recorded as the proportion of time the rats were in the target quadrant. In the space reversal experiment over days 7–9, training was almost the

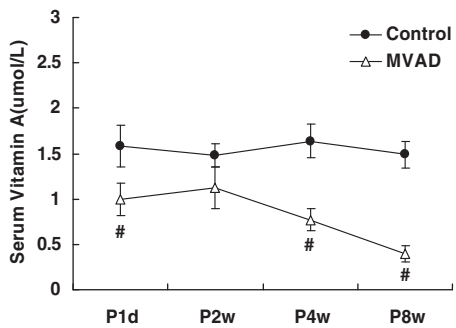

Fig. 1. Serum retinol levels of rat pups at P1d, P2w, P4w and P8w (μmol/l). Data are expressed as mean±S.E.M. ($^{\#}P<.01$ vs. control). At age P1d, P4w and P8w, the serum retinol level of pups in the MVAD group was significantly lower than that of the control ($P<.05$).

same as the space exploration element, but the platform was put in the opposite quadrant to that used in the initial space exploration. During day 10, a visible platform experiment was performed. The platform is kept submerged in the same way as that in the hidden platform version, except that a 'flag' is mounted, which extends above the water surface by approximately 12 cm. It is used to test the animals for their ability to learn to swim to a cued goal. Spatial learning, short- and long-term memory, as well as the ability to learn new things were all tested in the rats through the above four experiments.

2.4. Real-time fluorescence quantitative polymerase chain reaction

Extraction of hippocampal RNA was performed using the total RNA isolation system, EZgeno™ (Genemega, San Diego, CA) and the complementary DNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The complementary DNA quantification by real-time polymerase chain reaction was performed using LightCycle 2.0 instrument (Roche, Basel, Switzerland). Thermocycling was programmed as 5 min at 94°C for predegeneration, 35 cycles of 30 s at 94°C for degeneration, 30 s at annealing temperature (Table 1), 20 s at 72°C for extension and a final extension at 72°C for 10 min. Sequences of primers for the indicated genes are shown in Table 1.

2.5. Immunofluorescence and laser scanning confocal microscopy

Immunofluorescence staining was carried out as described [25]. Briefly, paraformaldehyde-fixed hippocampal tissue was soaked for 2 d in 15% and 30% sucrose separately, and frozen tissue was sectioned at 25 μm. The slices were blocked with 1%

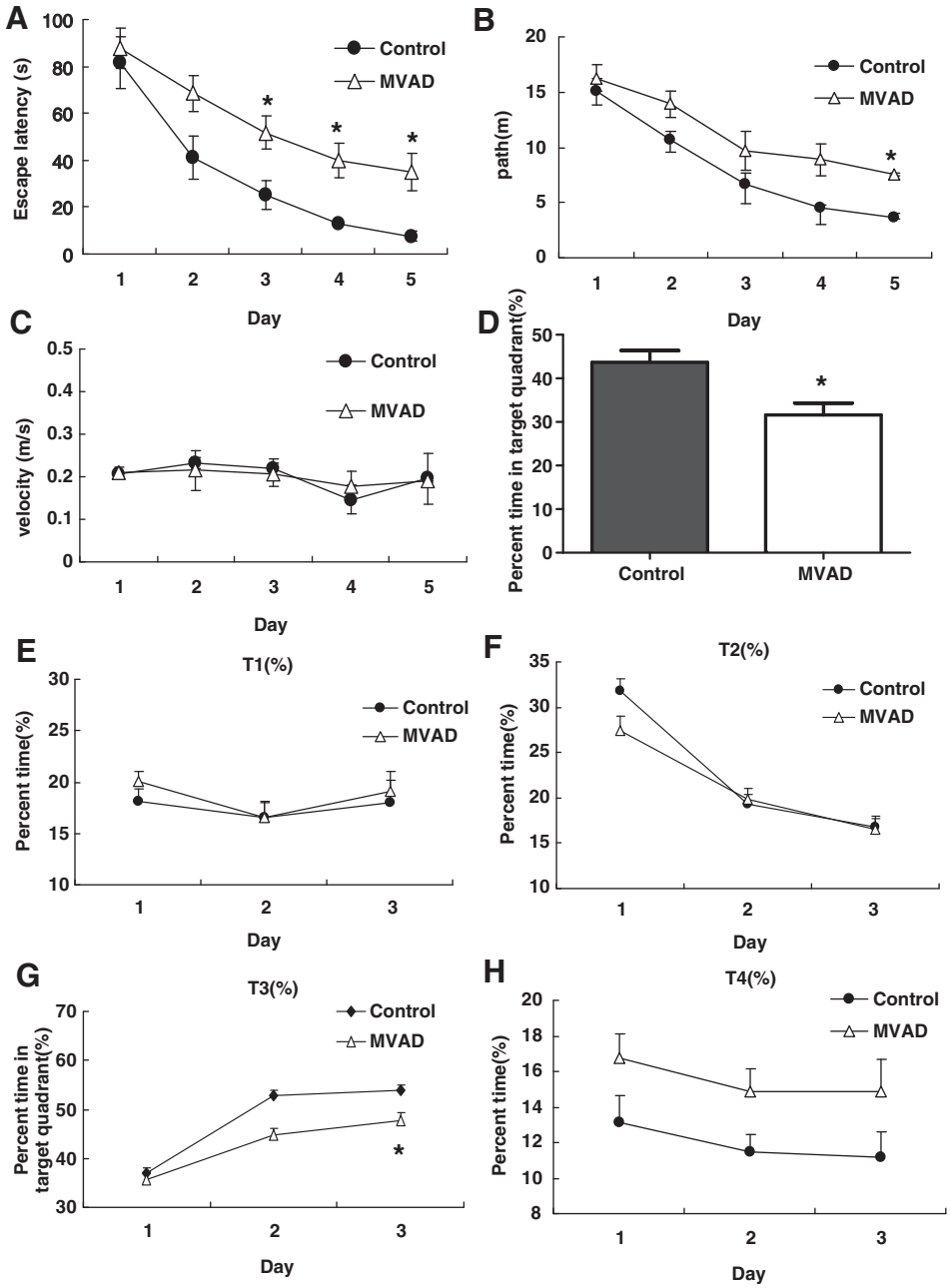

Fig. 2. Spatial acquisition test, probe trial test and spatial reversal test results of the two groups in Morris water maze test at 7 weeks of age. Data are expressed as mean±S.E.M. (n=8, *P<.05 vs. control). (A) The average escape latency in the MVAD group on test days 3, 4 and 5 was significantly longer than in the control group. (B) The distance to find the platform in each group was gradually reduced by training, and the difference between the two groups was almost consistent with that of latency. (C) There was no significant difference in swimming speed between the two groups during the training period. (D) Time proportion in target quadrant in the MVAD group was significantly lower than that in the control group (P<.05) in probe trial test. (E) Time proportion in quadrant 1 (T1) in both groups in spatial reversal test. (F) Time proportion in the former platform quadrant (T2) in both groups was gradually shortened in spatial reversal test. (G) On the third day, the time proportion of the control group in the new quadrant (T3) was significantly higher than that of the MVAD group in spatial reversal test (P<.05). (H) Time proportion in quadrant 4 (T4) in both groups in spatial reversal test.

bovine serum albumin, followed by incubation with 1:50 rabbit polyclonal subtype-specific RARα (C-20), RARβ (C-19) (Santa Cruz Biotechnologies, Santa Cruz, CA) or NMDAR1 (ab17345) (Abcam, Cambridge, MA) antibodies at 4°C overnight. After washing three times with phosphate buffered saline (PBS), the slices were incubated with a second antibody (a 1:100 dilution of fluorescein-conjugated affinipure goat anti-rabbit IgG [H+L] or rhodamine [TRITC]-conjugated affinipure goat anti-rabbit IgG pH+L]) for 30 min in a dark place. In addition, nuclei were stained using 4,6-diamidine-2-phenylindol-dihydrochloride (DAPI, Sigma, USA) for 5 min. Hippocampal slices on which the primary antibody was replaced by phosphate-buffered saline and cortex from hippocampal slices served as negative and positive controls, respectively. As for the colocalization of RARα and NR1, we first incubated the slices with primary antibody of NMDAR1 at 4°C overnight. After washing with PBS, the slices were incubated with a second antibody (FITC), then incubated with primary antibody of RARα at 4°C overnight. After washing with PBS, they were incubated with a second antibody (TRITC). After washing, nuclei were stained using DAPI for 5 min. The evaluation was performed using either a fluorescence microscope (TE2000-S; Nikon, Tokyo, Japan) or a laser scanning confocal microscope (LSCM; Leica, Heidelberg, Germany). Analysis of the sections was done using Image-Pro Plus 6.0 software. Six immunofluorescent histological sections were selected at each time point for statistical analysis of fluorescence expression intensity.

2.6. Western blotting

Western blotting was carried out as described [26]. Briefly, protein from either the nucleus or the cytoplasm or the total protein was extracted by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Worchester, MA) or the protein extraction kit (KeyGen Biotech, Nanjing, China). The protein samples were denatured by boiling and loaded onto a 4% SDS-polyacrylamide gel (Beyotime, Jiangsu, China) as 50 μg/lane. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) after electrophoretic separation. The membrane was blocked with 5% skimmed milk in the Tris-buffered saline with Tween buffer at 37°C for 2 h and probed with the primary antibodies, anti-RARα (Santa Cruz Biotechnologies), anti-RARβ (Santa Cruz Biotechnologies), anti-NMDAR1 (Abcam), anti-lamin B1(Santa Cruz Biotechnology) or anti-GAPDH (Santa Cruz Biotechnology) at 4°C overnight. This was followed by incubation with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at room temperature for 1 h. The staining was evaluated using an enhanced chemiluminescent kit (KeyGen Biotech). Image analysis was performed with the use of Quantity One software.

2.7. Statistical analysis

Statistical analysis was performed using SAS 8.1 statistical software. Data are expressed as mean±S.E.M. Serum vitamin A levels of dams and offspring and mRNA and protrin of the genes of two groups were compared using the Student's *t* test. ANOVA was used to compare the expression of genes at different time points. Tests of significance were two-tailed, and $P<.05$ was considered statistically significant.

3. Results

3.1. Serum retinol levels at different postnatal ages

The serum retinol level in the MVAD group was significantly lower than that of the control group (0.87±0.14 μmol/l, $n=16$ vs. 1.35±0.16 μmol/l, $n=16$, $P<.05$) before mating. No clinical manifestation of VAD was observed. Serum retinol concentrations at P1d, P2w, P4w and P8w in the offspring in both groups are shown in Fig. 1. At the age of 8 weeks, the serum retinol level in the MVAD group was significantly lower than that of the control group ($P<.05$).

3.2. Results of Morris water maze test

The results of the spatial acquisition test were recorded as latency and distance swam to find the platform in order to measure spatial learning and short-term memory in the rats. The results showed that the latency of the rats in each group became shorter with successive training days, suggesting that pups in each group were able to learn. Analysis between the two groups showed that the average latency in the MVAD group on test days 3, 4 and 5 was significantly longer than that in the control group (Fig. 2A). The distance to find the platform in each group was also gradually reduced by training, and the difference between the two groups was almost consistent with that of latency (Fig. 2B). In addition, there was no significant difference in swimming speed in each group (Fig. 2C).

The probe trial test was conducted to test the ability of long-term reference memory. The results showed that the proportion of time in the right quadrant in the MVAD group was significantly lower than that in the control group ($P<.05$), which suggested that long-term reference memory in the control group was better than that in the MVAD group (Fig. 2D).

Spatial reversal tested the ability to find a new target location. This usually takes 5 days. As all the offspring could find the platform quickly after 3 days of training, the training time was shortened to 3 days. The results showed that the time proportion in the former platform quadrant (T2) in both groups was gradually shortened. On the third day, the time proportion of the control group in the new quadrant (T3) was significantly higher than that of the MVAD group (Fig. 2G), which suggests that the ability of the control group to learn new things was stronger than that of the MVAD one.

The results of the visible platform test showed that, at the end of behavioral testing, all rats were able to swim to the visible platform and then find the platform, indicating there was no significant difference in basic capability between the rats, such as their vision, swimming ability, ability to learn to leave the pool wall, climb onto the platform and evade water. The above results,

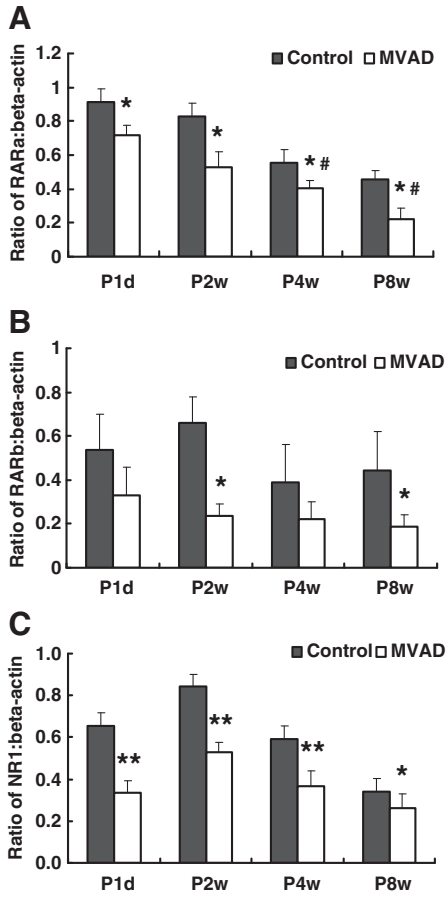

Fig. 3. mRNA expression of RARα, RARβ and NR1 in the hippocampus of pups at different ages. Data are mean±S.E.M. ($n=6$, *$P<.05$ vs. control, **$P<.01$ vs. control, #$P<.05$ vs. P1d). (A) The mRNA expression of RARα was significantly lower at the four time points in the MVAD group than in the control group ($P<.05$). (B) The expressions of RARβ at the four time points in the MVAD group were very low and were significantly lower than those in the control group at P2w and P8w ($P<.05$). (C) In the hippocampus of the control group, NR1 mRNA level was significantly elevated from P1d to P2w and then gradually decreased until P8w. The mRNA level of NR1 at four time points in the control group was significantly higher than that in the MVAD group ($P<.05$).

with regards to spatial acquisition, probe trial and spatial reversal, were reliable and comparable.

3.3. Effect of pregnancy-onset MVAD on mRNA expressions of RARα, RARβ and NR1

With the postnatal development of the hippocampus, mRNA expression of RARα in the control group decreased gradually from P1d to P8w. There was no significant difference between P1d and P2w or P4w and P8w in the control group ($P>.05$), while the expression was significantly lower in P4w and P8w compared with P1d and P2w ($P<.05$). The mRNA expression of RARα was significantly lower in the MVAD group at four time points when compared with that of the control group ($P<.05$; Fig. 3A).

The mRNA of RARβ was expressed at the four time points after birth but without a trend in the variation ($P>.05$). The expressions of RARβ in the MVAD group were very low at the four time points and were significantly lower than those in the control group at P2w and P8w ($P<.05$; Fig. 3B).

Fig. 3C shows that in the hippocampus of the control group, the NR1 mRNA level was significantly elevated at P1d and P2w and then gradually decreased until P8w. The mRNA level of NR1 at most time

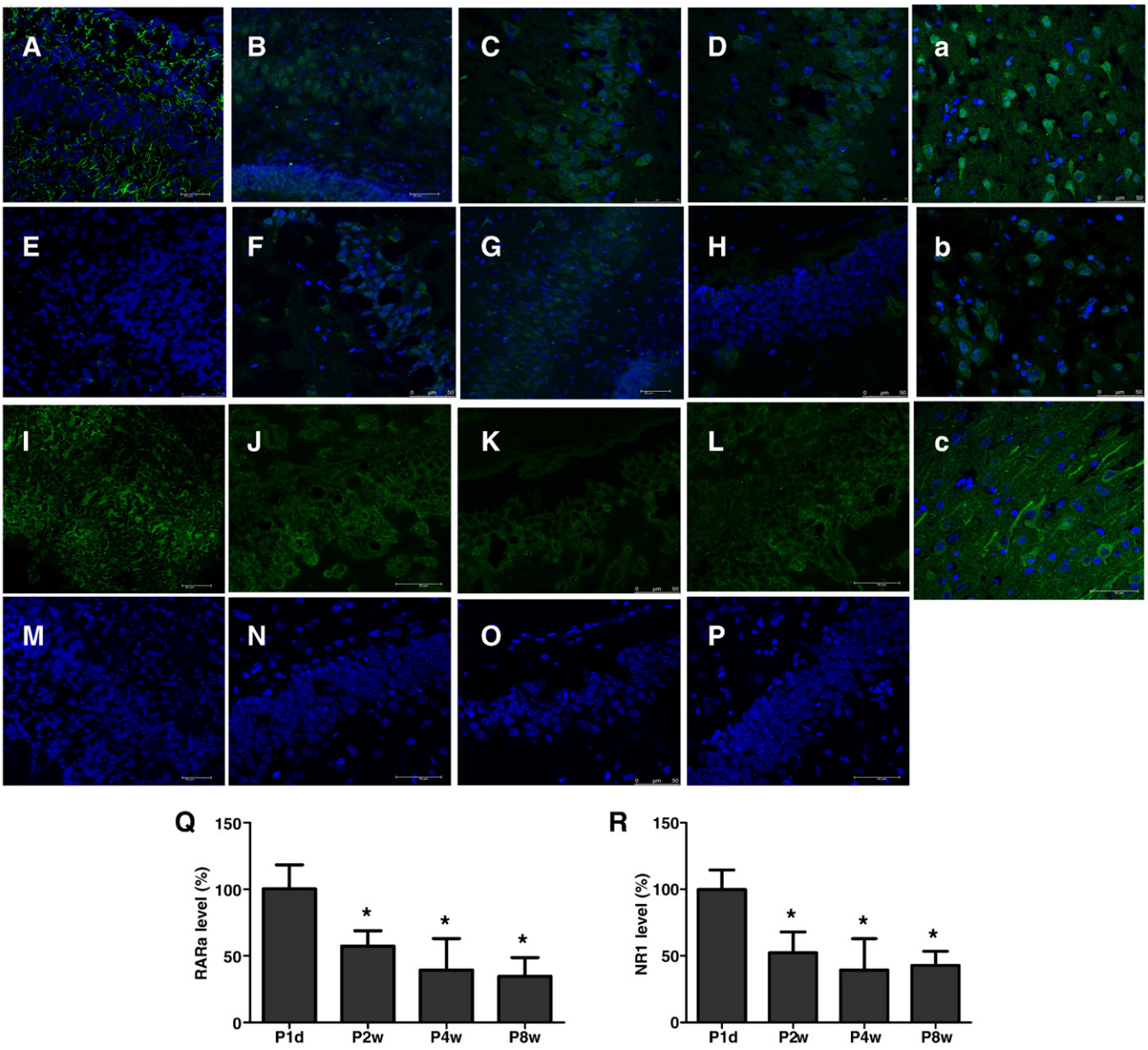

Fig. 4. Laser scanning confocal microscopy results of the subcellular localizations of RARα, RARβ and NMDAR1 in CA1 area of hippocampus. The scale bars are 50 μm. Green refers to target protein and blue refers to DAPI. (A–D) Immunofluorescent detection of expression and subcellular localization of RARα at P1d, P2w, P4w and P8w, respectively (original magnification ×400). At P1d, abundant RARα protein is located in the cytoplasm and neurites and then located in the nuclei of neurons by P2w. Later, it shifted to the cytoplasm gradually with expression in the cytoplasm and some neurites during P8w. (E–H) Immunofluorescent detection of expression and subcellular localization of RARβ at P1d, P2w, P4w and P8w, respectively (original magnification ×400). No distinct expression of RARβ was detected from P1d to P8w in hippocampus. (I–L) Immunofluorescent detection of expression and subcellular localization of NR1 at P1d, P2w, P4w and P8w, respectively (original magnification ×400). The expression of NR1 declined with development and located in the cytoplasm and neurites. (M–P) refer to nuclei stained with DAPI of the slices of NR1 at P1d, P2w, P4w and P8w, respectively. (Q and R) Quantitative analysis of fluorescence intensity of RARα and NR1, respectively. Data are mean±S.E.M. ($n=6$, *$P<.05$ vs. P1d). (a–c) Cortex from hippocampal slices served as positive control of RARα, RARβ and NR1, respectively.

points in the control group was significantly higher than that in the MVAD group ($P<.05$).

3.4. Protein expression levels and subcellular localization changes of RARα, RARβ and NR1 during postnatal hippocampal development

Fluorescence intensity of RARα in hippocampal CA1 pyramidal cells was analyzed. Fig. 4A–D,Q shows that RARα protein expression decreased gradually with hippocampal development. Expression of RARα in the hippocampus at P2w, P4w and P8w decreased significantly compared with those at P1d ($P<.05$), and there was no significant difference between P4w and P8w ($P>.05$), which was consistent with the change in mRNA. With LSCM, we found abundant RARα protein located at cytoplasm and neuritis at P1d and then at the nuclei of neurons at P2w. Later, it gradually shifted to the cytoplasm and was expressed in the cytoplasm and some neurites at P8w (Fig. 4A–D). Although there was mRNA expression of RARβ in the hippocampus, we hardly detected any expression by immunofluorescence from P1d to P8w (Fig. 4E–H).

Fig. 6 shows the Western blotting results of RARα and RARβ in two groups. RARα expression and subcellular localization changes at four time points were almost consistent with immunofluorescence results. In the control group, RARβ was almost absent at P1d and then weakly expressed mainly in the nucleus at P2w. When it came to P4w and P8w, the expression was enhanced, being more located in the nuclei than in the cytoplasm.

Fig. 4 I–P,R shows that there was a large amount of NR1 expression at P1d in the cytoplasm of hippocampal nerve cells and in the neurites of pyramidal cells in the radiation layer, which decreased at P2w and P4w ($P<.05$). Western blotting results were consistent with immunofluorescence. The strongest expression of NR1 was at P1d, which gradually decreased thereafter. There was no significant difference between expression levels at P2w, P4w and P8w ($P>.05$; Fig. 6).

3.5. Effects of pregnancy-onset MVAD on protein expression and subcellular localization of RARα, RARβ and NR1

The results of immunofluorescence showed that the protein expression of RARα and NR1 in the MVAD group at P1d were significantly lower than those in the control group and that expression of both was localized mainly in the cytoplasm (Fig. 5).

Fig. 6 shows that the subcellular localization of RARα at the four time points in the MVAD group was similar to control group, but the expression level was lower than control group ($P<.05$, Fig. 6A,C,D). At P1d, a very low expression of RARβ was expressed in the cytoplasm in the MVAD group. At P2w, the expression of RARβ remained low and was located mainly in the nucleus, while there was no cytoplasmic expression in the MVAD group. At P4w and P8w, the expression of RARβ in the MVAD group was higher than that in P1d and P2w, with more localization in the nuclei than in the cytoplasm. There was no significant difference in levels of RARβ compared with that of the control group ($P>.05$, Fig. 6B,E,F). The expression of NR1 in the MVAD group at P1d, P2w and P4w was lower than that in the control group ($P<.05$, Fig. 6G,H).

3.6. Colocalization of RARα and NR1 at P1d in the hippocampus

The fluorescence intensity and location of RARα and NR1 was detected by LSCM (Fig. 5). At P1d, the fluorescence intensities of RARα

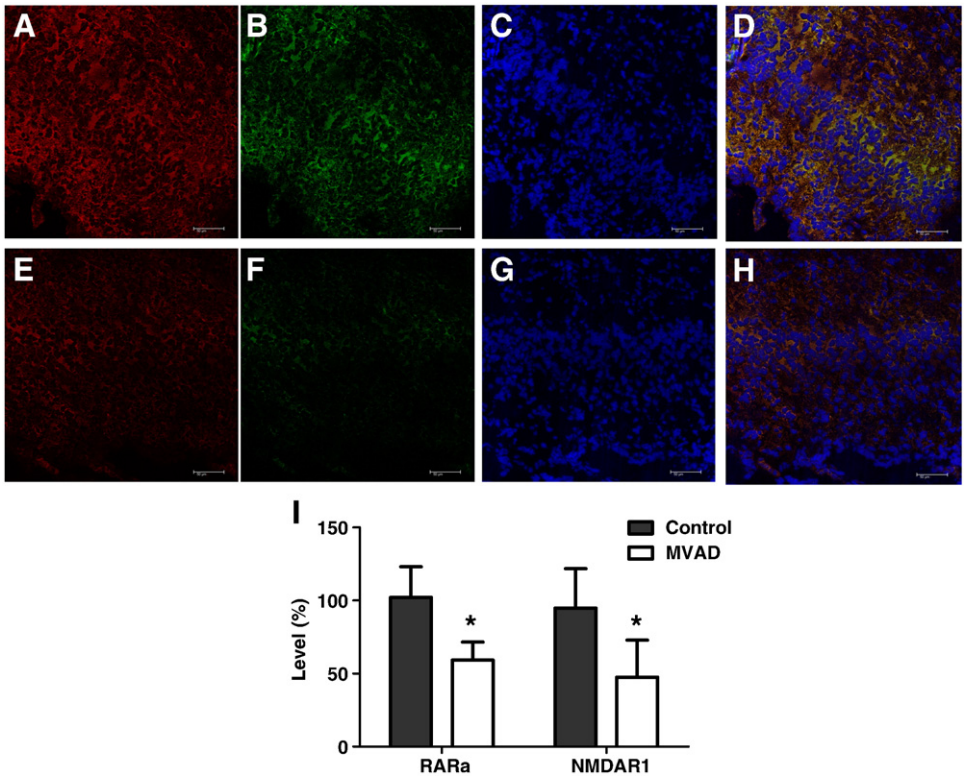

Fig. 5. Immunofluorescent detection of subcellular localization of RARα and NR1 in the hippocampus at P1d by LSCM (original magnification ×400). The scale bars are 50 μm. Red refers to expression of RARα. Green refers to expression of NR1 and blue refers to DAPI. (A–C) Expression of RARα and NR1 in the control group at P1d. (D) Merged panels A, B and C. (E–G) Expression of RARα and NR1 in the MVAD group at P1d. (H) Merged panels E, F and G. From D and H, we can see a mass of RARα and NR1 colocalized in the cytoplasm of cells in the hippocampus at P1d. (I) Quantitative analysis of fluorescence intensity showed that expression of RARα and NR1 in the MVAD group were significantly lower than those in the control group. Data are mean±S.E.M. ($n=6$, *$P<.05$ vs. control).

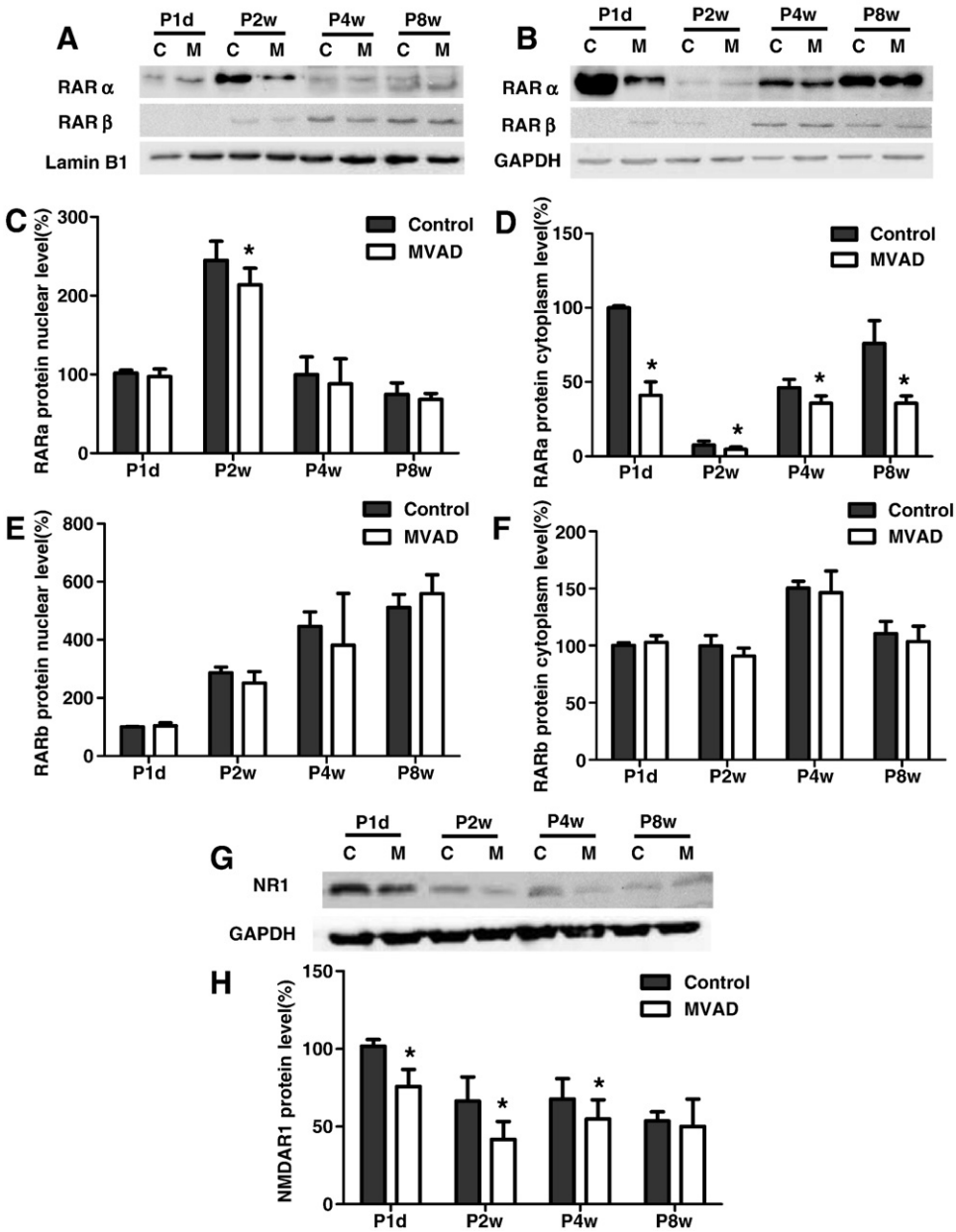

Fig. 6. The protein expressions and subcellular localizations of RARα, RARβ and NR1 in hippocampus by Western blotting at different ages after birth. Data are mean±S.E.M. (n=6, *P<.05 vs. control). (A) Nuclei expressions of RARα and RARβ in the control (C) and MVAD (M) group at four time points. Lamin B1 served as intercontrol. (B) Cytoplasm expressions of RARα and RARβ in the control (C) and MVAD (M) group at four time points. GAPDH served as intercontrol. (C and E) Quantitative analysis showed that nuclei expression of RARα in the MVAD group was lower than that in the control group, especially at P2w, while there was no significant difference of RARβ between the two groups. (D and F) Quantitative analysis showed that cytoplasm expression of RARα in the MVAD group was significantly lower than that in the control group at four time points, while there was no significant difference of RARβ between the two groups. (G) Expression of NR1 in the control (C) and MVAD (M) group at four time points. GAPDH served as intercontrol. (H) Quantitative analysis showed that expression of NR1 in the MVAD group was significantly lower than that in the control group at P1d, P2w and P4w.

and NR1 were very high, and both RARα and NR1 colocalized in neural cell cytoplasm in the hippocampus.

4. Discussion

In the present study, we investigated postnatal mRNA and protein expression of RARs and NMDAR1 and the impact of pregnancy-onset MVAD on the expression of those genes. The results indicate that MVAD beginning from embryonic stage can influence learning and memory in offspring in their adulthood. Pregnancy-onset MVAD can affect mRNA and protein expression of RARα and NR1 in the hippocampus after birth, which may be closely related to learning and memory function in hippocampus and the synaptic plasticity of the calcium signaling pathway.

4.1. Pregnancy-onset MVAD affected learning and memory of offspring at P7w

In the present study, the results of the Morris water maze test show that pregnancy-onset MVAD had a significant effect on spatial learning, short-term memory, long-term memory, and the ability to learn new things in the offspring at P7w. The synapse plays an important role in learning and memory. The period from mid–late embryo development to approximately P4w is critical for hippocampal

development [27], during which synapses undergo rapid proliferation and pruning. A recent study found that hippocampus and memory formation is linked to atRA [19]. The result of serum vitamin A concentration in our study showed that the level of vitamin A was lower in the MVAD group than that in the control for the first 4 weeks after birth, and this may lead to the absence of a critical period of development of hippocampus. However, the reason why pregnancy-onset MVAD affected learning and memory of offspring at P7w remains unclear. Then, we investigate mRNA and protein expression of RARs, which play important roles in RA signaling pathway.

4.2. Pregnancy-onset MVAD primarily affects mRNA and protein expression of RARα in the hippocampus of offspring postnatally

Retinoic acid, especially atRA, plays a key role in brain development and neuronal functional regulation. atRA regulates downstream gene expression by combination of nuclear receptors RARs (α, β, γ) and/or RXRs (α, β, γ) with the target gene response element [28]. Different forms of RARs localize in different regions of the mammalian brain [29,30]. RARα is expressed in the cortex and hippocampus, and RARβ is localized abundantly in the caudate nucleus head/putamen, caudate nucleus and olfactory tubercle, while RARγ is not found in the central nervous system [29]. Zetterström et al. [30] found that RARα is the only RAR that is expressed in the hippocampal CA1–CA3 area of mice and rats and in the rat dentate gyrus granule cells at mRNA and protein levels. It has been confirmed that LTP and LTD were impaired in RARβ or RARβ/RXRγ knockout mice [31], but in another study, RARβ was not detected in the hippocampus at mRNA level [30]. This seems to be contradictory and more studies are needed. Retinoid×receptors are not RA signaling pathway-specific [32,33], and they may play a role in the absence of RA [34]. Therefore, RARs may play a more direct and important role in the RA signaling pathway due to its combination with atRA. In the present study, we focused on the postnatal changes of RARα and RARβ at mRNA and protein levels, as well as the effect of pregnancy-onset MVAD on expression of those genes. The mRNA expression of RARα was relatively high in the first 2 weeks after birth, which is consistent with rapid hippocampal development during this period [35]. The mRNA of RARα decreased significantly from P4w to P8w compared with those of P1d and P2w, which is consistent with our preliminary research. The results suggest a transcriptional active phase of RARα in the hippocampus at an early postnatal stage. With respect to protein level, there was abundant expression of RARα at P1d, which began to decline from P2w. In our previous study, we found that the mRNA level of RARα remained stable in the fetal brain of the rats from E11d to E20d, suggesting that RARα may play an important role in the embryonic and early postnatal developmental period of the hippocampus.

The relationship between atRA and synaptic plasticity in RARβ homozygous mutant mice, in which the spatial learning ability was significantly impaired in the Morris water maze test, has already been reported [31]. In the present study, there was no significant change in mRNA expression of RARβ with postnatal hippocampal development. The expression of RARβ was significantly lower in the MVAD group than that in the control group at P2w and P8w. However, it was rarely detected by immunofluorescence in the hippocampus at four time points after birth. Western blotting results showed there was no significant difference between the MVAD group and the control group. These results suggest that there is differential expression of RARβ at mRNA and protein levels, and there may be posttranscriptional regulation. Krezel et al. [29] found that the expression levels of RARs at the transcriptional and protein levels are not necessarily the same in adult mice. The results of the present study indicate that pregnancy-onset MVAD may principally affect mRNA and protein expression of RARα gene in hippocampus postnatally, while there is no significant effect on expression of RARβ, especially at the protein level.

4.3. Effect of MVAD on expression of NMDAR1 in postnatal hippocampal development

Glutamate is one of the major excitatory neurotransmitters. Glutamate receptors are divided into metabolic receptors and ionotropic receptors. The latter include three types: NMDARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and KA receptors. NMDA receptors are the main regulators of neuronal differentiation, synaptogenesis, excitatory neurotransmission, synaptic plasticity, LTP and LTD in cortical and hippocampal neurons. NMDA receptor also provide a basis for learning and memory [14,15].

NMDAR1 is necessary for the function of NMDA receptors [16]. NR1 mRNA is widely distributed in the whole brain, especially in the hippocampus, cerebral cortex and cerebellum [36]. The expression of NR1 changes dynamically in the critical period after birth. Its expression reaches a peak at 2 to 3 weeks after birth and then reduces gradually to the adult level [37]. In our present study, the mRNA level of NR1 in the control group was significantly elevated from P1d to P2w and then decreased gradually. Franklin et al. [38] reported that, in the developing rat brain, the mRNA level of NR1 increases almost sixfold in the prefrontal cortex and hippocampus from P3d to adulthood (P67d), and the NR1 level increases by nearly threefold from P3d to P15d. This suggests that there is a rapid growth period of NR1 within 2 weeks after birth. The hippocampal NMDA receptor-dependent LTP reaches its peak in the 2 weeks after birth and then decreases gradually with synaptic pruning. At the protein level, there was abundant expression of NR1 at P1d. The expression of NR1 was lower at P2w and P4w than that at P1d. The contradictory expression of mRNA and protein of NR1 at P2w was interesting. It may be due to posttranscriptional or posttranslational modification, and it needs further research. In our study, mRNA and protein levels of NR1 in the MVAD group at P1d, P2w and P4w were significantly lower than that in the control group, suggesting that pregnancy-onset MVAD may affect expression of NR1 at this critical development period. This reduction in NR1 protein expression may lead to a decline in functional NMDA receptors [39], which may be related to learning and memory impairment in adulthood.

4.4. Relationship between RARα and NR1

Numerous *in vitro* experiments have shown that NR1 is one of the target genes of RA signaling pathway. For example, NR1 expression is dramatically increased in RA-induced neural differentiation of human adipose tissue multipotent stromal cells [18]. Beczkowska et al. [17] reported that RA could promote mRNA expression of NR1 in neuroblastoma–glioma NG108-15 cells. The present study found that protein levels of RARα and NR1 were very high at P1d, and they colocalized in the cytoplasm of nerve cells in the hippocampus; this suggests that RARα and NR1 may be related to hippocampal development, especially in the critical period of embryonic and early postnatal development. In addition, a large number of studies have shown that RA signaling pathway is related to some neurodegenerative diseases, such as Alzheimer's disease (AD), schizophrenia and depression. One of the common features of these diseases is impaired learning and memory ability [16,40–42]. Some types of receptors, such as RARα, RARγ, RXRβ and RXRγ, RA metabolism-related proteins and enzymes, such as retinol binding protein, RALDH and CYP26s, in RA signaling pathway are related to pathogenesis of AD-related gene [43,44]. RARs have been used as target genes of treatment for AD [45–47]. Many studies have confirmed that changes in the number and composition of the NMDA receptor subunits have a close relationship with cognitive function damage in AD, schizophrenia and other neurodegenerative diseases [48,49]. From this perspective, investigation about the relationship between RA signaling

pathway and NMDA receptor subunits could provide new clues to the mechanism of NMDA receptor-related cognitive degenerative diseases. Chen and Napolu [26] found that atRA played a role in translation regulation on AMPA receptor subunit GluR1 through RARα. NMDA receptors are important in LTP and LTD, but they also regulate the expression of AMPA receptors [50,51]. Questions remain as to whether atRA regulates the expression of GluR1 directly or indirectly and what the relationship is between RA signaling pathway and NMDA receptors. In further study, we will focus on the relationship between RARα and calcium ions and the relationship between RA signaling pathway and NMDA receptor subunits, with the use of *in vitro* experiments from hippocampal nerve cell culture as well as hippocampal slices.

In conclusion, the present study found that pregnancy-onset MVAD may have an effect on RARα expression during the early postnatal period, as well as on expression of NMDAR1, which is closely related to learning and memory function of hippocampus and calcium signaling pathway in the regulation of synaptic plasticity. In future studies, we will further explore changes in the expression and localization of RARα in the offspring of rats with MVAD and the interaction between RARα-mediated RA and NMDA receptor signaling pathways.

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