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Postnatal AVP treatments prevent social deficit in adolescence of valproic acid-induced rat autism model

Jing Wu [a,1], Yu-Chuan Dai [b,c,1], Xing-Yu Lan [b,d,e], Hong-Feng Zhang [f], Shu-Zhen Bai [b,d,e], Ying Hu [b,d,e], Song-Ping Han [g], Ji-Sheng Han [b,d,e], Rong Zhang [b,d,e,h,*]

[a] Medical and Health Analysis Center, Peking University, Beijing, 100191, PR China
[b] Neuroscience Research Institute, Peking University, Beijing 100191, PR China
[c] State Key Laboratory of Transducer Technology, Aerospace Information Research Institute, Chinese Academy of Sciences, Beijing 100190, PR China
[d] Key Laboratory for Neuroscience, Ministry of Education/National Health and Family Planning Commission, Peking University, Beijing 100191, PR China
[e] Department of Neurobiology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, PR China
[f] Fujian Provincial Key Laboratory of Neurodegenerative Disease and Aging Research, Institute of Neuroscience, School of Medicine, Xiamen University, Xiamen 361102, Fujian, PR China
[g] Wuxi Shenpingxintai Medical Technology Co., Ltd. Wuxi 214000, Jiangsu, PR China
[h] Autism Research Center of Peking University Health Science Center, Beijing 100191, PR China

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ABSTRACT

Studies have shown that arginine-vasopressin (AVP) is an important neuropeptide regulating social behaviors. The present work aimed to detect changes in the AVP numbers and level in a valproic acid (VPA)-induced rat model of autism and the underlying mechanism of its pathogenesis. Our results indicated that infants exposed to VPA showed obviously impaired communication and repetitive behaviors with reduced number of AVP-ir cells in paraventricular nucleus (PVN) and cerebrospinal fluid (CSF). The postnatal subcutaneous injection of AVP can alleviate social preference deficits and stereotyped behaviors, accompanied with the increase of the AVP concentrations in the CSF. We concluded that AVP system was involved in etiology of VPA-induced autism-like symptoms and postnatal AVP treatment rescued the behavioral deficits,which could be a promising treatment for autism.

1. Introduction

Autism spectrum disorder (ASD) is a serious lifelong neurodevelopmental disorder characterized by persistent deficits in social communication and social interaction, along with restricted and repetitive patterns of behavior, narrow interests and/or activities [1]. Although many researches have been carried out, the mechanism of ASD remains unclear. It was generally accepted that ASD was resulted from heterogeneous etiology including both genetic and environmental factors [2]. Results of several studies have indicated that the environmental factors related to the pathogenesis of ASD included the toxins, mental elements, inflammatory infection, and the uterine environment, etc. It has been attested that the biological environment of the uterus played a key role in the pathogenesis of ASD [3–5].

Arginine-vasopressin (AVP) is a 9 amino acid peptide which is synthesized in separate neuronal populations in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei as well as in the "accessory nuclei" (AC) that are situated between the PVN and SON [6]. AVP creates both hormonal effects, such as water balance, on peripheral target tissues and neuromodulatory actions in the central nervous system controlling social behavior and cognition, including social preference [7], social memory [8], social bonding [9], and aggression [10].

In recent years, a growing number of scientific reports have shown that AVP is a crucial factor in neurodevelopmental disorders, including the ASD [11,12]. However, as reviewed by Wilczyński et al. [13], literature regarding the levels of AVP in the ASD children vs neurotypical groups is extremely scarce and the available studies present contradictory outcomes. In the five studies comparing plasma AVP levels between autistic and typically developing children (TDC), one reported a decreased AVP levels in autistic children [14] and one

* Corresponding author at: Neuroscience Research Institute, Peking University, Beijing, PR China.
E-mail addresses: zhangrong@bjmu.edu.cn, zhangrongbjmu@163.com (R. Zhang).
[1] These authors have contributed equally to this work.

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reported an opposite result [15], while the other three reports showed no differences [11,16,17]. Thus, the relationship between AVP level changes with pathogenesis of autism needs further verification. Furthermore, a recent review by Hendaus et al. indicates that the AVP signaling pathway is one of the most encouraging conceivable signaling pathways for ASD therapeutic discovery [12]. There were also clinical treatment trials [18] that found intranasal vasopressin improved social deficits in children with autism, while in another trial [19], vasopressin V1a receptor agonist improved adaptive behaviors in men with ASD. But there is also no consensus regarding whether AVP or AVP agonists ameliorate behavior or social function problems of animal models of autism.

To verify the effect of AVP on ASD, we used VPA-induced ASD rat model to detect the influence of VPA on AVP levels in the brain and explored the effects of AVP treatments on core autism symptoms including social communication and social interaction deficits, as well as restricted and repetitive patterns of behavior.

2. Materials and methods

2.1. VPA-induced rat model of autism

Male and female Wistar rats used for the autism model were purchased from the Viton Lihua Experimental Animal Company and maintained on the standard laboratory specific pathogen-free (SPF) at the Department of Experimental Animal Sciences, Peking University Health Science Center. All experimental procedures were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and approved by the Committee on the Ethics of Animal Experiments of the Peking University Health Science Center (ethics approval ID, LA2015204).

Rats were housed individually under a regulated environment(light time 7:00~19:00, ambient temperature 23 ± 2 °C, humidity 50 ± 10 %) with sufficient diet.

After the male and female rats reached the age of 12 weeks, the male and female rats were allowed to mate overnight. The mating day was considered embryonic day 0.5 (E0.5) in the presence of a vaginal plug. The pregnant rats were randomly distributed into two groups: a VPA group and a control group. Then the VPA group was intraperitoneally injected with VPA (Sigma: P4543, diluted with NS to a concentration of 200 mg/mL) at a dose of 450 mg/kg body weight on E12.5, and the control group received the same volume of NS at E12.5.

The progeny birth day was recorded as the 0th day (PND0). After weaning at postnatal day 21 (PND21), the offspring of the same sex were housed separately with 3–4 per cage. In this study, only male offspring were selected as research subjects.

Ultimately, there were three offspring cohorts used in this study to assess the social behaviors, development of the central AVP system and the effect of the postnatal AVP treatment (7 daily subcutaneous injections beginning from PND0) on autistic-like behaviors of VPA rats.

2.2. Behavioral tests

2.2.1. Isolation-induced ultrasonic vocalizations (USVs)

When rats are separated from mother rats, they can send ultrasonic waves with a frequency of about 40 kHz. Studies have shown that the infant rat can emit the highest number of ultrasounds on the 7th day after birth (PND7). The number is often used as an indicator to check whether the early social function of the rat is well developed.

Changes in social communication of infant rats were tested by analyzing isolation-induced USVs as reported by Zhang et al. Isolation-induced UAVs were recorded on PND7 from 18:00 to 22:00 in a quiet environment with dim light. Briefly, infant rats were individually removed from the home cage and gently transferred to the test cage on a heating pad (37 °C). USVs were recorded for 300 s for each rat and collected by an ultrasonic microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) hung 25 cm above the cage floor. The connected amplifier (AUSG-116H, Avisoft Bioacoustics, Berlin, Germany) was set at a sampling frequency of 250 kHz with a 125 kHz low-pass filter. The recorded files were analyzed with Avisoft SASLab Pro (Version 4.52) using fast Fourier transform (512 FFT-length, 100 % frame size, hamming window, 50 % time-window overlap) [20].

2.2.2. Three-chamber sociability test

Social preference and social novelty responses of adolescent rats were evaluated by the three-chamber sociability test on PND 35-40, as documented in the previous studies [21,22]. Briefly, each rat was subject to a three session test including a habituation stage of 5 min, a social preference stage of 10 min and a social novelty stage of 10 min. The time spent by each subject in every chamber of the apparatus was automatically recorded. The entire activities of subjects were also videoed for further analyses of the movement tracks and social index.

2.2.3. Light/dark box test

Generally, rats prefer to move around in dark environments and would like to explore the light environments because of their natural instincts. Rats with a high level of anxiety rarely explored the light chamber and stayed in the dark chamber for a long time during the light/dark box test.

According to a previous study [20], rats were subject to a light/dark box test for 5 min, the level of anxiety of the rats was evaluated by the time spent in the light side of the apparatus.

2.2.4. Self-grooming behavior

The self-grooming test entails setting an empty cage similar to the rats' home cage in a dark room, and placing the camera right above the cage. During the habituation, rats were encouraged to explore the cage for 5 min. After habituation rat behavior was video-taped for 10 min and the total self-grooming time was calculated.

2.3. AVP treatment

To assess the long-term effects of postnatal AVP treatment, young VPA rats were randomly divided into two groups. Within 7 days after birth, one group of infant rats received a daily subcutaneous injection of AVP (3 μg/20 μL in NS), and the other group were set as control which received a subcutaneous injection of 20 μL NS during the first week after birth instead. Isolation-induced USVs of young rats was evaluated on PND7. After weaning, offspring from the same litter were housed together (3–4 rats per cage) and were subjected to the rest of the experiments from PND35 to PND40.

2.4. Tissue collection

For determining AVP peptide levels, rats were anesthetized by an intraperitoneal injection with 10 % chloral hydrate (300 mg/kg body weight) before sampling. Briefly, 150 μL of CSF were collected from the cisterna magna of each rat using insulin syringe of 1 mL. The trunk blood (5 mL) was subsequently collected following decapitation and the serum was then separated after being maintained at room temperature and centrifuged at 4 °C. All samples were stored at −80 °C.

2.5. Detection of AVP levels in the CSF and serum

AVP content in the CSF and serum was assayed using a commercially available ELISA kit (Enzo life sciences, ADI-900-153A-0001). Extraction of AVP was performed with acetone and petroleum ether according to the product manual prior to the assessment. The ELISA kit was highly specific to AVP (detecting limit: 15.0 pg/mL) with little cross-reactivity (less than 7.5 %) with oxytocin. The intra-assay CV for AVP is 10.2 %.

2.6. Determination of mRNA levels of AVP and their receptors

Total mRNA was extracted from the brain tissue micropunches using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The expression level of the AVP mRNA and AVP receptor 1a (V1aR) was estimated by TaqMan® Gene Expression Assays on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) as documented in the previous studies [21].

2.7. Immunohistochemistry

Six sections containing the PVN or SON (between −0.8 and 2.1 mm from the Bregma) were selected every 150 μm for AVP immunohistochemical analysis. In order to examine which subpopulations of AVP

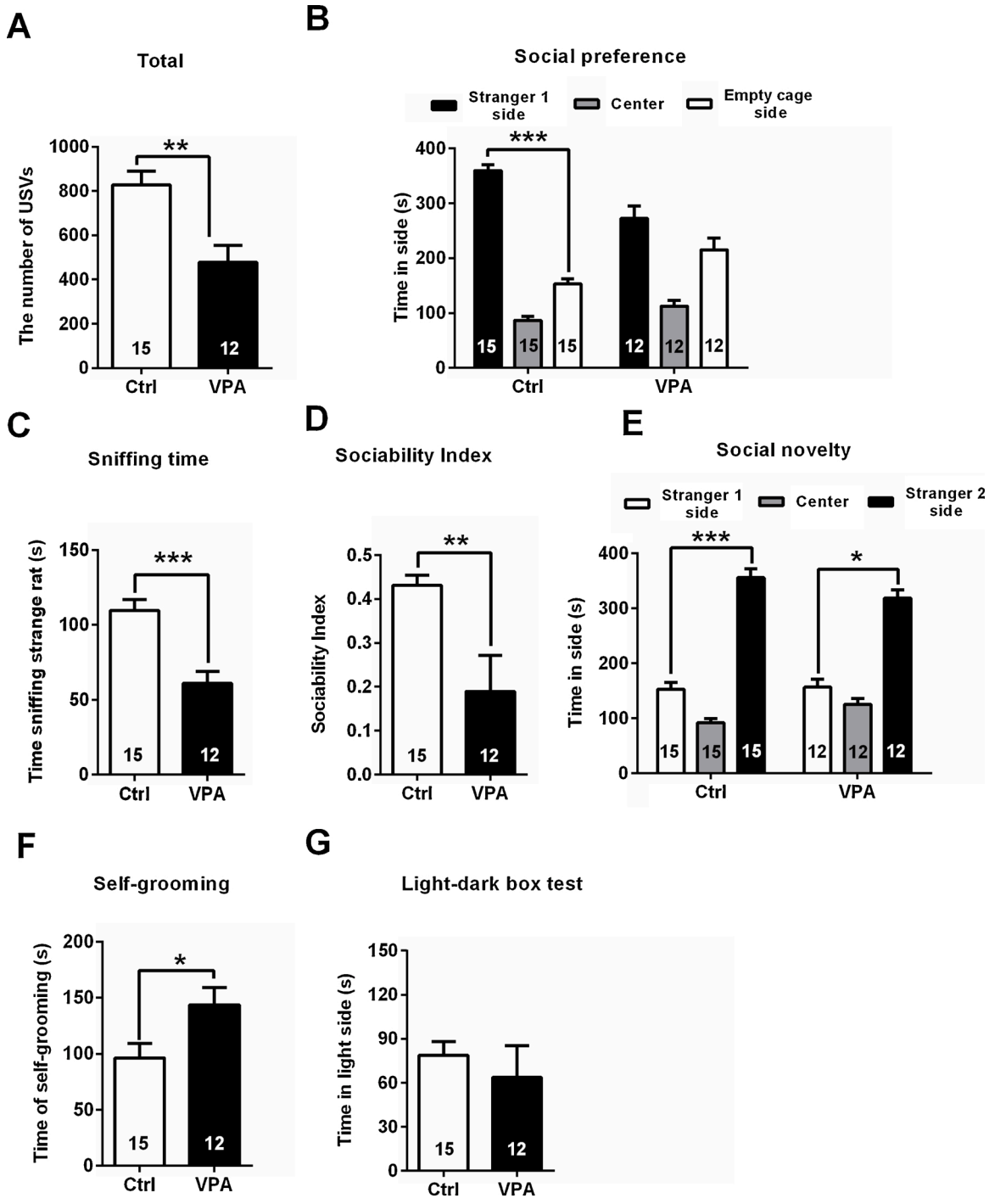

Fig. 1. Behavioral comparison of VPA progeny and normal progeny.
(A) Isolation-induced USVs; the total number of USVs in VPA rats was significantly reduced; (B) the three-box social preference test; there was no significant difference in the residence time of the VPA rats in both boxes. In addition, VPA rats sniffed strange rat time (C) and (D) the sociability index was significantly lower than the control group; (E) VPA rats showed normal social novelty; (F) The self-combing test, the self-combing time of VPA rats was significantly higher than that of the control group; (G) In the light and dark box test, there was no statistical difference in the residence time between the control group and VPA rats on the side of the light box.
*$P < 0.05$, ** $P<0.01$, *** $P <0.001$. USVs: ultrasonic vocalizations.

neurons wereaffected in VPA rat infants, parvocellular or magnocellular AVP-ir cells were counted as the data for each sample under a Leica automated DMI-inverted microscope (Leica DMI 4000B). Referring to previous study [23,24], the sections of magnocellular and parvocellular subpopulations were selected for counting in the corresponding locations of brain slices after the calculation of the control rat brain atlas. Only male offspring were used in second and third cohorts because the VPA rats had no gender differences in their phenotypes.

2.8. Statistics

The numbers of animal used in each experiment are mentioned in figures. Statistical analyses were performed using IBM SPSS Statistics 19 (SPSS, Inc., an IBM Company) and GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) generated graphs. All results are expressed as mean ± SEM. Behavioral and biochemical comparisons between groups were analyzed by an unpaired *t* test or Mann-Whitney *U* test The three-chamber social test was analyzed with paired *t* test to determine within-group side preference. One-way ANOVA followed by a LSD *post hoc* test was used to correct for multiple comparisons. Statistical significance was set at $P < 0.05$ (two-tailed) in all tests.

3. Results

3.1. VPA rats displayed social impairments

In the isolated-induced USVs tests, results showed that the number of USVs the VPA group emitted was significantly less than from the control group ($P < 0.01$, Fig. 1A).

The time the control rats spent in the stranger rat side was significantly longer than that in the empty cage side ($P < 0.001$, Fig. 1B). However, the VPA-induced rats showed no preference between the two sides. Furthermore, we found that the sniffing time spent in the VPA-induced rats offsprings was significantly less than that in the control rats ($P < 0.001$, Fig. 1C). Compared with the control group, the social index of the VPA-rats was also significantly lower ($P < 0.01$, Fig. 1D).

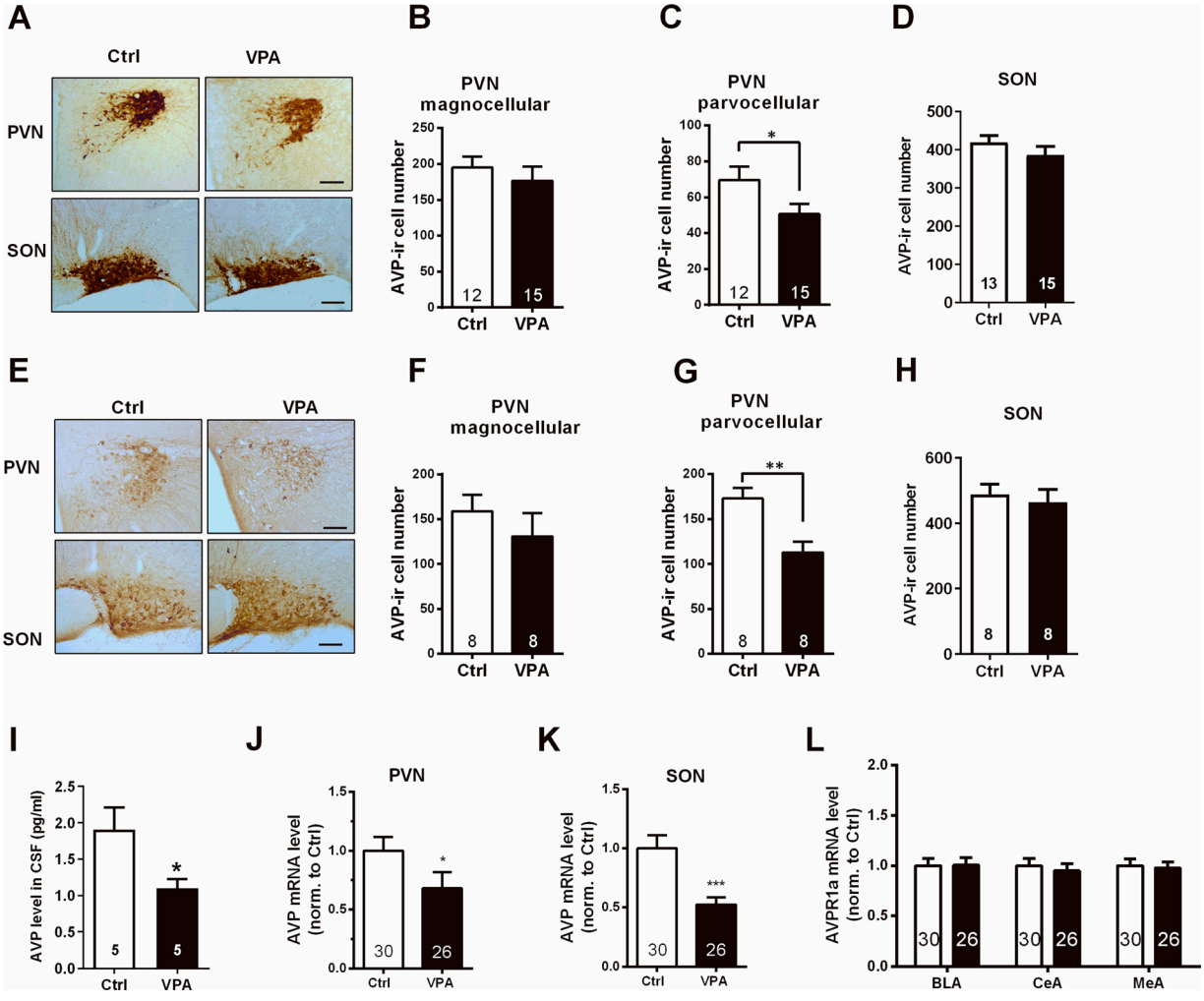

Fig. 2. The AVP-ir cells number and the AVP concentration in CSF in control and VPA rats.
(A) Immunochemical illustration of AVP-ir cells of infant rats in control and VPA groups; In PVN, (B) there was no statistically significant differences in the number of magnocellular AVP-ir cell betweenVPA infant rats and the control group and (C) the number of parvocellular AVP-ir cell was significantly lower than the control group; (D) In SON, the difference in the number of AVP neurons between the VPA infant rats and the control group was not statistically significant; (E) Immunochemical illustration of AVP-ir cells of adolescent rats in control and VPA groups; In PVN, (F) there was no statistically significant differences in the number of magnocellular AVP-ir cell between adolescent VPA rats and the control group and (G) the number of parvocellular AVP-ir czxxell was significantly lower than the control group; (H) In SON, the difference in the number of AVP neurons between the VPA rats and the control group was not statistically significant; (I) Compared with the control group, the concentration of AVP in CSF of VPA rats was significantly reduced; (*J* and K) AVP mRNA levels were significantly decreased in PVN and SON of VPA rats; (L) V1aR mRNA levels remained unchanged in BLA, CeA or MeA of VPA rats.
Bar =100 μm. * P <0.05, ** P<0.01. PVN: paraventricular nucleus, SON: Srpraoptic nucleus, CSF: cerebrospinal fluid.

And the second stage of the three-chamber tests indicated that both the VPA rats and the control exhibited intact social novelty, as they spent more time in the chamber with the novel rat than that with familiar rat ($P < 0.001$ and $P < 0.05$, Fig. 1E). The self-grooming test and the light/dark box test illustrated the level of stereotyped behavior and anxiety of the testing rats. The VPA rats spent more time grooming themselves, which indicated that the VPA rats displayed more stereotyped behaviors (unpaired t test; $P < 0.05$, Fig. 1F). With the light/dark box test, there was no significant difference between the VPA rats and the control group ($P = 0.1520$, Fig. 1G).

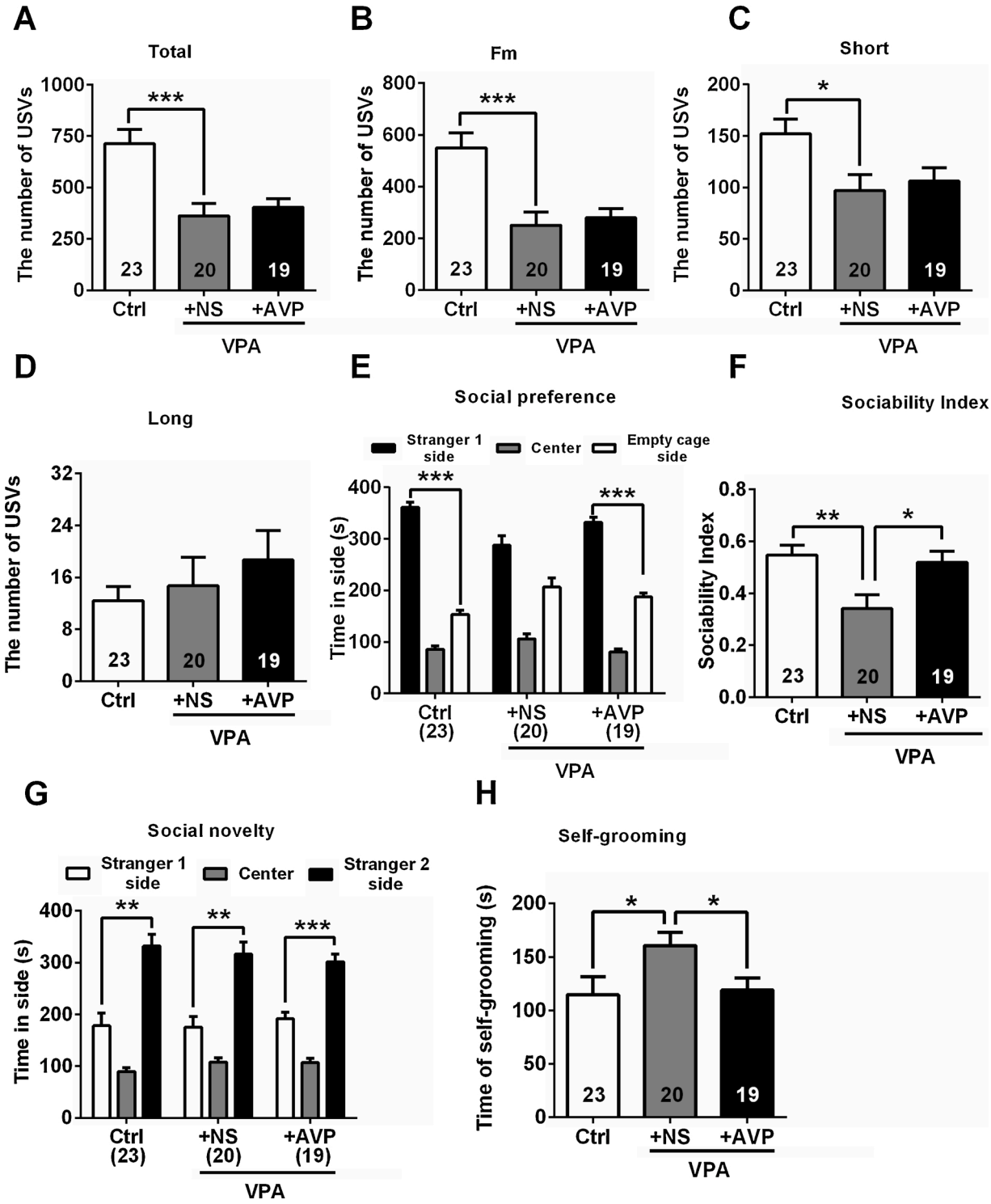

Fig. 3. Behavior test of VPA offspring after AVP treatment.
(A) The total number of USVs calls of control group infant rats, NS-treated VPA infant rats and AVP-treated VPA infant rats; (B) the number of Fm waves; (C) the number of short waves; (D) the number of long waves; (E and F) Social preference of VPA rats was normal following early AVP treatment; (G) Social novelty of VPA rats was not affected; (H) Time spent in self-grooming by VPA rats was lowered following early postnatal AVP administration.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. USVs: Ultrasound calling, Ctrl, control.

3.2. Number of AVP neurons in VPA rats

In PVN, the numbers of AVP neurons subpopulations (magnocellular and parvocellular) were counted. As shown in Fig. 2, in both infant rats ($P = 0.4761$, Fig. 2B) and adolescent rats ($P = 0.3908$, Fig. 2F), there were no statistically significant difference in the numbers of magnocellular AVP-ir cell between VPA group and the control group. However, the numbers of parvocellular AVP-ir cell in both infant rats ($P < 0.05$, Fig. 2C) and adolescent rats ($P < 0.01$, Fig. 2G) were significantly lower in the VPA group than those in the control group.

In SON of both infant rats ($P = 0.2842$, Fig. 2D) and adolescent rats group($P = 0.6422$, Fig. 2H), there were no statistically significant differences in the numbers of AVP neurons in the VPA group and the control group.

In addition, the AVP level in the CSF significantly decreased in the VPA rats ($P < 0.05$, Fig. 2I). Furthermore, as shown in Fig. 2L ($P < 0.05$) and Fig. 2M ($P < 0.001$), the AVP mRNA levels in the PVN and SON were significantly decreased. However, the V1aR mRNA levels remained unchanged in BLA ($P = 0.9545$), CeA ($P = 0.6297$) and MeA ($P = 0.8056$) of VPA offspring (Fig. 2L).

3.3. Effect of acute AVP treatment on behavioral tests

We applied constant AVP treatment to VPA offspring for a week after their birth to detect whether the treatment can rescue the social deficits of VPA rats. And the control group was injected with equivalent NS subcutaneously.

Statistics show that the total number of UVSs emitted from VPA rats was significantly decreased, due to reduction of Fm waves and short waves which are critical waves for rat's communication. But there was no statistically significant difference in the long waves between the two groups.

The AVP injection did not increase the total number of UVSs after the VPA infant rats separated from their mother rats, but it did to some extent improve the number of Fm waves and short waves (Fig. 3A–D).

As shown in Fig. 3E and F, results of the three chambers test showed the therapeutic effect of early AVP treatment on the rats, with the postnatal AVP supply group spending more time around the model rat. In addition, compared with VPA + NS rats, postnatal AVP treatment significantly reduced the duration of self-grooming in VPA rats and enabled them to return to a normal level (Fig. 3H).

3.4. Effect of AVP treatment on AVP neurons and AVP concentration

In PVN, the numbers of both the magnocellular and the parvocellular AVP-ir cell were shown an increased tendency after AVP treatment although there was no statistically significant difference between VPA + NS rats and VPA + AVP rats (Fig. 4B–C). However, in SON, the development of AVP neurons in control rats and VPA + NS rats remained normal, and postnatal AVP treatment did not alter the development of AVP neurons, as the number of AVP-ir cells had no significant changes in the VPA + AVP group compared with the VPA + NS group (Fig. 4D). In

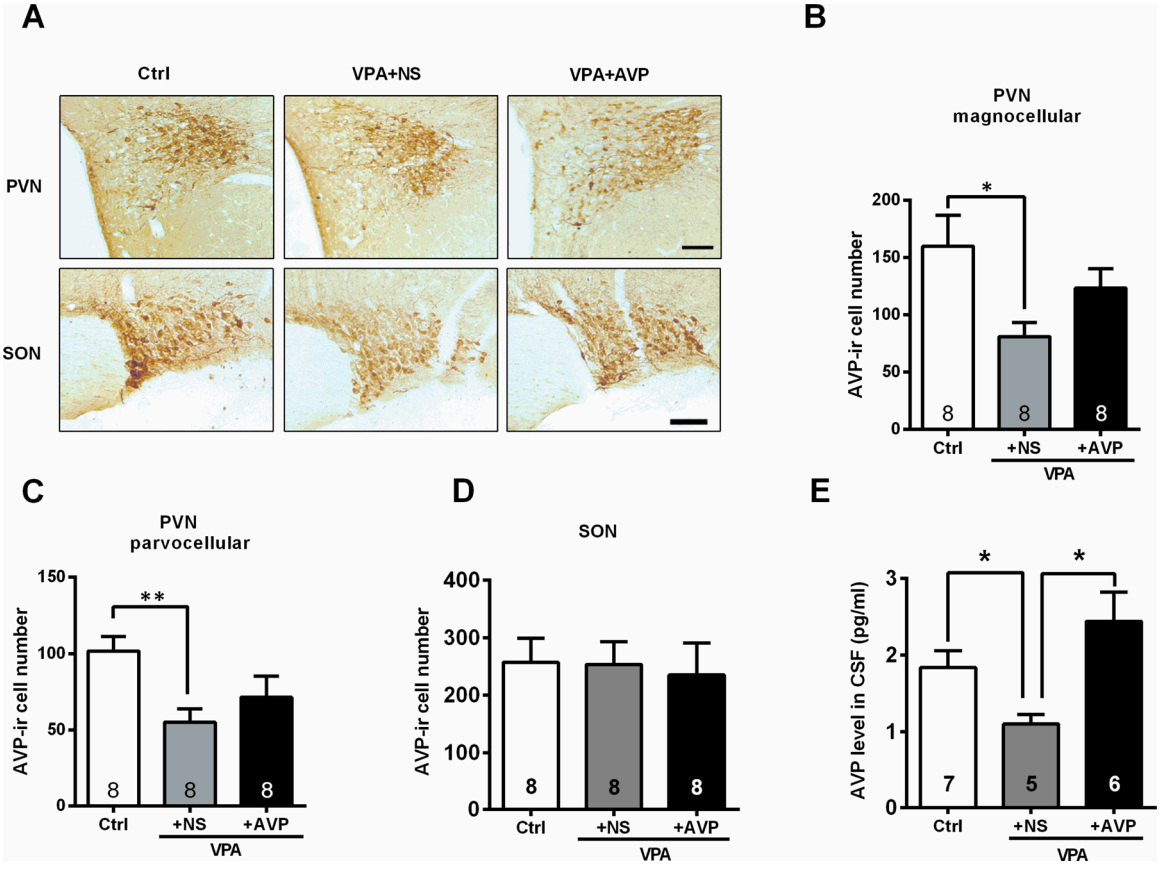

Fig. 4. Development of rat AVP system after AVP treatment.
Schematic diagram of immunohistochemical staining of AVP neurons in the brains of VPA rats and control rats; (B and C) In PVN, the number of both the magnocellular and the parvocellular neurons were shown an increased tendency after AVP treatment although there was no statistically significant difference between VPA + NS rats and VPA + AVP rats; (D) In SON, the development of AVP neurons in control rats and VPA + NS rats remained normal, and AVP treatment after birth neither increased nor inhibited the number of AVP neurons; (E) After AVP treatment, the level of AVP in CSF of VPA + AVP rats was significantly higher than that of VPA + NS rats.
Bar =100 μm. * $P < 0.05$. PVN: paraventricular nucleus, SON: Srpraoptic nucleus. CSF: cerebrospinal fluid.

addition, it is noted that the deficits of AVP levels in CSF were renovated in the VPA + AVP group compared with the VPA + NS group (Fig. 4E).

4. Discussion

VPA is widely used to induce ASD animal models [25,26]. There are many types of animals and methods used for VPA modeling. Previous studies demonstrated that Wistar rats exposed to prenatal VPA showed symptoms of ASD [22], such as defects in social preference, increased repetitive behavior, sensory abnormalities, anxiety, etc. The VPA model established in our study exhibited a reduced number of ultrasonic vocalization calls during infancy, which is consistent with previous research results [27]. Ultrasonic vocalization represents the language communication ability of young rats, and impaired ultrasonic vocalization ability corresponds to the language communication disorder which is one of the main symptoms of clinical ASD patients [28,29].

In order to examine which AVP neurons were affected in VPA rats, VPA neurons in SON and subpopulations of AVP neurons including magnocellular and pavocellular AVP-ir cells in PVN were counted in present study. The results showed that the number of parvocellular AVP-ir cell in both infant rats (Fig. 2C) and adolescent rats (Fig. 2G) were significantly lower in the VPA group than in the control group and the mRNA showed similar trendency (Fig. 2J). Meanwhile there were a decreased tendency in numbers of magnocellular AVP-ir cells in both infant rats (Fig. 2B) and adolescent rats (Fig. 2F) compared with control group, although there was no statistically significant difference between groups. In SON, there was no statistically significant difference in the number of AVP neurons between groups in both infant rats (Fig. 2D) and adolescent rats (Fig. 2H) although the mRNA showed obvious decrease (Fig. 2K). We still cannot explain the contradictory phenomenon in SON. In brief, it indicated that the impaired of AVP neurons especially the parvocellular AVP-ir cell in PVN of VPA-induced autism rats, which is more susceptible, occurred at an early age after birth and lasted into adolescence. We speculated that this was the key reason for obviously impaired communication and repetitive behaviors in VPA rats and the decrease of AVP level in CSF.

The administration of the AVP treatment includes intranasal and injection and we selected treatment by injection in the present study. Generally, a long enough time is needed for the treatment to work. AVP treatment will not have a significant effect on the development of the nervous system during a short term. Although the AVP treatment had no immediate effect on the neural development in early stage after birth, the postnatal treatment played a long-term role in the improvement of AVP concentrations in the brains of VPA rats. It can be seen from the results that after postnatal AVP treatment, the numbers of both the magnocellular and the parvocellular AVP-ir cells in adolescent rats were shown an increased tendency although the level of AVP in CSF of VPA + AVP rats was significantly higher than that of VPA + NS rats (Fig. 4).

In addition, the results indicated that the autism-like behaviors of VPA rats were improved by postnatal AVP treatment, including improved social preferences and reduced repetitive behaviors. Consistently, the results of clinical studies showed that the plasma AVP concentration was negatively correlated with repetitive stereotyped behavior [11]. We speculated that a postnatal subcutaneous injection of AVP could increase the AVP concentrations in the CSF and then alleviate the impairment of AVP-ir cells in PVN as well as induce recovery from social preference deficitsand stereotyped behaviors.

In previous studies, there were a few reports exploring the association between the AVP system and the autistic behaviour induced by VPA. Štefánik et al. found that VPA had no effect on the mRNA expression of AVP [30]. In recent years, new research [31] on prairie voles has found that prenatal VPA exposure downregulated the vasopressin receptor (V1aR) mRNA expression in the prefrontal cortex. In present study, the AVP mRNA levels in the PVN and SON significantly decreased but with no change of V1aR in amygdala. We concluded that AVP system involved in etiology of VPA-induced autistic rats model and postnatal AVP treatment rescued the behavioral deficits and could be a promising treatment for autism.

In conclusion, the VPA rat model exhibited typical autism-like behavior, accompanied by a decrease in the number of the AVP neurons especially the parvocellular AVP-ir cell in PVN, and a decrease in the content of peptides in the CSF, as well as a decrease AVP mRNA levels in the PVN and SON. Early postnatal AVP treatment could improve the social defects and reduce repetitive behaviors of VPA rats by increasing the AVP concentration in CSF. Due to the therapeutic effects of postnatal AVP treatment on the autism-like symptoms of VPA rats, it is believed that the AVP supplement during the early life stage could be a promising treatment for autism induced by environment factors.

CRediT authorship contribution statement

Jing Wu: Investigation, Validation, Writing - original draft. **Yu-Chuan Dai:** Investigation, Validation, Writing - original draft. **Xing-Yu Lan:** Methodology. **Hong-Feng Zhang:** Methodology. **Shu-Zhen Bai:** Methodology. **Ying Hu:** Methodology. **Song-Ping Han:** Supervision. **Ji-Sheng Han:** Supervision. **Rong Zhang:** Formal analysis, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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