

Combined Maternal and Offspring Vitamin D3 Supplementation Ameliorates Autism-Like Behaviors via VDR Pathway Activation, Neuroinflammatory Suppression, and Metabolic Homeostasis Restoration

Qingxi Li^{1,2}, Jing Zhang³, Xiaohaoran Lyu¹, Le Zou¹, Mingyang Yuan¹, Jierui Yan³, Xing Wan¹, Yuting Yang¹, Wenming Xu³, Jiang Xie¹

¹Department of Pediatrics, The Third People's Hospital of Chengdu (Affiliated Hospital of Southwest Jiaotong University), College of Medicine, Southwest Jiaotong University, Chengdu, Sichuan, 610031, People's Republic of China; ²Department of Pediatrics, The Seventh People's Hospital of Chenghua District, Chengdu, Sichuan, People's Republic of China; ³Joint Laboratory of Reproductive Medicine (SCU-CUHK), Key Laboratory of Birth Defects and Related Diseases of Women and Children of Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu, Sichuan, People's Republic of China

Correspondence: Jiang Xie, Department of Pediatrics, Affiliated Hospital of Southwest Jiaotong University, The Third People's Hospital of Chengdu, 82 Qinglong Road, Qingyang District, Chengdu, Sichuan, 61003L, People's Republic of China, Email xiejiang@swjtu.edu.cn; Wenming Xu, Joint Laboratory of Reproductive Medicine (SCU-CUHK), Key Laboratory of Birth Defects and Related Diseases of Women and Children of Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu, Sichuan, People's Republic of China, Email xuwenming@scu.edu.cn

Background: Autism Spectrum Disorder (ASD) is a widespread neurodevelopmental disorder with no approved medications targeting its core symptoms. Based on the “dual-hit” hypothesis combining Maternal Immune Activation (MIA) and Maternal Separation (MS), this study investigated whether combined maternal and offspring vitamin D3 supplementation could ameliorate autism-like behaviors by modulating the VDR pathway, neuroinflammation, and metabolic homeostasis.

Materials and Methods: A “dual-hit” ASD mouse model was established in C57BL/6 offspring by combining maternal immune activation (MIA) with maternal separation (MS). Pregnant mice were randomly allocated into three groups (n = 5 per group): Control, Model (MIA+MS), and Intervention (MIA+MS + high vitamin D diet). Offspring in the intervention group continued to receive vitamin D supplementation post-weaning (from 3 weeks of age) until 8 weeks. Offspring behavioral tests were conducted on postnatal days 42–56, with the litter serving as the unit of analysis. Serum, brain tissue, colon contents, and liver samples were subsequently collected and analyzed using ELISA, Western blot, LC-MS, and immunohistochemistry.

Results: Compared with controls, MIA+MS offspring exhibited significant social deficits, anxiety, and repetitive behaviors. Combined vitamin D supplementation markedly improved social preference (P<0.0001), reduced anxiety (P<0.001), and decreased repetitive behaviors (P < 0.05). It also upregulated VDR expression in the brain (P<0.01), reduced neurotoxic metabolites (indoxyl sulfate, 6-phosphogluconic acid, and kynurenine pathway intermediates), lowered pro-inflammatory cytokines (IL-1β (P<0.001), IL-6 (P<0.001), TNF-α (P<0.001)), and restored carnitine metabolic homeostasis by modulating TMLHE expression and function.

Conclusion: Combined maternal and offspring vitamin D3 supplementation significantly improves autism-like behaviors in a “dual-hit” ASD model. The observed protective effects may involve activation of the VDR pathway, reduction of neuroinflammation and neurotoxic metabolites, and systemic restoration of immune and metabolic homeostasis. These findings suggest the potential utility of vitamin D in ASD prevention and intervention, warranting further investigation.

Keywords: autism spectrum disorder, vitamin D, VDR, TMLHE

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by significant clinical heterogeneity. Its diagnosis is primarily based on two clusters of core symptoms: deficits in social interaction, and the presence of stereotyped, repetitive patterns of behavior, interests, or activities. The continuous rise in ASD prevalence worldwide has made it a serious public health concern.¹ It is widely accepted that ASD results from complex interactions between genetic susceptibility and environmental risk factors, such as prenatal infection, nutritional deficiencies, and early-life stress.^{2,3} In experimental models, maternal immune activation (MIA) during pregnancy can effectively induce autism-like behaviors and neuroinflammation in offspring, and is considered one of the core environmental risk factors.⁴ Specifically, MIA leads to fetal exposure to high levels of cytokines that promote inflammation, which programmatically alter the development of the fetal immune system, resulting in dysregulated immune responses after birth that are characterized by a transition to a pro-inflammatory phenotype. Concurrently, the development of gut-associated lymphoid tissues and the enteric nervous system may also be impaired. After birth, the intestinal immune system of the offspring remains in a “hypersensitive” state, prone to excessive inflammatory reactions in response to normal flora or mild stimuli.⁵ Meanwhile, postnatal maternal separation (MS), as a significant early-life stressor, leads to persistently elevated levels of glucocorticoids in the offspring. Prolonged high levels of glucocorticoids can suppress protective immune responses and disrupt intestinal immune homeostasis.^{6–8} Consequently, impairment of the intestinal barrier allows harmful microbial metabolites to translocate across the intestinal mucosa into the bloodstream, thereby activating the systemic immune system and leading to chronic low-grade inflammation. These inflammatory signals can be transmitted to the brain via circulation or the vagus nerve, triggering neuroinflammation, which in turn affects neural development and behavior. The resulting central stress and inflammatory responses can, in turn, feedback to the gut through neural and endocrine pathways, further exacerbating intestinal dysfunction and forming a self-reinforcing vicious cycle.⁹

Notably, early life represents a dynamic and vulnerable window during which postnatal stressors such as maternal separation (MS) may act as a “second hit”, synergizing with MIA to exacerbate neurodevelopmental abnormalities—a concept known as the “multiple-hit” hypothesis.¹⁰ However, effective intervention strategies for such “dual-hit” (MIA + MS) models and their systemic mechanisms of action remain poorly understood.

Epidemiological and clinical research has demonstrated a strong association between maternal vitamin D (VD) insufficiency throughout gestation and an elevated likelihood of Autism Spectrum Disorder (ASD) in the offspring, drawing increasing focus to VD’s potential neuroprotective functions.^{11,12} The neurosteroidal properties of 1,25-dihydroxyvitamin D₃, underlie its non-classical actions. Its binding to the ubiquitously expressed vitamin D receptor (VDR) in the central nervous system enables the regulation of gene transcription, which is pivotal for neurodevelopment, synaptic plasticity, immune regulation, and the management of oxidative stress.^{13,14} Notably, vitamin D exerts immunomodulatory and anti-inflammatory effects and helps maintain intestinal barrier integrity, thereby indirectly fostering a healthy gut environment. As a result, VD supplementation not only alleviates intestinal symptoms but also lays a solid foundation for improving autism spectrum disorder (ASD)-like behavioral phenotypes.^{15,16}

Based on this evidence, VD supplementation holds promise for the treatment of ASD. However, existing studies have largely focused solely on either prenatal supplementation or postnatal supplementation to the offspring. The efficacy of a “combined maternal and offspring supplementation” regimen—spanning pregnancy, lactation, the juvenile period, and adolescence—in mitigating the effects of a “dual-hit” challenge, along with its holistic impact on cerebral VDR signaling, neuroimmune function, and peripheral metabolic networks, remains inadequately explored and lacks systematic empirical investigation.

Therefore, this study attempts to utilize a mouse model of autism-like behaviors induced by MIA combined with MS to investigate the behavioral improvements resulting from combined maternal and offspring vitamin D₃ supplementation. In addition, based on previously published findings from our research group, dietary supplementation containing 5000 IU/kg of vitamin D has been shown to ameliorate aberrant immune status in the offspring following MIA.¹⁷ Furthermore, we seek to elucidate the underlying mechanisms at molecular and systemic levels, including: 1) the expression and activation of VDR protein in offspring brain tissue; 2) levels of neurotoxic substances and peripheral inflammatory cytokines; and 3) changes in the amino acid metabolic profile in intestinal contents and the carnitine metabolic profile in

blood. This research aims to provide new experimental evidence clarifying the pleiotropic protective effects of VD in the nervous system and try to establish a compelling theoretical basis for translating VD supplementation strategies into early interventions for ASD.

Materials and Methods

Experimental Animals

The mice housing was maintained at 22 ± 1 °C with a 12 h/12 h light/dark cycle. Specific pathogen-free (SPF) C57BL/6J mice (6–8 weeks old, 20–25 g) were acclimatized with free access to food and water.

Autism Model Establishment (MIA + MS Dual-Hit)

Maternal Immune Activation (MIA)

Preparation of poly(I:C): Polyinosinic-polycytidylic acid (Sigma, P1350) was reconstituted in sterile phosphate-buffered saline (PBS) to a final concentration of 1 µg/µL and prepared freshly before use. Administration: On gestational day (GD) 12.5, dams in the model and experimental groups received an intraperitoneal injection of poly(I:C) solution at a dose of 20 µL/g (equivalent to 20 mg/kg);¹⁸ Control group dams were administered sterile PBS (pH 7.4) at an equivalent volume. Maternal body temperature and activity were monitored for 48 hours post-injection.

Maternal Separation (MS)

From postnatal day (PND) 2, pups in the model and experimental groups were separated from their dams and individually placed in clean cages for 3 hours daily during random time intervals.^{19,20} Dams remained in the home cage. This procedure continued for two weeks until PND 15. Pups in the control group remained with their dams throughout the experiment.

Experimental Grouping and Intervention Scheme

Group design: Pregnancy-confirmed female mice were randomly to three groups (n = 5 per group): Control group: PBS injection + AIN-93G diet; Model group: poly(I:C) injection + AIN-93G diet; Experimental group: poly(I:C) injection + high VD diet (vitamin D 5000 IU/kg).¹⁷ From each litter, 2–3 male offspring were randomly selected for subsequent behavioral testing. All subsequent data analyses were performed with the litter as the statistical unit. Male offspring were selected based on two key considerations: (1) the well-documented male predominance in ASD diagnosis in humans;²¹ (2) the more pronounced and reproducible autism-like behavioral phenotypes consistently reported in male rodent models.

Intervention timeline: Dams: The experimental group received the high VD diet from the day of plug confirmation until weaning (PND 21). Offspring: The experimental group continued on the high VD diet after weaning until PND 56 (8 weeks of age).

Behavioral Assessments (PND 42–56)

All tests were conducted in a quiet environment (illuminance: 40 lux) with intervals of ≥ 48 hours to minimize carry-over effects.

Three-Chamber Social Test

To determine the levels of sociability and social novelty preference, the classic three-chamber test was employed. The apparatus was a transparent, partitioned rectangular box (60 × 40 × 22 cm) with connecting doorways (5 × 8 cm). Wire cages placed in the side chambers served to confine stranger mice. After 30 minutes of habituation to the test room, the subject mouse underwent a three-phase protocol: 1) Habituation: A 5-minute free exploration of all chambers, each containing an identical object. 2) Sociability: A 10-minute session allowing the mouse to choose between a novel mouse (Stranger 1) and an inanimate object, with exploratory behavior quantified. 3) Social Novelty: A final 10-minute session presenting a choice between the now-familiar Stranger 1 and a new stranger mouse (Stranger 2). Testing occurred in a quiet setting, and the apparatus was decontaminated with 75% ethanol following each trial.

Elevated Plus Maze

The elevated plus maze was used to assess anxiety-like behavior. The apparatus, elevated 50 cm, featured a central platform (10 × 10 cm) from which two open arms and two enclosed arms (each 30×6 cm; wall height 15 cm) extended. Following a 30-minute acclimation to the testing room, individual mice were positioned on the central platform was aligned with an open arm and granted a 10-minute free exploration period. The duration, distance, and frequency of entered the open and closed arms were recorded. The apparatus was thoroughly swept with 75% ethanol after each trial.

Open Field Test

The open field test was conducted to evaluate anxiety-like behavior and locomotor activity. Mice were placed in a gray open-top arena (40 × 40×40 cm) under dim light. After 30 min of acclimation to the room, each mouse was set gently in the arena center and a 10-minute free exploration session was allowed. The floor was virtually divided into 16 equal squares, with the central 4 squares defined as the center zone. Total distance traveled, time spent in the center zone, and locomotor patterns were captured and processed with tracking software. The arena was sanitized with 75% ethanol between trials.

Marble Burying Test

The marble burying test was employed to assess repetitive and stereotyped behaviors. In this assay, a clean cage (40 × 30×22 cm) was prepared with a 5-cm deep layer of fresh corncob bedding. Twenty black glass marbles (diameter: 14–15 mm) were then arranged into a 4×5 grid on the leveled surface. An individual mouse was introduced into a corner of the cage and given a 30-minute exploration period. A marble was scored as buried if at least two-thirds of its volume was submerged under the bedding material. The final count of buried marbles was documented, with an increased count reflecting elevated repetitive digging behavior. Following each trial, the used bedding was replaced, and all marbles were disinfected with 75% ethanol.

Metabolite Detection in Mouse Brain Tissue

Sample Collection: Following behavioral testing, offspring mice were returned to the housing facility for at least 48 hours. At 8 weeks of age, mice were sacrificed by cervical dislocation. Brain tissues were harvested, snap-frozen in liquid nitrogen, and maintained at −80°C. Metabolites in the samples were analyzed using liquid chromatography-mass spectrometry (LC-MS). Brain Tissue Metabolomics Analysis was performed on a Vanquish UHPLC/Q Exactive™ HF system (Thermo Fisher). A pooled QC sample was prepared from equal volumes of all samples. Metabolites were identified at three levels: Level 1 (confirmed by matching MS1, MS2, and retention time to reference standards), Level 2 (putatively annotated by matching MS1 and MS2 spectra), and Level 3 (tentatively assigned by MS1 match only). Differential metabolites were screened by VIP >1.0, FC >1.2 or < 0.833, and p-value < 0.05.

Metabolite Detection in Colonic Content

Sample Collection: After behavioral tests, offspring mice were returned to the housing facility for ≥48 hours. At 8 weeks of age, mice were euthanized by cervical dislocation. Colonic contents were aseptically collected between 9:00 AM and 12:00 PM, snap-frozen in liquid nitrogen, and stored at −80°C. Metabolites were detected using high-resolution LC-MS/MS. Colonic Content Metabolomics Analysis was performed on a Vanquish Flex/QE-HF-X system (Thermo). QC was prepared by pooling equal supernatant volumes from extracted samples. Metabolites were identified at Level 2 (putative annotation based on MS1 and MS2 matches). Differential metabolites were selected with p-value <0.05, VIP >1.0, and FC >1.0.

Western Blot Analysis of Protein Expression

Sample Collection and Processing: Upon completion of behavioral assessments, offspring mice were maintained for a minimum of 48 hours prior to tissue collection. At 8 weeks of age, the mice were euthanized via cervical dislocation.

Brain and liver tissues were aseptically harvested, immediately snap-frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

Western Blotting Procedure: Tissue lysates were prepared using RIPA buffer containing a protease inhibitor cocktail (Beyotime, Beijing, China). The extracted proteins were resolved by SDS-PAGE on 10–12% gels and subsequently electrophoretically transferred to PVDF membranes (ISEQ10100, Millipore). Following blocking with a rapid blocking buffer (G2052-500ML, Servicebio), the membranes were probed with primary antibodies at 4°C overnight. The antibodies used were: anti-VDR (1:6000, 67192-1-Ig, Proteintech), anti-TMLHE (1:500, 16621-1-AP, Proteintech), and anti-GAPDH (1:5000, 60004-1-Ig, Proteintech). After extensive washing with TBST, the membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies: goat anti-rabbit IgG (1:5000, SA00001-2, Proteintech) and goat anti-mouse IgG (1:5000, SA00001-1, Proteintech). Protein bands were visualized using an ECL detection system (E-Blot, Touch Imager S), and the band intensities were quantified with ImageJ software, with GAPDH serving as the loading control.

Immunohistochemistry (IHC)

After induction of anesthesia using 10% pentobarbital (10 mL/kg), mice underwent perfusion with PBS for 6 minutes followed by 4% PFA. The collected brain tissues were then processed through post-fixation in 4% PFA for 72 h, standard dehydration, paraffin embedding, and were finally sectioned at $5\ \mu\text{m}$. Following deparaffinization and rehydration through a graded alcohol series, sections underwent antigen retrieval via microwave heating in citrate buffer (20 min). After cooling and washing, endogenous peroxidases were blocked with 3% H_2O_2 (25 min, RT). Non-specific sites were blocked with BSA, followed by incubation with the primary antibody at 4°C overnight. After subsequent washes, sections were incubated with an HRP-conjugated secondary antibody (37°C , 30 min). Signal development was performed using DAB, followed by hematoxylin counterstaining, dehydration, and mounting with neutral resin. Finally, images were captured using a confocal microscope (BA400Digital).

Serum Analysis

Serum levels of vitamin D (25(OH)D, ng/mL), inflammatory cytokines (IL- 1β , IL-6, TNF- α , pg/mL), and L-carnitine were measured using commercial ELISA kits according to the manufacturers' instructions. Each assay used 10 μL of serum.

Histopathological Examination

All the offspring mice were sacrificed at the end of the behavioral test, and their liver and kidney tissues were excised to assess the safety of VD. Mice were anesthetized and perfused as described in IHC. For histological analysis, liver and kidney tissues were fixed, embedded in paraffin, sectioned, and stained with H&E. After dehydration and clearing, sections were mounted with neutral resin. Images were acquired using a microscopy imaging system.

Data Processing

Data analysis and visualization were performed in GraphPad Prism 9.0. Quantitative results are expressed as mean $\pm$ SD. Differences between two groups were evaluated by an unpaired *t*-test. For comparisons more than two groups, one-way ANOVA with Bonferroni's post-hoc correction was applied. Two-way ANOVA was applied in the behavioral analysis of social ability and social novelty. A *p*-value < 0.05 was considered statistically significant.

Results

Animal Model Validation

Mice subjected to the combined “dual-hit” of maternal immune activation (MIA) and maternal separation (MS) exhibited autism spectrum disorder (ASD)-like behaviors. To investigate whether prenatal poly(I:C)-induced MIA and early-life adversity caused by postnatal MS led to neurobehavioral abnormalities and ASD-like behavioral deficits in offspring, we assessed core autism-like symptoms—including social ability, social novelty preference, and stereotyped behavior—in

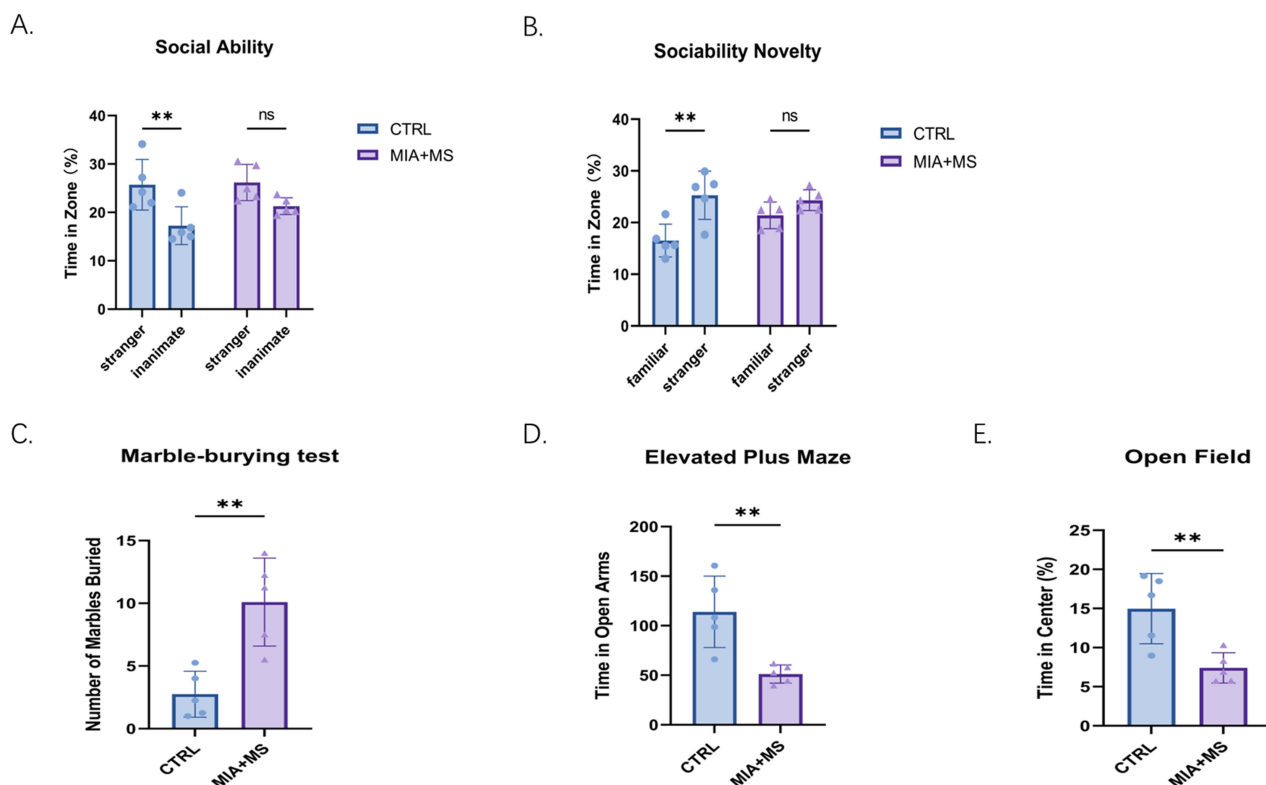


Figure 1 (A) Sociability test, showing the proportion of time spent by the test mouse in the chamber containing a stranger mouse (social stimulus) versus the chamber with an inanimate object in both the control (CTRL) ($n = 5$ litters, 2–3 mice per litter) and MIA+MS ($n = 5$ litters, 2–3 mice per litter); (B) Social novelty test, illustrates the proportion of time spent by the test mouse in the chamber with a novel stranger mouse versus the chamber with the familiar mouse in the CTRL ($n = 5$ litters, 2–3 mice per litter) and MIA+MS ($n = 5$ litters, 2–3 mice per litter); (C) Marble-burying test, indicates the number of marbles buried by mice in the CTRL ($n = 5$ litters, 2–3 mice per litter) and MIA+MS ($n = 5$ litters, 2–3 mice per litter); (D) Elevated plus maze test, represents the time spent in the open arms by mice in the CTRL ($n = 5$ litters, 2–3 mice per litter) and MIA+MS ($n = 5$ litters, 2–3 mice per litter); (E) Open field test, displays the proportion of time spent in the center zone of the arena by mice in the CTRL ($n = 5$ litters, 2–3 mice per litter) and MIA+MS ($n = 5$ litters, 2–3 mice per litter). (Proportion of time spent = time spent in a specific zone/total test time $\times 100\%$; Data are presented as mean $\pm$ SD; ** $P < 0.01$; ns, not significant.).

the model group. Social ability and social novelty were evaluated using the three-chamber social test. Compared with the control (CTRL) group, offspring in the MIA+MS group showed significant deficits in social behavior (Figure 1A and B). During the sociability phase, CTRL mice displayed a clear preference for the social stimulus (stranger mouse) over the non-social object, indicating normal social ability. In contrast, MIA+MS mice showed no significant preference between the stranger and the inanimate object, demonstrating impaired sociability. In the social novelty phase, CTRL mice preferred the novel stranger over the familiar mouse, indicating intact social novelty recognition. MIA+MS mice, however, exhibited no significant preference between the familiar and novel mouse, suggesting a deficit in social novelty. Stereotyped behavior was assessed using the marble-burying test. As shown in Figure 1C, MIA+MS mice buried significantly more marbles than CTRL mice, indicating increased repetitive and stereotyped behaviors. To determine whether the model mice also exhibited anxiety- and depression-like behaviors, we performed the elevated plus maze and open field tests. The results indicated that MIA+MS mice displayed significant anxiety- and depression-like phenotypes (Figure 1D and E).

Combined Maternal and Offspring Vitamin D3 Supplementation Ameliorates Autism-Like Behaviors in Offspring

To evaluate the interventional effect of combined maternal and offspring vitamin D3 supplementation on offspring ASD-like phenotypes, pregnancy-confirmed dams were randomly assigned to three groups ($n = 5$ per group): Control group: PBS injection + AIN-93G diet; Model group (MIA+MS): poly(I:C) injection + maternal separation (14 days) + AIN-93G

diet; Experimental group (MIA+MS-VD): poly(I:C) injection + maternal separation (14 days) + high VD diet (vitamin D₃, 5000 IU/kg). The VD intervention protocol was as follows: Dams in the experimental group received the high VD diet from the day of vaginal plug detection until offspring weaning (postnatal day [PND] 21). Offspring in the experimental group continued on the high VD diet after weaning until PND 56 (8 weeks of age). Maternal separation was performed from PND 2 to PND 15 (14 days in total).

Behavioral tests were conducted on offspring between PND 42 and PND 56 to assess the potential amelioration of core autism-like symptoms and anxiety/depression-like behaviors. As shown in Figure 2A and B, in the three-chamber sociability test, offspring in the MIA+MS-VD group showed a significant preference for the social stimulus (stranger mouse) over the non-social object (inanimate), indicating that VD intervention restored normal sociability impaired by the “dual-hit” (MIA+MS). Figure 2C demonstrates that in the social novelty test, the MIA+MS-VD group spent significantly more time interacting with the novel stranger mouse than with the familiar mouse, confirming that VD supplementation rescued the social novelty preference deficit induced by MIA+MS. In the marble-burying test (Figure 2D), the MIA+MS-VD group buried significantly fewer marbles compared to the MIA+MS model group, indicating a reduction in repetitive and stereotyped behaviors following VD intervention. Furthermore, in the elevated plus maze test (Figure 2E and F), the MIA+MS-VD group spent notably more time in the open arms than the MIA+MS model group, suggesting an alleviation of anxiety-like behavior. Similarly, in the open field test (Figure 2G and H), the MIA+MS-VD group exhibited a significantly higher proportion of time spent in the center zone compared to the MIA+MS model group, further supporting the anxiolytic effect of VD supplementation.

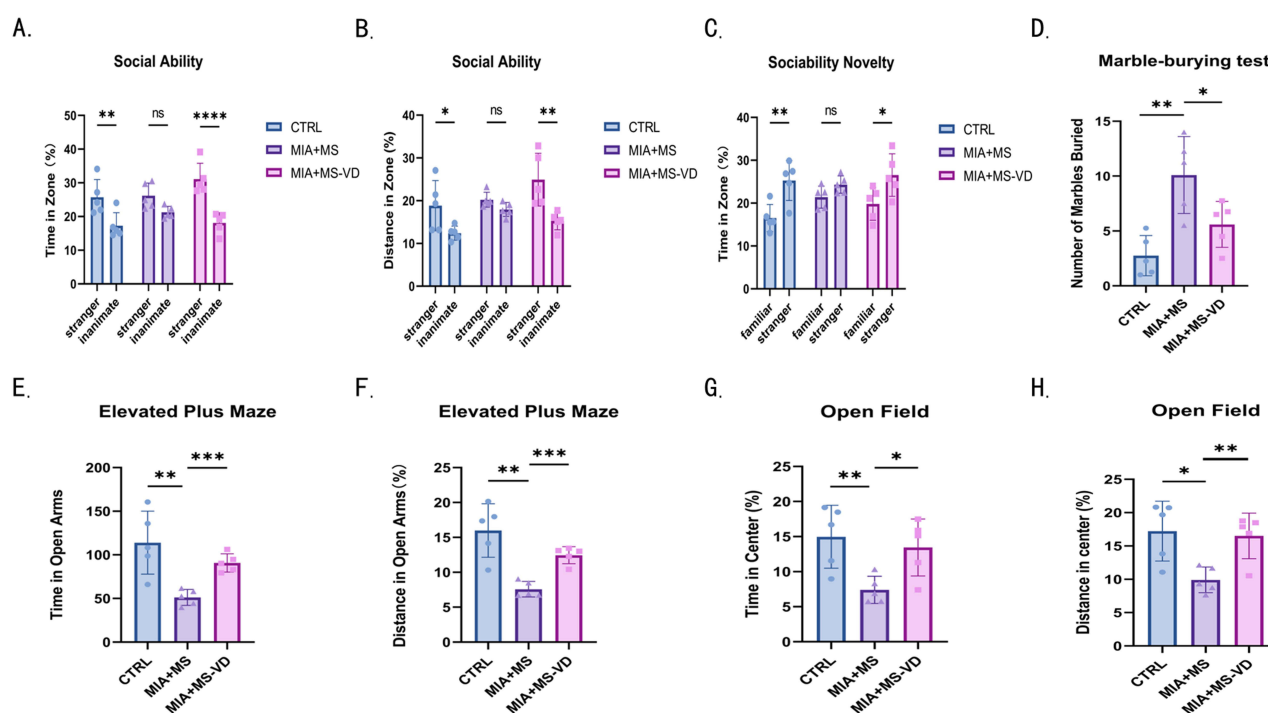


Figure 2 (A) Sociability test: Proportion of time spent in the chamber containing a stranger mouse (social stimulus) vs the chamber with an inanimate object by offspring in the CTRL (n = 5 litters, 2–3 mice per litter), MIA+MS (n = 5 litters, 2–3 mice per litter), and MIA+MS-VD (n = 5 litters, 2–3 mice per litter). (B) Sociability test: Proportion of distance traveled in the stranger vs inanimate object chambers across the CTRL (n = 5 litters, 2–3 mice per litter), MIA+MS (n = 5 litters, 2–3 mice per litter), and MIA+MS-VD (n = 5 litters, 2–3 mice per litter). (C) Social novelty test: Proportion of time spent in the chamber with a novel stranger mouse vs the familiar mouse across the CTRL (n = 5 litters, 2–3 mice per litter), MIA+MS (n = 5 litters, 2–3 mice per litter), and MIA+MS-VD (n = 5 litters, 2–3 mice per litter). (D) Marble-burying test: Number of marbles buried by mice in the CTRL (n = 5 litters, 2–3 mice per litter), MIA+MS (n = 5 litters, 2–3 mice per litter), and MIA+MS-VD (n = 5 litters, 2–3 mice per litter). (E and F) Elevated plus maze test: Time spent in the open arms (E) and proportion of distance traveled in the open arms (F) among the three groups. CTRL (n = 5 litters, 2–3 mice per litter), MIA+MS (n = 5 litters, 2–3 mice per litter), and MIA+MS-VD (n = 5 litters, 2–3 mice per litter). (G and H) Open field test: Proportion of time spent (G) and proportion of distance traveled (H) in the center zone by mice in the CTRL (n = 5 litters, 2–3 mice per litter), MIA+MS (n = 5 litters, 2–3 mice per litter), and MIA+MS-VD (n = 5 litters, 2–3 mice per litter). (Proportion of time spent = time in a specific zone/total test time × 100%; Proportion of distance traveled = distance in a specific zone/total distance traveled × 100%. Data are presented as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, not significant).

“Dual-Hit” Challenge Upregulates VDR Protein Expression in Offspring Brain, and Combined Maternal-Offspring VD Supplementation Further Enhances VDR Levels via Ligand-Induced Activation

VD and VDR Function: Vitamin D (VD) and its receptor (VDR) play roles far beyond the regulation of calcium and phosphorus metabolism; they act as crucial neurosteroids in the brain. VDR is widely distributed in multiple brain regions, and involved in modulating neurodevelopment, immune inflammation, energy homeostasis, and mitochondrial function.^{14,22,23}

Regulation of VDR: VDR expression is dynamically regulated by extracellular and intracellular cues, and can be modulated by inflammatory stimuli and ligand exposure. Upregulation of VDR in response to inflammatory challenge represents a compensatory or reactive mechanism—wherein the brain increases the availability of VDR as a preparatory “tool” to mount anti-inflammatory programs when needed.^{24,25} Conversely, high-dose vitamin D intake can induce a self-amplifying, ligand-guided upregulation of VDR. High levels of VD ligand not only activate existing VDR but also promote further receptor synthesis, thereby amplifying downstream biological signaling. Specifically, 1,25-(OH)₂D binds to VDR, facilitating the formation of a VDR/RXR heterodimer. This complex translocates to the nucleus, where it not regulates target genes (eg, the antimicrobial peptide CAMP) but also binds directly to vitamin D response elements (VDREs) within the promoter region of the VDR gene itself. This binding initiates transcription of VDR, resulting in increased VDR protein synthesis—a positive feedback loop that profoundly enhances transcriptional activity and biological effects such as immune regulation and neuroprotection.^{26,27}

To explore the potential mechanism underlying these behavioral improvements, we thus measured serum vitamin D levels using ELISA. Compared with the CTRL group, the MIA+MS group showed notably reduced serum VD levels, which were significantly elevated after combined maternal and offspring VD supplementation (Figure 3A). Additionally,

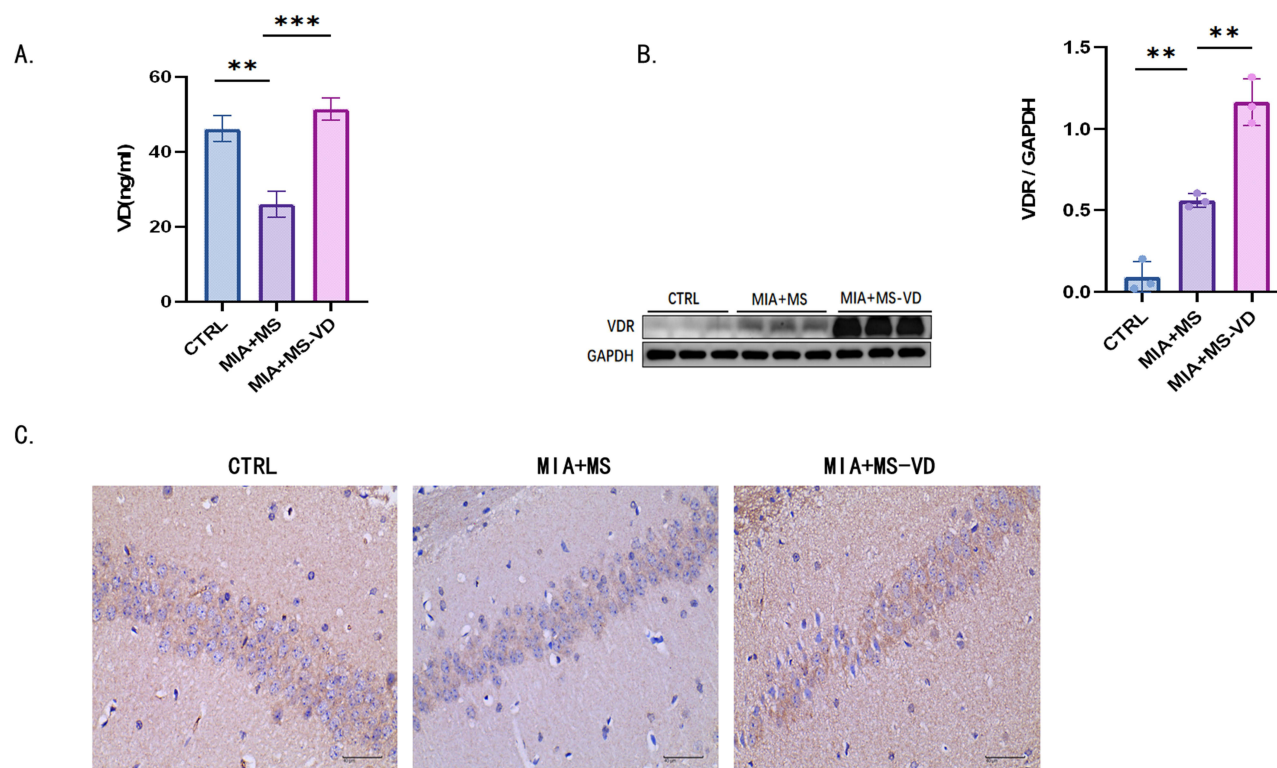


Figure 3 (A) Serum vitamin D levels in offspring mice measured by ELISA. Compared to the CTRL group, the MIA+MS group showed significantly reduced serum VD levels. Combined maternal and offspring VD supplementation significantly increased serum VD concentration. (B) Western blot analysis of VDR protein expression in brain tissue. VDR protein levels exhibited an increasing trend across the CTRL, MIA+MS, and MIA+MS-VD groups. (C) Immunohistochemical staining of VDR in paraffin-embedded sections of the hippocampal region. The percentage of VDR-positive area showed an increasing trend among the CTRL, MIA+MS, and MIA+MS-VD groups. Scale bar = 40 μm. (Data are presented as mean ± SD; **P < 0.01, ***P < 0.001; ns, not significant.).

as demonstrated in the [supplementary data](#), increased serum vitamin D levels did not induce any hepatic or renal injury in the offspring ([Figure S1](#)). Furthermore, Western blot analysis of offspring brain tissue revealed that VDR protein expression was increased in the MIA+MS model group compared to the CTRL group. Notably, combined maternal-offspring VD supplementation further enhanced VDR protein levels in the brain ([Figure 3B](#)). Consistently, immunohistochemical staining of the hippocampal region demonstrated higher VDR expression in the MIA+MS group relative to controls, and this expression was further elevated following VD supplementation ([Figure 3C](#)).

Dual-Hit” Challenge Increases Neurotoxic Metabolites in Offspring Brain, and Combined Maternal-Offspring VD Supplementation Attenuates Their Levels

Patients with ASD often exhibit gut microbiota dysbiosis. Increased activity of certain microbiota can shift tryptophan metabolism toward the indole pathway, leading to elevated production of indoxyl sulfate.^{28,29} This metabolite exhibits neurotoxicity, induces oxidative stress and inflammatory responses, and may compromise blood-brain barrier integrity, thereby adversely affecting neurodevelopment and function.^{30,31} 6-Phosphogluconic acid is an intermediate in the pentose phosphate pathway, which is crucial for generating NADPH—a key molecule for cellular defense against oxidative stress. Abnormal levels of 6-phosphogluconic acid may reflect compensatory activation of this pathway in response to increased oxidative stress; conversely, impaired pathway function may lead to insufficient NADPH production, exacerbating oxidative damage to the nervous system.³² Dysregulated tryptophan metabolism is a notable feature of ASD. Pro-inflammatory cytokines can induce indoleamine 2,3-dioxygenase (IDO), diverting tryptophan catabolism toward the kynurenine pathway. This disrupts the balance of neuroactive metabolites, which can alter NMDA receptor activity, generate oxidative stress, and cause neurotoxicity. Altered levels of (R)-N-formyl-beta-hydroxy-L-kynurenine, an intermediate of this pathway, reflect such dysregulation.³³ In this study, compared to the CTRL group, metabolomic analysis of brain tissue revealed elevated levels of indoxyl sulfate, 6-phosphogluconic acid, and (R)-N-formyl-beta-hydroxy-L-kynurenine in the MIA+MS group. Notably, combined maternal and offspring VD supplementation reversed this trend, leading to a significant reduction in the levels of these neurotoxic metabolites ([Figure 4](#)).

“Double Hit” Alters Offspring Inflammatory Cytokine Levels in Blood and the Content of Certain Amino Acid-Related Substances in Intestinal Contents

Consistent with the known effects of MIA and MS on systemic inflammation,^{5–9} we found that the MIA+MS group exhibited significantly elevated serum levels of pro-inflammatory mediators (IL-1 β , IL-6, TNF- α). This heightened inflammatory state was markedly reduced by combined maternal and offspring vitamin D (VD) supplementation ([Figure 5A](#)), which aligns with VD’s documented immunoregulatory and anti-inflammatory properties and its efficacy in reducing systemic inflammation.^{15,34} Furthermore, metabolomic analysis of intestinal contents revealed that the same MIA+MS intervention significantly increased the levels of specific amino acid-related metabolites. The levels of these metabolites were also significantly normalized by VD supplementation ([Figure 5B](#)). This metabolic amelioration is likely linked to VD’s crucial role in maintaining intestinal barrier integrity,³³ demonstrating that VD supplementation effectively counteracts both the systemic inflammatory and gut metabolic disruptions induced by MIA and MS ([Figure 5B](#)).

Dual-Hit” Challenge Disrupts Carnitine Metabolism in Offspring, and Combined Maternal-Offspring VD Supplementation Restores Metabolic Homeostasis

Interestingly, in the ASD mouse model induced by MIA combined with MS, we found a marked decrease in the levels of two medium-chain acylcarnitines (MCACs), (2E,5Z,7E)-decatrienoylcarnitine and (3,8)-decadienoylcarnitine, in brain tissue ([Figure 4](#)), along with an increase in hydroxytrimethyllysine (HTML) in intestinal contents ([Figure 5B](#)). Consistently, serum L-carnitine also showed a decreasing trend ([Figure 6A](#)), indicating a systemic carnitine deficiency. Metabolomic analysis revealed an abnormal accumulation of HTML—a precursor in carnitine biosynthesis—in intestinal contents, suggesting a potential blockade in the carnitine synthesis pathway. Following vitamin D (VD) intervention, HTML levels were significantly reduced, and serum L-carnitine was markedly increased. Given that the liver is the

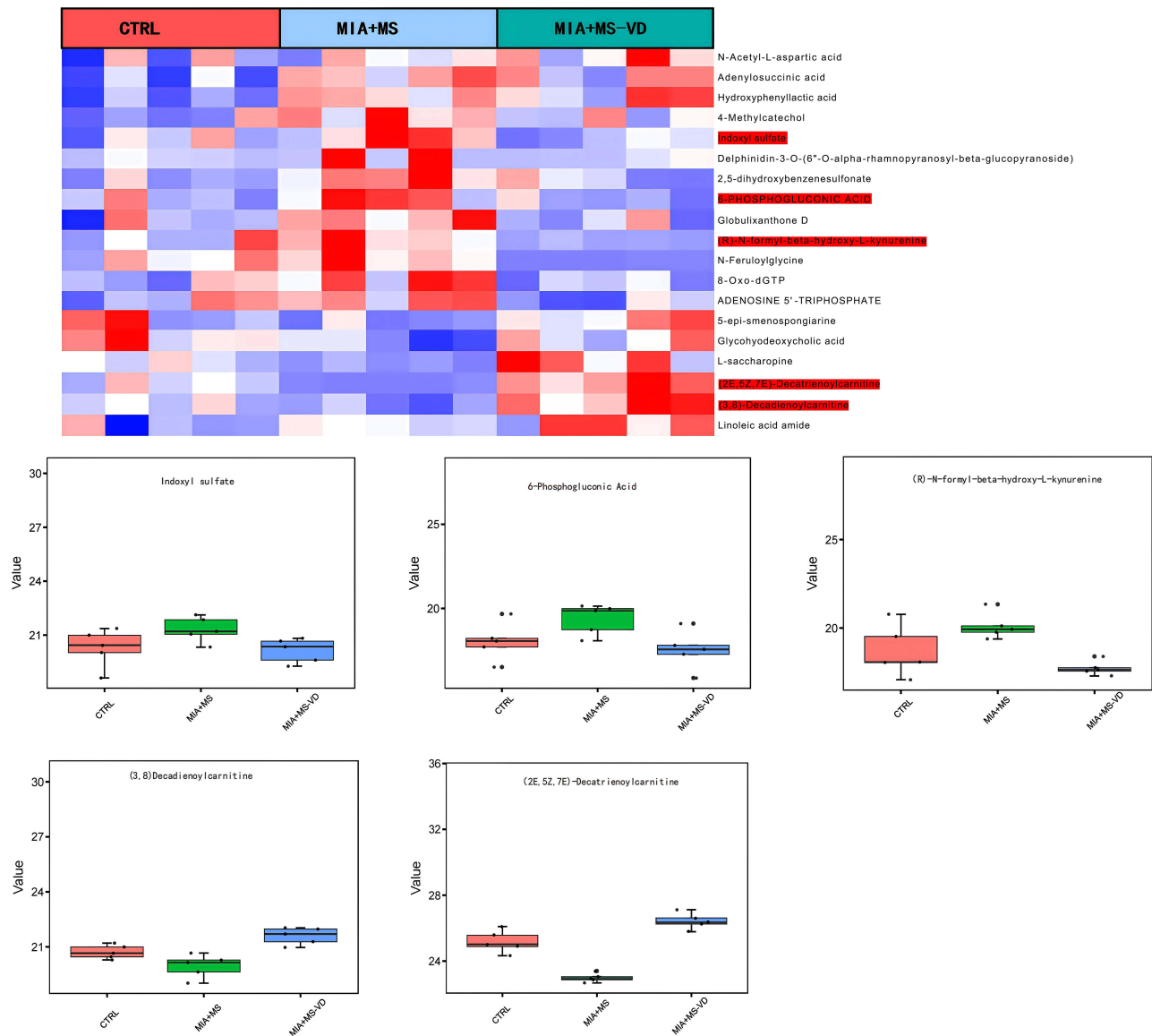


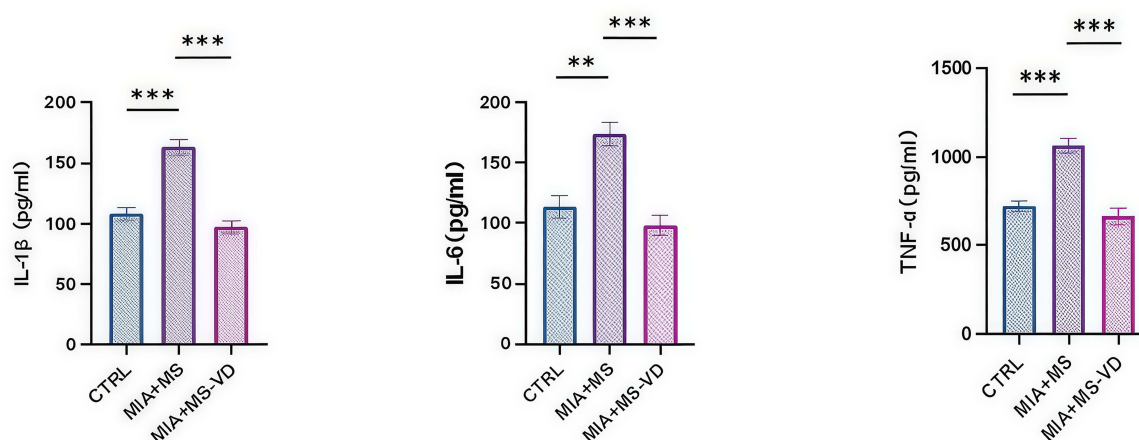
Figure 4 Metabolite profiling in brain tissue. As indicated by the red marks in the heatmap, the levels of the following substances—indoxyl sulfate, 6-phosphogluconic acid, (R)-N-formyl-beta-hydroxy-L-kynurenine, (2E,5Z,7E)-decatrienoylcarnitine, and (3,8)-decadienoylcarnitine—exhibited the following intergroup differences: Compared to the CTRL group, the MIA+MS group showed increased levels of the neurotoxic metabolites indoxyl sulfate, 6-phosphogluconic acid, and (R)-N-formyl-beta-hydroxy-L-kynurenine. Combined maternal and offspring VD supplementation reduced their concentrations. In contrast, the levels of two acylcarnitine species—(2E,5Z,7E)-decatrienoylcarnitine and (3,8)-decadienoylcarnitine—were decreased in the MIA+MS group relative to controls, and were restored following VD supplementation.

primary site of endogenous carnitine synthesis, we further examined the expression of trimethyllysine hydroxylase (TMLHE), the rate-limiting enzyme in this pathway. Western blot and immunohistochemical staining results showed that TMLHE protein levels were significantly elevated in the liver of MIA+MS mice. However, after VD intervention, TMLHE expression was significantly downregulated, even below normal levels (Figure 6B and C).

Discussion

The robust systemic inflammation and oxidative stress induced by the MIA+MS model likely disrupt the hepatic metabolic microenvironment (eg, depleting essential cofactors or generating inhibitory molecules), leading to significantly inhibited TMLHE enzyme activity despite increased protein expression.³⁵ We propose that a plausible mechanism underlying these findings may involve the compensatory upregulation and functional inactivation of TMLHE. As the key enzyme catalyzing the first step of carnitine synthesis—converting trimethyllysine (TML) to HTML—TMLHE protein

A.



B.

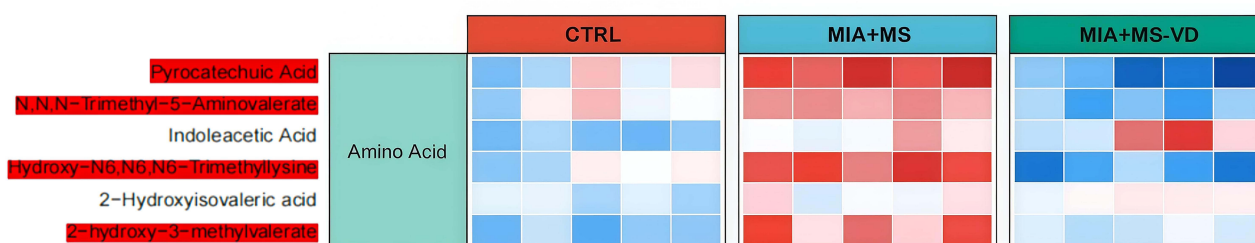


Figure 5 (A) Serum inflammatory cytokines: Compared to the CTRL group, the serum levels of inflammatory cytokines IL-1 β , IL-6, and TNF- α were significantly elevated in the MIA+MS group. These levels were markedly reduced following combined maternal and offspring VD supplementation. **(B)** Metabolite analysis of intestinal contents. As shown by the red marks in the heatmap, the levels of the substances Pyrocatechuic Acid, N,N,N-Trimethyl-5-Aminovalerate, 2-hydroxy-3-methylvalerate, and Hydroxy-N6,N6,N6-Trimethyllysine exhibited the following intergroup differences: The levels of amino acid-related metabolites—Pyrocatechuic Acid, N,N,N-Trimethyl-5-Aminovalerate, 2-hydroxy-3-methylvalerate, and Hydroxy-N6,N6,N6-Trimethyllysine—were significantly higher in the MIA+MS group than in the CTRL group. These metabolites were also significantly decreased after combined VD supplementation. (Data are presented as mean $\pm$ SD; **P < 0.01, ***P < 0.001; ns, not significant.).

upregulation in the model group is likely a compensatory response to systemic carnitine deficiency and HTML accumulation. However, this compensation appears ineffective since TMLHE is an iron- and ascorbate-dependent dioxygenase whose activity is highly sensitive to the cellular redox state.^{36,37} Notably, after combined VD intervention, although TMLHE protein levels were reduced below normal, HTML decreased and serum carnitine increased, indicating that VD does not simply suppress the pathway but restores its overall metabolic efficiency. Potential mechanisms include: VD, via its nuclear receptor (VDR), exerts potent anti-inflammatory and antioxidant effects,^{38,39} thereby restoring the cellular microenvironment and cofactor homeostasis necessary for TMLHE enzymatic activity. Carnitine synthesis is a multi-step process. VD may also upregulate the expression or activity of downstream enzymes (eg, ALDH9A1, BBOX).⁴⁰ As downstream flux becomes more efficient, the demand for high TMLHE protein levels decreases, allowing even lower expression to support higher overall metabolic throughput, potentially further enhanced by reduced feedback inhibition. It is proposed that the inflammatory and oxidative milieu induced by the MIA+MS model leads to a dissociation between compensatory high expression and functional inactivation of TMLHE in the hepatic carnitine synthesis pathway. Vitamin D's therapeutic effect does not lie in simply increasing or decreasing a single enzyme; rather, it remodels a healthier hepatic metabolic environment (anti-inflammatory, antioxidant) and potentially synergistically optimizes the entire synthesis pathway. This restores TMLHE activity, enhances downstream steps, and ultimately

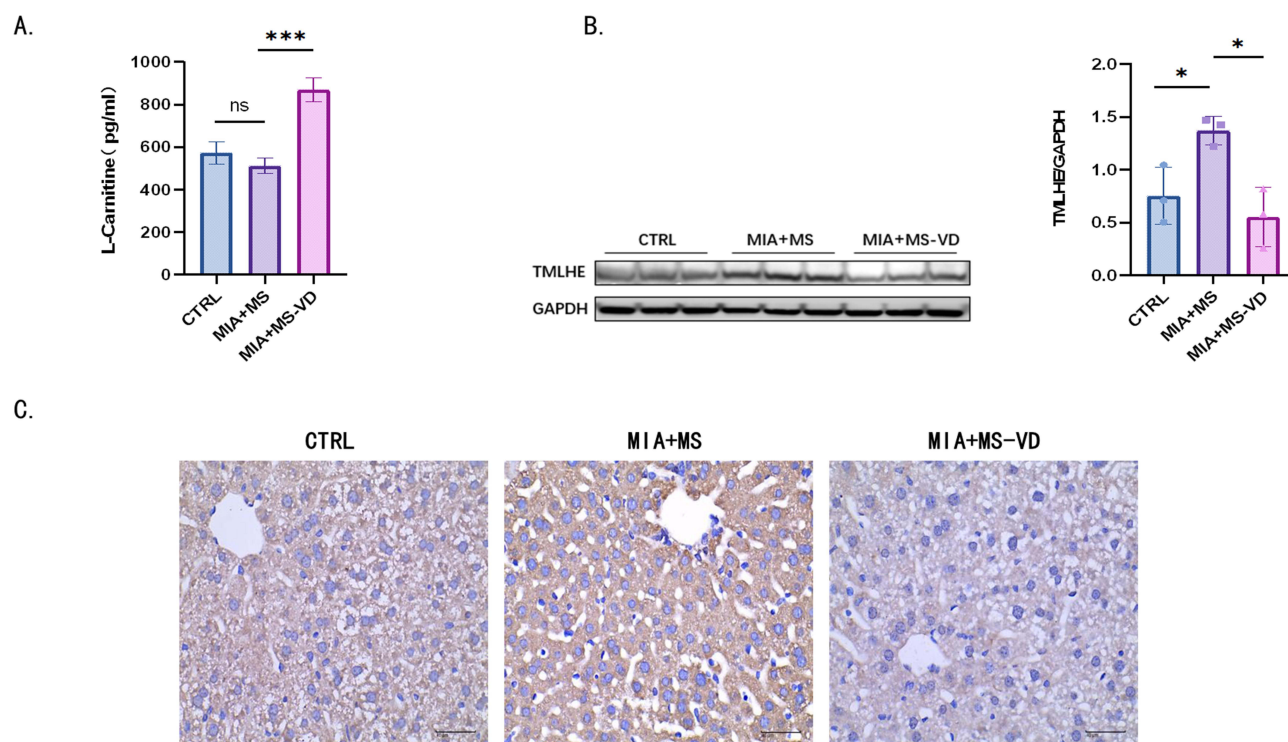


Figure 6 (A) Serum L-Carnitine levels showed a decreasing trend in the MIA+MS group compared to the CTRL group. Maternal and offspring combined vitamin D (VD) supplementation significantly increased serum L-Carnitine levels. (B) Western blot analysis revealed an upward trend in hepatic TMLHE protein expression in the MIA+MS group relative to the CTRL group. Maternal and offspring combined VD supplementation significantly reduced TMLHE protein levels. (C) Immunohistochemical staining of TMLHE in paraffin-embedded liver sections demonstrated an increase in the percentage of TMLHE-positive area in the MIA+MS group compared to the CTRL group. Maternal and offspring combined VD supplementation significantly decreased the TMLHE-positive area, Scale bar = 40 μ m. (Data are presented as mean $\pm$ SD; *P < 0.05, ***P < 0.001; ns, not significant.).

reverses carnitine deficiency in a more efficient and physiological manner. These results present novel mechanistic perspective into the neuroprotective effects of VD and suggest new potential therapeutic strategies for ASD.

Conclusion

The results of this study demonstrate that combined maternal and offspring supplementation with vitamin D3 significantly ameliorates autism-like behaviors in the “dual-hit” offspring model (induced by maternal immune activation combined with maternal separation). The underlying mechanism may be related to the multi-system regulatory effects mediated by vitamin D through the vitamin D receptor (VDR). Specifically, the “dual-hit” stimulus upregulated VDR protein expression in the offspring’s brain tissue, and vitamin D supplementation further enhanced VDR protein levels via ligand-induced activation, suggesting that VDR signaling pathway activation may be key to its neuroprotective effects. At the molecular level, the “dual-hit” model increased the production of neurotoxic substances in the offspring’s brain, which were effectively reduced by vitamin D intervention. Furthermore, this model induced disturbances in the amino acid metabolic profile in the offspring’s intestinal contents, elevated systemic inflammatory cytokine levels, and disrupted carnitine metabolism. Notably, this study uncovers disruptions in carnitine and TMLHE metabolism in the model mice and demonstrates that combined maternal-offspring vitamin D supplementation rectifies these disturbances—a finding that offers a novel perspective.

While these preclinical findings highlight a promising therapeutic strategy, their translation requires future validation in human studies to confirm the efficacy and safety of this specific supplementation regimen and to elucidate the integrated multi-system mechanisms involved.

Data Sharing Statement

The data supporting the findings of this study are available within the article and its [supplementary materials](#).

Ethics Approval and Informed Consent

All procedures carried out in this research were compliant with the ethical standards approved by the Animal Ethics Committee of West China Second University Hospital, Sichuan University (Approval number: (2025) Animal Ethics Approval No. 001). The welfare of laboratory animals is in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no competing interests in this work.

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