Vitamin A deficiency induces autistic-like behaviors in rats by regulating the RARβ-CD38-oxytocin axis in the hypothalamus

Xi Lai\textsuperscript{1,2*}, Xiaofeng Wu\textsuperscript{1,2*}, Nali Hou\textsuperscript{1,2}, Shu Liu\textsuperscript{1,2}, Qing Li\textsuperscript{1,2}, Ting Yang\textsuperscript{1,2}, Jingkun Miao\textsuperscript{2,3}, Zhifang Dong\textsuperscript{2,4}, Jie Chen\textsuperscript{1,2*}, and Tingyu Li\textsuperscript{1,2,4*}

\textsuperscript{1}Children’s Nutrition Research Center, Children’s Hospital of Chongqing Medical University, Chongqing 400014, China

\textsuperscript{2}Ministry of Education Key Laboratory of Child Development and Disorders, China

International Science and Technology Cooperation base of Child Development and Critical Disorders, Chongqing 400014, China

\textsuperscript{3}Certer for Clinical Molecular Medicine, Children’s Hospital of Chongqing Medical University, Chongqing 400014, China

\textsuperscript{4}Chongqing Key Laboratory of Translational Medical Research in Cognitive Development and Learning and Memory Disorders, Chongqing 400014, China

\textsuperscript{*}These authors contributed equally to this work.

Received: 01-Sep-2017; Revised: 24-Nov-2017; Accepted: 04-Dec-2017

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mnfr.201700754.

This article is protected by copyright. All rights reserved.
To whom correspondence should be addressed: Children’s Nutrition Research Center, Children’s Hospital of Chongqing Medical University, Chongqing 400014, China. E-mail: jchen010@foxmail.com or tyli@vip.sina.com.

Abbreviations

VA: vitamin A
ASD: autism spectrum disorders
VAD: vitamin A deficiency
VAS: vitamin A supplement
VAN: vitamin A normal
RA: retinoic acid
atRA: all-trans retinoic acid
RAR: retinoic acid receptors
RARE: retinoic acid receptor element
OXT: oxytocin
CD38: cluster of differentiation 38
RFP: red fluorescent protein

FST: forced swim test

Key words Vitamin A, autism, retinoic acid receptor beta, oxytocin

Abstract

Scope: Vitamin A (VA) is an essential nutrient for the development of the brain. We previously found that children with autism spectrum disorder (ASD) have a significant rate of VA deficiency (VAD). In the current study, we aim to determine whether VAD is a risk factor for the generation of autistic-like behaviors via the transcription factor retinoic acid receptor beta (RARβ)-regulated cluster of differentiation 38 (CD38)-oxytocin (OXT) axis.

Methods and results: Gestational VAD or VA supplementation (VAS) rat models were established, and the autistic-like behaviors in the offspring rats were investigated. The different expression levels of RARβ and CD38 in hypothalamic tissue and serum retinol and OXT concentration were tested. Primary cultured rat hypothalamic neurons were treated with all trans retinoic acid (atRA) and recombinant adenoviruses carrying the rat RARβ (AdRARβ) or RNA interference virus RARβ-siRNA (siRARβ) were used to infect neurons to change RARβ signal. Western blotting, chromatin immunoprecipitation (ChIP) and intracellular Ca^{2+} detections were used to investigate the primary regulatory
mechanism of RARβ in the CD38-OXT signaling pathway. We found that gestational VAD increased autistic-like behaviors and decreased the expression levels of hypothalamic RARβ and CD38 and serum OXT levels in the offspring. VAS ameliorated these autistic-like behaviors and increased the expression levels of RARβ, CD38 and OXT in the gestational VAD pups. In vitro, atRA increased the Ca²⁺ excitability of neurons, which might further promote the release of OXT. Different CD38 levels were induced in the neurons by infection with different RARβ adenoviruses. Furthermore, atRA enhanced the binding of RARβ to the proximal promoter of CD38, indicating a potential up-regulation of CD38 transcriptional activity by RARβ.

**Conclusions:** Gestational VAD might be a risk factor for autistic-like behaviors due to the RARβ signal suppression of CD38 expression in the hypothalamus of the offspring, which improved with VAS during the early-life period. The nutritional status during pregnancy and the early-life period is important in rats.

**Introduction**
Autism spectrum disorders (ASDs) constitute a set of neurodevelopmental disorders that are characterized by early-onset difficulties in social communication and unusually restricted, repetitive behaviors and interests. Considerable comorbidities, including anxiety disorders (42–56%) and depressive disorders (12–70%), are observed in ASD [1]. The underlying alterations in the brain occur long before the ASD symptoms become obvious [1, 2]. Gestational risk factors that could affect neurodevelopment have been suggested to increase the risk for autism [3, 4].

VAD is a serious public health problem throughout the developing world, especially affecting pregnant and lactating women, infants, and young children [5]. Vitamin A (VA) plays important roles in embryonic development, the maintenance of epithelial surfaces, immune competence, reproduction, brain development, etc. [6–10]. All-trans retinoic acid (atRA) is the functional form of VA in the body, and atRA plays an important role in the maturation of the central nervous system (CNS) during embryonic development by activating transcriptional factors, including retinoic acid nuclear receptors (RARs/RXRs) [11]. Our previous studies have shown that gestational VAD impaired learning and memory skills in gestational VAD pups and that marginal VAD in early life could facilitate the pathogenesis of Alzheimer's disease [12]. Our studies indicate that VA is involved in the pathogenesis of neurological diseases.
Although VA is indispensable during embryonic development, it remains unclear whether gestational VAD could be a risk factor for ASD. Some studies have reported that children with ASD consume less than the recommended amounts of certain nutrients from food, such as VA, due to food aversions and stereotyped eating behaviors\cite{13, 14}. Our previous study on nutritional status in ASD children found that the greatest deficiency was observed in VA (77.9%) followed by iron deficiency. The VA concentration was also found to be negatively correlated with the Childhood Autism Rating Scale (CARS) score\cite{15}. Recently, retinoic acid (RA) pathways, which are mediated by the retinoid orphan receptor alpha (RORA), have been implicated in the underlying pathobiology of ASD\cite{16, 17}. In this paper, we first hypothesized that gestational VAD might lead to autistic-like behaviors in offspring.

Oxytocin (OXT) is a 9-amino-acid peptide hormone that is produced mainly by midline neurons in the hypothalamus\cite{18, 19}. OXT can promote maternal behaviors, social memory and social bonding in both non-human mammals and humans\cite{19}. In recent years, OXT has received considerable attention as a potential treatment for the social deficits in ASD because of its role in the regulation of social behavior\cite{20-22}. CD38 is a transmembrane glycoprotein with ADP-ribosyl cyclase activity, which plays a critical role in OXT release by increasing the calcium excitability in hypothalamic neurons\cite{23-26}. RARs have been identified as transcriptional regulators of the CD38 gene, which has retinoic acid receptor elements.
(RAREs) in its promoter region \cite{27,28}. atRA has recently been shown to up-regulate the reduced CD38 transcription in lymphoblastoid cell lines from ASD patients \cite{29}. Therefore, we propose that atRA may regulate the CD38-OXT axis through RARs.

To investigate this hypothesis, we established the gestational VAD rat model, which has been patented in its country of origin, to analyze the effects of VAD on autistic-like behaviors and RARs-CD38-OXT signaling in the offspring. Second, the gestational VAD pups were supplemented with VA to investigate the improvement effects on the autistic-like behaviors and RARs-CD38-OXT signaling. Finally, primary cultured neurons extracted from the rat hypothalamus were infected with recombinant adenoviruses carrying the rat RAR\(\beta\) (AdRAR \(\beta\)) or RNA interference virus RAR\(\beta\)-siRNA (siRAR\(\beta\)) to reveal the possible molecular mechanism underlying ASD-related proteins \textit{in vitro}.

\textbf{Methods and Materials}

\textbf{Reagents and antibodies}

The retinol standards, atRA and retinyl acetate were purchased from Sigma-Aldrich, UK. The anti-RAR\(\beta\) and anti-CD38 primary antibody were obtained from Abcam, USA (ab53161, ab199137). The anti- RAR\(\beta\) primary antibody for ChIP-test was obtained from Santa Cruz Biotechnology, USA(C-19X). The OXT-ELISA Kit was obtained from Enzo Life Sciences,
GER. The ChIP kit was obtained from Millipore, USA. The SYBR green mix kit was purchased from QIAGEN, GER.

**Animals**

Sprague-Dawley rats (obtained from Chongqing Medical University Animal Care Centre, Chongqing, China) were individually housed in the laboratory colony of the Children’s Hospital of Chongqing Medical University (permission number -SCXK(Yu)2012-0015). All rats were housed in standard animal cages under specified pathogen-free (SPF) laboratory conditions.[30]

**Methods used to establish the VA normal (VAN), VAD and VA supplementation (VAS) rat models**

The VAN, VAD and VAS rat models were generated following previously described protocols[31,32] and have been patented in their country of origin (patent number: ZL201010233032.8). All female rats were randomly fed with VAN diet (a basic diet with 7000 IU retinol/kg) or VAD diet (a basic diet with 300 IU retinol/kg) for 3 weeks after 1 week of acclimatization and then mated with males. Some of the VAN or VAD female rats were randomly selected and sacrificed to test the serum retinol concentration to confirm the success of the VAD animal models. Other female rats were administered to the VAN or VAD diet during the pre-pregnancy phase, pregnancy and the lactation period. New pups
were collected as gestational VAN or VAD subjects of our study. And pups born before 5:00 pm were considered born on postnatal day 0 (PND 0). After weaning, new pups were still fed with the VAN or VAD diets throughout the study. Furthermore, 17 VAD pups (8 males, 9 females) were supplemented with vitamin A (retinyl acetate, 83.33 UI/day) through oral gavage from PND 1 to PND 7. Retinyl acetate was dissolved in edible oil at the required concentrations, and the oral gavage procedure was carried out as described by Deacon RM [30]. After PND 7, VAS pups were fed with the VAN diet throughout the study. The body weights of the rats were monitored every 7 days until PND 42, the time point at which the behavioral tests were performed.

**Behavioral tests**

The behavioral tests were conducted in the offspring between PND 42 and PND 56 main through ANY-Maze Video Tracking System (ANY-Maze, USA). The sequence of the behavioral tests was the open-field test, the three-chambered sociability test, the elevated plus maze (EPM) and the forced swim test (FST). All the behavioral test contains accommodation periods before testing day except open-field test. Each behavioral test was performed at least 24 hours after the previous test. Detailed information regarding the behavioral assays is provided in Additional File 1.
The open field test is a useful and simple test for the assessment of locomotor activity and general behavior in rodents\textsuperscript{[30]}. The total time of self-grooming (defined as paw licking, washing the nose or face or scratching the fur with any foot), the total number of fecal boli and urination, and the average motor speed during the ten-minute test were recorded.

The sociability test was performed in a three-chambered apparatus\textsuperscript{[30]}. Subject rats were individually tested in the apparatus for 5 min, and the number of entries and time spent in each chamber were automatically monitored.

The EPM and the forced swim tests were used to assess the anxiety- and depressive-related behaviors of the rats, respectively\textsuperscript{[30]}. The number of entries and time spent on the enclosed and open arms of the EPM were monitored for 5 min. In the FST, the duration of immobility was defined as floating or the least amount of movement to maintain the rat’s head above the water during the five-minute test. The latency to first immobility was defined as the time to first immobility period after the rat was placed in the water.

**Animal tissue collection and serum retinol detection**

After the completion of the behavioral tests, the offspring rats (approximately 9 weeks of age) were anesthetized, and their femoral arterial blood was then collected for serum through centrifuging at 4°C at 1600×g for 15 minutes. The rats were subsequently decapitated, and their brain tissues were rapidly removed from the skull and transferred onto ice. Dissection of
rat hypothalamus was performed according to the stereotaxic atlas of Paxinos and Watson (2007)\cite{30}. The serum and brain tissue were stored at -80°C until assayed.

The concentration of retinol in the serum was tested by high-performance liquid chromatography (HPLC, DUG-HPLC-20AT, Japan) following a previously described protocol\cite{31}. Briefly, 200 μl of serum was needed for the extraction. The residue was dissolved in a 100-μl mobile phase and injected into a column installed in the HPLC apparatus. The mobile phase was a methanol-DH₂O mixture (97:3). The concentration of retinol was determined by spectrophotometry at 315 nm. All procedures were performed by the same operator in a dark room without light.

**Enzyme-linked immunosorbent assay (ELISA)**

The serum was collected to determine the concentration of OXT using a commercial OXT ELISA kit (ENZO, GER). The procedure was performed according to the manufacturer’s protocols. The color optical density absorbance was measured at a wavelength of 405 nm. The sample concentrations were calculated using a microtiter plate reader (Thermo, USA) according to the relevant standard curves, and each assay was carried out in triplicate.

**Primary cultured rat hypothalamic neurons and treatments**
Primary cultured rat hypothalamic neurons were prepared and cultured as previously described with some modifications [31]. Primary hypothalamic neurons were prepared from rats at embryonic days 17-18 (E17-18) according to the stereotaxic atlas of Paxinos and Watson (2007) [30].

After assessing the purity through neuron-specific enolase (NSE) staining, the primary hypothalamic neurons were treated with vehicle (pure ethanol) or atRA (1 mmol/L, dissolved in pure ethanol) on day 5. Twenty-four hours after atRA treatment (on day 6), recombinant adenoviruses carrying the rat RARβ (AdRARβ) or RNA interference virus RARβ-siRNA (siRARα) were used to infect neurons. Ad-RFP, with red fluorescent protein (RFP), was used as a control adenovirus. All three viruses had similar interference conditions. The cell culture medium was not replenished for the duration of the treatment. On day 7, the total protein of the neurons was isolated and stored at -80°C. Each treatment group contained at least 3 biological replicates.

**Isolation of total protein and Western blotting**

The total protein in the brain tissue of VAN/VAD/VAS offspring and the primary hypothalamic neurons was extracted using a radio immunoprecipitation assay (RIPA) lysis buffer (KeyGEN Biotech, China) containing 0.1% protease inhibitor cocktail (KeyGEN...
Biotech, China). The protein concentrations in the homogenates were determined using the BCA protein assay kit (ATGene, USA) with a microtiter plate reader (Thermo, USA).

We performed Western blotting as previously described with some adjustments [31], including the use of the manufacturer’s anti-RARβ antibody (1:2000) and an anti-CD38 antibody (1:800) according to the manufacturer’s recommendations. The protein expression was tested using an ECL kit (Millipore, USA) and an ECL Imaging System (Syngene G: BOX, UK). The images were converted to digital files, and the intensity of the bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland).

**Intracellular Ca2+ detection**

We tested the calcium excitability in primary hypothalamic neurons treated with atRA (atRA(-): n=19; atRA(+): n=23). Before intracellular Ca2+ detection, primary hypothalamic neurons were cultured and treated with vehicle (pure ethanol) or atRA for 24 hours. The calcium excitability of the primary hypothalamic neurons was detected by a calcium imaging instrument as described in our previous studies, but some adjustments were performed. Briefly, the cells were incubated with Fluo-4 at 37°C in the dark for 30 minutes. The Fluo-4-labeled cells were captured with an intensified CCD camera (Cool-SNAP HQ2, USA) every 5s through excitation wavelengths of 340 nm and 380 nm with emission detected at 520 nm for a total of 5 minutes. After the baseline measurements during the first minute, 75
μl of KCl solution was added to the wells to induce changes in the intracellular free Ca$^{2+}$, and images of the Fluo-4 fluorescence were obtained for an additional 4 minutes. The ratio of the fluorescence intensities (F340 nm/F380 nm), which is an indicator of the intracellular free Ca$^{2+}$ concentration, was calculated using the appropriate software (NIS element AR3.1). The variance wave values between the baseline (the first 20 s) and the peak were compared for all groups.

**Chromatin immunoprecipitation and quantitative polymerase chain reaction**

(ChIP-qPCR)

ChIP was performed using a ChIP kit purchased from Millipore. The operation procedure was performed following the manufacturer’s protocol. Briefly, the chromatin from hypothalamic neuron was sheared to 200-1000 bp after sonication (medium power, 20 cycles of 30s with 30s between pulses) and incubated overnight at 4°C with 8 μg of the RARβ primary antibody or negative control IgG antibody. Q-PCR was carried out using the SYBR Green mix with the Bio-Rad CFX Connect Teal-Time system. The primer sequences for the rat CD38 and GAPDH gene promoters are shown in Table 1. Fold enrichment was calculated over IgG using $2^{(-\Delta\Delta CT)}$, where $\Delta\Delta CT = (\text{normalized } Ct_{p} - \text{Ct}_{IgG})$.

**Statistical analysis**
All data are represented by the mean ± SEM and were analyzed by GraphPad Prism 6.0 software. Growth curves were analyzed by two-way ANOVA. The behavioral tests, serum retinol and OXT levels and the Western blotting, ELISA and ChIP-qPCR data were analyzed by one-way ANOVA with the appropriate post hoc tests. The intracellular Ca^{2+} test was analyzed using the unpaired t-test. The significance level was set at $P<0.05$.

**Results**

**Gestational VA deficiency (VAD) decreased the serum retinol levels of offspring rats, and VAS increased their serum retinol levels**

A total of 21 VAN pups (9 males, 12 females) from VAN pregnant rats, 22 VAD pups (11 males, 11 females) and 17 VAD pups (8 males, 9 females) finished all the behavioral tests and serum retinol concentration detection. No abnormal death or loss of rat was found during the study.

Retinol is one of the main forms in the process of VA metabolism and has become the diagnostic criteria for assessing the VA level in many clinical applications. The serum retinol concentrations in the offspring VAN, VAD and VAS pups are shown in Fig. 1A. The retinol levels in the VAD group were significantly lower than that in the VAN ($P<0.001$) and VAS groups ($P<0.001$). This result indicated that we had successfully established the VAN, gestational VAD and VAS offspring rat models. The growth curves of these rats are shown in
Fig. 1B (male) and Fig. 1C (female). A repeated measure two-way ANOVA showed there were no statistically significant differences among the three groups (VAN/VAD/VAS). Additionally, no visible ocular manifestations of VAD were detected in any of the experimental rats.

**Gestational VAD impaired social interactions and increased repetitive behaviors in offspring rats and VAS ameliorated the impaired behaviors.**

Between PND 42 and PND 56, a series of behavioral tests were performed with the pups to determine whether gestational VAD could produce autistic-like behaviors in the offspring rats. In the behavioral tests, there was no difference between the genders among the three groups (VAN/VAD/VAS). Therefore, we combined male and female rats within the same VA intervention group for the subsequent analyses.

In the three-chambered test, rats with lower retinol concentrations spent less time in the rat chamber ($P<0.001$, vs. VAN; $P<0.01$, vs. VAS) and more time in the object chamber ($P<0.001$, vs. VAN; $P<0.001$, vs. VAS) than the VAN (21 rats) and VAS (17 rats) groups (Fig. 2A). No significant differences were observed between the VAN and the VAS groups.

We used the open-field test to assess the degree of anxiety and stereotyped behavior in rats. Repetitive behavior is associated with normal patterns, but unusually long periods of time spent performing self-grooming behavior are considered abnormal [33]. As shown in Fig. 2B,
no obvious difference was found in movement speed, suggesting that locomotor activity was not impaired in the VAN/VAD/VAS rats. On the basis of this finding, we found significant increases in self-grooming time in the VAD group offspring compared to those offspring in the VAN ($P<0.001$) group, as shown in Fig. 2C. The VAS offspring rats spent less time performing self-grooming behavior than did the VAD rats. However, this trend was not statistically significant. No significant differences were observed between the VAN and the VAS groups ($p<0.05$).

**Gestational VAD increased anxiety- and depression-related behaviors and VAS ameliorated VAD-induced anxiety and depression.**

Comorbidity in autism is common, particularly anxiety disorders and depressive disorders. In the open field test, defecation frequency is a simple way to measure fear and anxiety in rats [30]. The defecation frequency of the rats in this study is shown in Fig. 2D. VAD rats had a higher defecation frequency than the VAN ($P<0.001$) or VAS rats ($P<0.05$).

The EPM test was conducted to evaluate the anxiety-related behaviors of the pups [30]. The more time that the rats spent in the open arms, the lower their anxiety levels. Fig. 3A shows that VAD rats spent less time in the open arms than the VAN group; however, the difference was not statistically significant ($P>0.05$). However, the VAS rats spent significantly more
time in the open arms than did the VAD rats \( (P<0.001) \). No significant differences were observed between the VAN and the VAS groups.

To evaluate the effects of VAD on the depression-related behaviors of the rats, the forced swimming paradigm was used to record their latency to first immobility and the total immobility time \[^{30}\]. In the forced swim test, the VAD rats showed a significantly decreased latency to first immobility \( (P<0.001, \text{Fig. 3B}) \) and increased duration of immobility \( (P<0.001, \text{Fig. 3C}) \) compared to those shown by the VAN pups. Furthermore, statistically significant changes in the latency to first immobility \( (P<0.001, \text{Fig. 3B}) \) and the duration of immobility \( (P<0.05, \text{Fig. 3C}) \) were observed after the VAS treatment administration to the gestational VAD pups. And VAS rats also showed longer immobile times than VAN rats \( (P<0.05, \text{Fig. 3C and 3D}) \).

Overall, the behavioral tests suggested that the offspring rats with gestational VAD demonstrated more autistic-like behaviors than the VAN rats, and these behaviors were alleviated by the VAS during early life.

**Gestational VAD decreased the expression levels of hypothalamic RARβ and CD38 and serum OXT in offspring rats**

To investigate the molecular mechanisms underlying VAD-induced autistic-like behaviors in the pups, we detected the expression levels of RARβ and CD38 protein in the hypothalami of
the rats in the VAN, VAD and VAS groups. As shown in Fig. 4A and 4B, the level of RARβ protein in the VAD rats was significantly lower than that in the VAN rats (P<0.01), and it was significantly higher in the VAS rats than that in the VAD group (P<0.05). Similar changes were found about the CD38 protein (P<0.001, vs. VAN; P<0.05 vs. VAS) (Fig. 4C). Meanwhile, we also found that the serum OXT concentrations were significantly lower in the VAD group than those in the VAN (P<0.05) or the VAS groups (P<0.01). No significant differences were observed between the VAN and the VAS groups (p<0.05).

**Hypothalamic neurons treated with atRA showed a higher calcium excitability when KCl was activated**

Fig. 5A shows that most primary cultured neurons were stained by NSE, suggesting that the purity of the primary hypothalamic neurons was satisfactory for the subsequent study. We used Ca\(^{2+}\) excitability as an indirect index of OXT release because CD38 regulates OXT secretion through mobilizing intracellular Ca\(^{2+}\) pools to extracellular pools\([23, 24, 34, 35]\). The results shown in Fig. 5B-C indicate that the calcium excitability in the primary hypothalamic neurons was significantly higher in the atRA group than that in the untreated group following KCl activation (P<0.001), indicating that atRA may stimulate the release of OXT by activating calcium signals in the hypothalamic neurons.
RARβ up-regulated the expression levels of CD38 and OXT in primary hypothalamic neurons after the atRA treatment

To further investigate whether RARβ is involved in the regulation of the expression levels of CD38 and OXT, we used atRA and recombinant adenoviruses to alter the RARβ signaling pathway in primary hypothalamic neurons. After the infections with the adenoviruses siRARβ or Ad-RARβ, the expression levels of the RARβ and CD38 proteins were tested in the primary neurons following atRA treatment by Western blotting in Fig. 5D (Control: n=12; RFP+atRA: n=12; AdRARβ+atRA: n=12; siRARβ: n=12). As the levels of RARβ increased or decreased in primary neurons infected by the Ad-RARβ or siRARβ adenoviruses (RFP vs. AdRARβ, P<0.01; AdRARβ vs. siRARβ, P<0.001; control vs. AdRARβ, P<0.001, Fig. 5E), the expression levels of the CD38 protein were, simultaneously, elevated or reduced, respectively, in the primary neurons following the atRA treatment (RFP vs. AdRARβ, P<0.05; AdRARβ vs. siRARβ, P<0.001; control vs. AdRARβ, P<0.01, Fig. 5F).

RARβ up-regulated the transcription activity of CD38 by binding to its proximal promoter after the atRA treatment

To further investigate the interaction between RARβ and the CD38 proximal promoter, we conducted a ChIP-qPCR test using the anti-RARβ antibody. The ChIP-qPCR analysis clearly showed that RARβ was enriched on the promoter of the CD38 gene in the hypothalamic
neurons after the atRA treatment, which was statistically significantly greater than that without the atRA treatment (atRA(-) vs. atRA (+), P<0.05, Fig. 5G). **Discussion**

An appropriate nutritional status during the embryonic stage is essential for the growth and development of a fetus[^36,^37]. Gestational nutritional deficiencies, such as folic acid deficiency and vitamin D deficiency, have been demonstrated as risk factors for autism[^38].

As one of the important micronutrients for growth and development, VA has multiple physiological functions during embryonic development[^39]. However, it remains unclear whether gestational VAD could be related to the brain pathological changes in autism and lead to autistic-like behaviors in offspring. In the current paper, we established a gestational VAD rat model to investigate whether gestational VAD could be an environmental risk factor for autistic behaviors or social behavioral problems in offspring.

In our previous study, we found that gestational VAD resulted in poor performance on spatial learning and memory skills tasks in offspring rats, which was accompanied by significantly decreased mRNA and protein expression levels of RARα in the hippocampus[^31]. In the present study, we found that although gestational VAD offspring rats didn’t possess any growth disorder or locomotor activity impairment, they showed significant social deficits and increased repetitive behaviors. Furthermore, VAD increased anxiety- and depression-like
behaviors in the offspring rats, as measured by the EPM test and forced swim test, respectively.

Our previous studies suggested that the early postnatal period is an efficient therapeutic window during which VAS improves the learning-memory dysfunction and decreases the ratio of intestinal immune cells in VAD rats [32]. In the current study, we supplemented VAD offspring rats with VA and found that VAS ameliorated the autistic-like behaviors, i.e., increased the sociability and decreased the repetitive self-grooming time and depressive- and anxiety-related behaviors. Our behavioral tests revealed that gestational VAD could induce autistic-like behaviors in the offspring, demonstrating that VAD during pregnancy may be a risk factor for ASD.

OXT plays an important role in social behaviors. Many studies have indicated that the CD38-OXT pathway is significantly associated with ASD or autistic-like behaviors either in human or experimental animals [26, 40, 41]. As atRA has been identified as a transcriptional regulator of the CD38 gene through RARα in myeloid leukemia cell lines and atRA also can up-regulated the reduced CD38 transcription in the lymphoblastoid cell lines from ASD patients [27-29][42], it is conceivable that VAD and RA signals may be involved in the regulation of the CD38-OXT pathway for the generation of autistic-like behaviors. In our current study, the protein expression levels of hypothalamic RARβ were significantly lower in the VAD
pups than those in the VAS pups, which is consistent with previous investigations \[^{[31]}\].

Moreover, the offspring rats with gestational VAD showed greater decreases CD38 protein in the hypothalami and the OXT levels in the serum. Our findings showed a decrease in one RAR subtype, RARβ, in the hypothalamus, which is different from our previous study in hippocampus. This finding could be attributable to the different distributions of RARs in respective brain areas \[^{[43]}\].

An intranasal or subcutaneous OXT treatment has been shown to enhance social cognition, empathy, attunement, and reciprocity and reduce repetitive behaviors in ASD patients and autistic-like mice \[^{[20, 22, 44, 45]}\]. Compared to OXT treatment, VA intervention has obvious advantages because of its hypotoxicity and easy administration. Meanwhile, the levels of the RARβ and CD38 proteins in the hypothalamus and plasma OXT release are simultaneously increased in the VAS offspring rats, further indicating that the CD38-OXT pathway in the hypothalamus may be regulated by RARβ signaling during the generation of the autistic-like behaviors in gestational VAD pups. Our in vitro atRA treatment on neurons promotes Ca\(^{2+}\) excitability further indicated the regulating potential of atRA on CD38 and further OXT release in vitro.

To further study the mechanism by which atRA-RARs signaling regulates the CD38-OXT axis, we performed experiments in primary cultured hypothalamic rat neurons. First, we
treated the hypothalamic neurons with atRA and detected the expression levels of different subtypes of RARs. The qPCR data showed a significantly increased mRNA expression level of RARβ but not of the RARα or the RARγ subtypes. Additionally, the mRNA expression level of CD38 increased (Additional File 2). These results are consistent with those obtained by Riebold [29, 46]. Second, the protein expression levels of RARβ and CD38 also increased dramatically after the atRA treatment in the hypothalamic neurons, suggesting that atRA could activate RARβ and CD38 transcription. Third, alterations in RARβ signaling through the si-RARβ or Ad-RARβ adenoviruses produced synchronous changes in the RARβ and CD38 in the neurons, further supporting our previous hypotheses and findings regarding the regulatory role of RARβ in the CD38-OXT pathway and autistic-like behaviors in vitro. We also found that the RARβ subtype is the pivotal regulator of CD38 in primary hypothalamic neurons, which is inconsistent with other studies reporting that the RARα subtype is the pivotal regulator in myeloid leukemia cells [27, 46]. This is consistent with our in vivo study. In the brain, RARα is highly expressed in the hippocampus, and RARβ is highly expressed in the hypothalamus [43, 47, 48]. Finally, the ChIP-qPCR test further demonstrates that the atRA stimulates RARβ binding to the proximal promoter of the CD38 gene. The in vitro data suggest that RARβ regulates the expression level of CD38 in the hypothalamus to influence the level of OXT release in the serum. In future studies, we aim to focus on the effects of
RARβ signaling *in vivo* on autistic-like behaviors in rats with adeno-associated viral infections.

**Conclusion**

Altogether, our data clarify that gestational VAD is a risk factor for autistic-like behaviors in offspring. VA supplementation from the early-life period can improve the autistic-like behaviors caused by VAD. The molecular mechanism involves RARβ regulation of the expression level of CD38 in the hypothalamus to influence the level of serum OXT release, which plays an important role in autistic-like behaviors. Although ASD includes disorders with high heritability and a simple VAD may not cause the ASD phenotype, early-life VAD could still be an environmental trigger for individuals with potential ASD genotypes. This novel understanding of the VAD effects suggests that nutritional status during gestation or early life is important to rats.

**Authors contributions**

Xi Lai, Xiaofeng Wu, Nali Hou, Ting Yang, Jingkun Miao, Zhifang Dong, Jie Chen, and Tingyu Li designed the research. Xi Lai and Xiaofeng Wu performed the experiments and contributed equally to this work. Nali Hou, Ting Yang, Jingkun Miao, and Zhifang Dong helped perform the experiments. Xi Lai, Xiaofeng Wu, and
Ting Yang analyzed the data. Xi Lai, Jie Chen, and Tingyu Li wrote the paper. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

All procedures were approved by the Chongqing Medical University Animal Care Committee and complied with the Guidelines for Animal Research, Chongqing Science and Technology Commission.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

**Competing interests**

The authors declare that they have no competing interests.

**Acknowledgements**

This research was funded by the National Nature Science of Foundation of China (81471518, 81771223, 81770526, 81401747, 81601359).
References


This article is protected by copyright. All rights reserved.


This article is protected by copyright. All rights reserved.


Legends

Graphical Abstract.

Gestational VAD or VA supplementation (VAS) rat models were established, and the autistic-like behaviors in the offspring rats were investigated. The different expression levels of RARβ and CD38 in hypothalamic tissue and serum retinol and OXT concentration were tested by Western blotting. Blood was collected from the offspring to determine the serum retinol and OXT concentration. Primary cultured rat hypothalamic neurons were treated with all trans retinoic acid (atRA) and recombinant adenoviruses carrying the rat RARβ (AdRARβ) or RNA interference virus RARβ-siRNA (siRARβ) were used to infect neurons to change RARβ signal. Western blotting, chromatin immunoprecipitation (ChIP) and intracellular Ca²⁺ detections were used to investigate the primary regulatory mechanism of RARβ in the CD38-OXT signaling pathway.
Figure 1.  A) Serum retinol levels of the VAN (9 male, 12 female), VAD (11 male, 11 female) and VAS (8 male, 9 female) pups (aged 8 weeks) using HPLC. B) The growth curves of weight in male VAN/VAD/VAS rats. C) The growth curves of weight in female VAN/VAD/VAS rats. The values are the means±SEM, *P<0.05, **P<0.01, ***P<0.001, ns=not significant in multiple comparisons.
This article is protected by copyright. All rights reserved.
Figure 2. Three chambered test and open field test in VAN/VAD/VAS rats. A) The time spent in different chambers in the VAN, VAD and VAS groups. B) Locomotor activity (speed of movement) of rats tested in open field test. C) The time spent in self-grooming (open-field test). D) The defecation frequency in the VAN, VAD and VAS offspring rats. The values are the means±SEM, *P<0.05, **P<0.01, ***P<0.001, ns=not significant in multiple comparisons.
Figure 3. * Gestational VAD increased the offspring’s anxiety and depression levels, which were improved by early-life VAS. A) VAD decreased the time spent in the open arms of the EPM test compared to that in the VAN and VAS groups. B) VAD rats spent more time in the closed arms. C) The changes in the latency to first immobility in the offspring rats during the forced swimming test. D) The changes in the duration of immobility in the three groups during the forced swimming test.

The values are the means±SEM, *P<0.05, **P<0.01, ***P<0.001, ns=not significant in post hoc tests.
Figure 4. The expression levels of the RARβ and CD38 proteins in the offspring’s hypothalami and serum OXT concentrations. (VAN: n=16; VAD: n=22; VAS: n=10) A) The expression levels of the RARβ and CD38 proteins were assessed by Western blotting. B) The relative quantity of the RARβ protein expression levels was normalized to actin. C) The relative quantity of CD38 protein expression levels was normalized to actin. D) Concentration changes in the serum OXT in the three offspring rat groups (VAN: n=21; VAD: n=22; VAS: n=17). The values are the means ± SEM, *P<0.05, **P<0.01, ***P<0.001, ns=not significant in post hoc tests.
Figure 5. atRA up-regulated the expression levels of CD38 and OXT through the transcriptional regulation of RARβ in the primary hypothalamic neurons. A) Identification of primary cultured hypothalamic neurons using NSE. B-C) In the intracellular Ca2+ detection test, atRA increased the intracellular calcium excitability of the hypothalamic neurons. atRA (-): n=19; atRA (+): n=23. D) The expression levels of the RARβ and CD38 proteins in the primary hypothalamic neurons with the atRA treatment following the infection by AdRARβ or siRARβ adenovirus. Control: n=12; RFP+atRA: n=12; AdRARβ+atRA: n=12; siRARβ: n=12. E) Relative quantity of RARβ expression level was normalized to β-actin in the hypothalamic neurons. F) Relative quantity of CD38 expression level was normalized to β-actin in the hypothalamic neurons. G) RARβ bonded to the proximal promoter of CD38 in the ChIP-qPCR test. (IgG: n=7; positive control: n=9; atRA (-): n=9; atRA (+): n=9). The values are the means±SEM, *P<0.05, **P<0.01, ***P<0.001, ns=not significant in post hoc tests.
**Table 1**  Primer sequence of rat CD38 and GAPDH gene promoter

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD38 sense</td>
<td>CTGCTTTCCCACTCCTTAA</td>
</tr>
<tr>
<td>CD38 antisense</td>
<td>CACAACGCTGGATCTTCA</td>
</tr>
<tr>
<td>GAPDH sense</td>
<td>GCAAGTTCAACGGCACAGTC</td>
</tr>
<tr>
<td>GAPDH antisense</td>
<td>TGGTGGTGAAGACGCCAGTA</td>
</tr>
</tbody>
</table>