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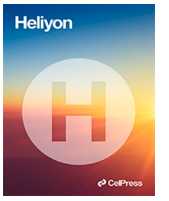

Research article

Establishment of a two-hit mouse model of environmental factor induced autism spectrum disorder

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A R T I C L E I N F O

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A B S T R A C T

Autism spectrum disorder (ASD) is a group of developmental diseases characterized by social dysfunction and repetitive stereotype behaviors. Besides genetic mutations, environmental factors play important roles in the development of ASD. Valproic acid (VPA) is widely used for modeling environmental factor induced ASD in rodents. However, traditional VPA modeling is low-inefficiency and the phenotypes often vary among different batches of experiments. To optimize this ASD-modeling method, we tested "two-hit" hypothesis by single or double exposure of VPA and poly:IC at the critical time points of embryonic and postnatal stage. The autistic-like behaviors of mice treated with two-hit schemes (embryonic VPA plus postnatal poly:IC, embryonic poly:IC plus postnatal VPA, embryonic VPA plus poly: IC, or postnatal VPA plus poly:IC) were compared with mice treated with traditional VPA protocol. The results showed that all single-hit and two-hit schemes produced core ASD phenotypes as VPA single treatment did. Only one group, namely, mice double-hit by VPA and poly:IC simultaneously at E12.5 showed severe impairment of social preference, social interaction and ultrasonic communication, as well as significant increase of grooming activity and anxiety-like behaviors, in comparation with mice treated with the traditional VPA protocol. These data demonstrated that embryonic two-hit of VPA and poly:IC is more efficient in producing ASD phenotypes in mice than the single-hit of VPA, indicating this two-hit scheme could be utilized for modeling environmental factors induced ASD.

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1. Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder which affects approximately 1 % of children around the world [1]. Deficits in social communication and social interaction, and repetitive behaviors are the core syndromes of ASD [2]. It is widely recognized that genetic and environmental factors and their interactions contribute to the development of ASD. Previous studies estimate that heritability accounts for approximately 38–55 % ASD cases [3]. Recent twin and family studies indicate a greater role for accumulative early environmental factors in inducing ASD [4]. Relative to genetic factors, how environmental factors elicit ASD has been poorly investigated.

Modeling environmental factor induced ASD is the first step for investigating the underlying mechanisms and assessing new treatments. Currently, several protocols have been adopted to mimic environmental interventions, which include prenatal exposure to sodium valproate (VPA), maternal autoantibodies and maternal immune activation [5]. Among them, VPA-induced ASD has been mostly adopted. VPA is originally used as an antiepileptic drug, also being used for treatment of bipolar disorders, migrain, headaches and neuropathic pain [6]. One of the first clinical observations reported that among four children–two sibling pairs–who were exposed in utero to valproic acid, 3 children showed fetal valproate syndrome and 1 showed infantile autism [7]. Later retrospective human studies documented that prenatal exposure to VPA, especially during the first trimester of pregnancy, is associated with high risk of ASD development [8]. In rodents, since the first report by Rodier et al. [9], extensive studies have been carried out on VPA-induced rodent model with the aim of uncovering molecular, electrophysiological, neuroanatomical mechanisms and testing new drugs [10, 11]. However, in our hands, we found this method is not as efficient as expected, either showing highly abortive in pregnant mice or inapparent ASD-like phenotype in offsprings.

In the real world, it is reasonable that single hit of certain environmental factor, whatever toxic chemical or inflammatory infection, even at high dosage or upon severe attack, is not sufficient for faithfully inducing ASD. Recently, a two-hit or multi-hit hypothesis was proposed to explain the development of some psychiatric disorders, such as schizophrenia [12]. Thus, it is possible that this two-hit or multi-hit hypothesis may be appliable to ASD. In the present study, we compared the effects of combined exposure of VPA with Poly:IC, a synthetic analog of double-stranded RNA used to mimic virus infection [13], with that of traditional single exposure of VPA, wishing to set up a more efficient model of environmental factor induced ASD.

2. Methods and materials

2.1. Mice

C57 mice were purchased from the Fourth Military Medical University's animal facility. The mice were allowed unrestricted access to food and water, and they were kept at room temperature with a 12-h light and dark cycle. The Fourth Military Medical University's Animal Care and Use Committee authorized all animal operations, which were carried out in accordance with ARRIVE principles. All mice in the same experimental group were randomly assigned to experimental treatment.

2.2. VPA/Poly:IC exposure

For the timed pregnancies, mice were set up in the late afternoon and plugs were checked in the next morning. The day when plugs were detected were designated as E0.5. Eight groups of experiments were set as the followings:

(1) E-VPA: pregnant mice were exposed to VPA at E12.5 (i.p, 500 mg/kg). Offsprings were raised to adult and ASD-like behaviors were evaluated.
(2) E-Poly: IC: pregnant mice were exposed to Poly: IC at E12.5 (i.p, 20 mg/kg). Offsprings were raised to adult and ASD-like behaviors were evaluated.
(3) E-VPA/P- Poly: IC: pregnant mice were exposed to VPA at E12.5 (i.p, 500 mg/kg), and the progenies were treated with poly:IC at P10 (i.p, 20 mg/kg) and raised to adult.
(4) E-Poly: IC/P-VPA: pregnant mice were exposed to poly:IC at E12.5 (i.p, 20 mg/kg). The progenies were treated with VPA at P10 (i.p, 166 mg/kg) and raised to adult.
(5) E-VPA/E-Poly: IC: pregnant mice were exposed to VPA (i.p, 250 mg/kg) and poly:IC (i.p, 20 mg/kg) at E12.5. Offsprings were raised to adult and ASD-like behaviors were evaluated.
(6) P-VPA/P-Poly: IC: neonatal pups were exposed to VPA (i.p, 166 mg/kg) and poly:IC (i.p, 20 mg/kg) at P10, and raised to adult.
(7) P-VPA: neonatal pups were exposed to VPA (i.p, 166 mg/kg) at P10, and raised to adult.
(8) P-Poly:IC: neonatal pups were exposed to poly : IC (i.p, 20 mg/kg) at P10, and bred up for experiments.

2.3. Three-chamber assay

As previously described [14], the test apparatus consisted of an opaque acrylic box (65 cm × 45 cm × 25 cm) containing three chambers (65 cm × 43 cm × 23 cm). Following habituation, a stimulus mouse was placed in the cylinder in the 'social chamber,' while the cylinder in the 'non-social chamber' remained empty. The test mice's time spent in the social and non-social rooms was assessed. The activity of each test mouse was recorded. Each chamber was cleaned with 75 % ethanol between testing. The behavior was

examined using the SMART 3·0 software (Panlab Harvard Apparatus, Spain). The preference score was calculated as (Time in social chamber - Time in non-social chamber)/(Time in social chamber + Time in non-social chamber).

2.4. Resident-intruder assay

The test was performed as previously described with minor modification [15]. Briefly, the resident mouse (referred to as the test mouse) was given the freedom to explore its home cage. Then, a novel mouse (around 3–4 weeks old) was introduced into the cage as an intruder. The test mouse was given 10 min to freely interact with the intruder mouse. Juvenile intruder mouse was chosen to prevent mutual aggression. The different intruder mouse was used in each test. The duration and frequency of direct contacts between the two mice were recorded.

2.5. Ultrasonic vocalization

Neonatal mice at P14 were placed inside a square transparent container (32 cm × 20 cm × 30 cm). An ultrasonic microphone was inserted through the top of the container. The sounds emitted by the mice were recorded using Ultramic 384K BLE (France) ultrasound microphones which had a flat frequency response ranging from 0 to 192 kHz. The sampling rate was 384 kHz, and the data was recorded in a 16-bit format. SeaWave 2.0 software was utilized for data acquisition and Deep SqueaK 3.0 software for data analysis. Our analysis focused on three aspects: (1) the number of ultrasonic vocalizations (USVs) between 25 and 90 kHz, (2) the duration of the calls measured in milliseconds, and (3) the principal frequency of the calls.

2.6. Grooming assay

A transparent acrylic cage (20 cm × 20 cm × 25 cm) was used as the grooming apparatus, with three opaque walls, one clear wall, and an opaque bottom. The recording began immediately after placing the mouse inside the cage and continued for a duration of 30 min. Following each test, the chamber was thoroughly cleaned using a 75 % ethanol. The number of grooming sessions, total grooming time and latency time of the first session were manually recorded and tallied by an experienced researcher who was blind to the treatment.

2.7. Marble burying assay

As previously described [16], the cage was prepared with wood chip bedding that was lightly tamped down to create a flat surface. Mice were provided with a habituation session and then 20 regular glass marbles were placed on the surface, each approximately 4 cm apart. Then, the mouse was placed in the cage, and recorded for 20 min. When the test was finished, the video was analyzed to determine the number of marbles buried.

2.8. Open field assay

Mice were placed in the center of a cuboid chamber (40 cm × 40 cm × 40 cm). Each test lasted for 10 min, during which the time spent in the center and outer zone, as well as the number of entries, were measured using an automated analysis system called SMART 3.0. Following each test, the chamber was cleaned using 75 % ethanol.

2.9. Elevated plus maze test

The apparatus consisted of two open channels (50 cm × 10 cm), and two same closed channels with 40 cm high walls, and a center platform (10 cm × 10 cm) connecting four channels. At the beginning of the test, the mice were placed in the central area, facing one of the open arms. Their behaviors were recorded for 5 min, and the travel path was measured using SMART 3.0. The apparatus was cleaned using 75 % ethanol after each test.

2.10. Rotarod test

Before testing, mice were trained for 3 days. Formal experiments were conducted on day 4. Mice were lowered slowly onto the rotating roller (with a grooved surface) and the timer was started when the mouse's tail was released. The starting speed of the rod was set at 4 rpm, accelerated to 40 rpm in 90 s, and lasted for 300 s. The latency to fall in each trial was recorded. Three trials were performed and the average falling time was adopted. If mice did not fall, the time was recorded as 300 s.

2.11. Statistical analysis

All the behavioral recordings were analyzed by a researcher blind to the experimental design. 7–21 mice were included for each group. Data were expressed as means ± standard error (S.E.) and analyzed by using GraphPad Prism 10.0 and SPSS 20.0 software. The homogeneity of variance test was assessed with Levene's test and the normality was assessed with the Shapiro-Wilk test. For the data which met the normality and homogeneity of variance: Student' t-test and One-way ANOVA were adopted. For those not: Mann-

Whitney *U* test or Kruskal-Wallis H test with Dunn's multiple-comparisons test, Welch's *t*-test, or Wilcoxon Signed-Rank test was conducted. Statistical significance was assessed at levels of $P < 0.05$.

3. Results

3.1. Experimental design

In traditional single risk factor protocols, 400–600 mg/kg VPA or 10–60 mg/kg Poly:IC has been used to induce ASD by different labs [11,17,18]. We adopted 500 mg/kg VPA or 20 mg/kg Poly:IC, the most widely used dosage in literatures, as the starting dose of single-exposure. To establish an optimal two-hit procedure, we set up the experiments with 8 groups at embryonic day 12.5 (E12.5, the time point around neural tube closure) and postnatal day 10 (P10, the starting point of cortical synaptogenesis): embryonic single exposure (E-VPA, E-Poly:IC), postnatal single exposure (P-VPA, P-Poly:IC), embryonic 2-hit exposure (E-VPA/E-Poly:IC), postnatal 2-hit exposure (P-VPA/P-Poly:IC), embryonic/postnatal 2-hit exposure (E-VPA/P-Poly:IC, E-Poly:IC/P-VPA) (Fig. 1A).

We first evaluated if the treatments were toxic. Mice in most group survived well. In E-VPA/E-Poly:IC group, treatment with 500 mg/kg VPA plus 20 mg/kg Poly:IC resulted in death of all 11 pregnant mice at 2 days post exposure, while 80 % of mice treated with 250 mg/kg VPA plus 20 mg/kg Poly:IC survived (Fig. 1B). Thus, we adopted 250 mg/kg VPA in this group.

In the procedures of postnatal VPA exposure, both 500 mg/kg and 250 mg/kg VPA were lethal (All mice died within 2 days of VPA exposure. Fig. 1C). 7 out of 8 mice treated with 166 mg/kg VPA survived. We thus adopted 166 mg/kg VPA in all postnatal procedures (Fig. 1A and C).

We next evaluated if the two-hit exposure had other long-term toxicities which may interfere behavior analysis by examining the motor function of these animals. Rotarod test revealed no difference of the running time among all 4 two-hit groups, E-VPA-treated group and saline control, suggesting that the two-hit protocol is relatively safe (Supplementary Fig. 1).

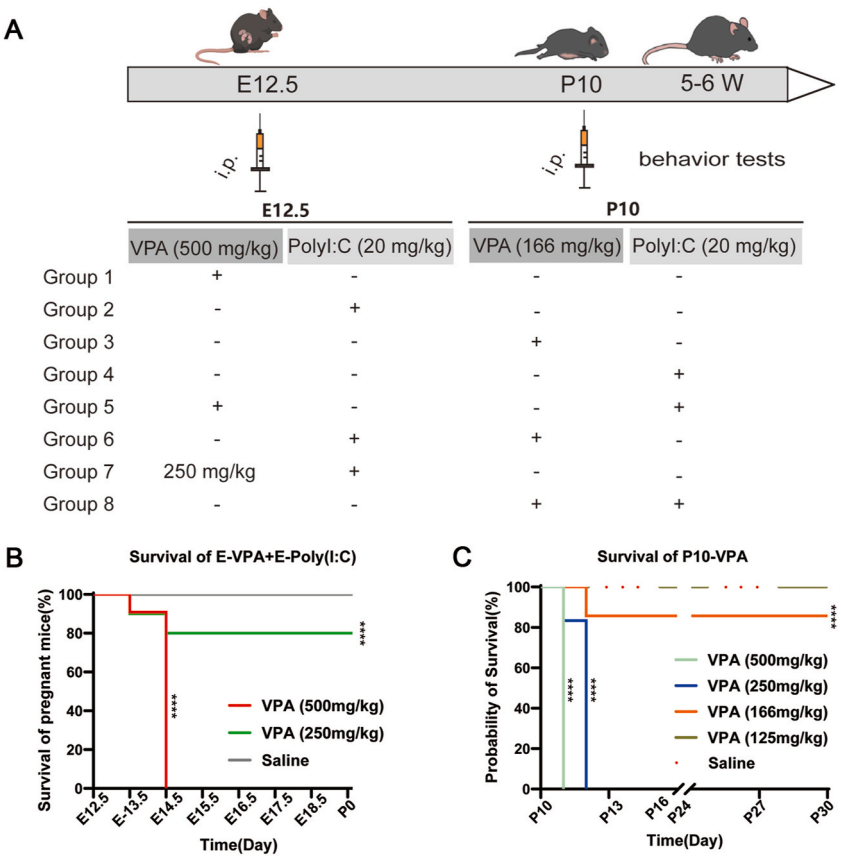

Fig-1. Scheme of experimental design and survival analysis. (A) The time and dose of VPA/poly:IC for each experimental group. (B) Survival analysis of pregnant mice in E-VPA/E-poly:IC group upon different doses of VPA exposure. N = 11 mice in 500 mg/kg VPA, 10 mice in 250 mg/kg VPA and 8 mice in saline treated group. (C) Survival analysis of mice in P-VPA group upon different doses of VPA exposure. N = 6 mice in 500 mg/kg VPA, 6 mice in 250 mg/kg VPA, 7 mice in 166 mg/kg, 8 mice in 125 mg/kg and 8 mice in saline treated group. ****$P < 0.0001$.

3.2. ASD-like phenotypes in VPA or Poly:IC single-hit models

All ASD-associated behaviors in this study were examined according to the following timeline. Ultrasonic vocalization (USV) of offsprings was evaluated at P14. Social novelty, social preference and social interaction were assessed at 5–6 weeks post birth. Grooming and marble-burying behaviors were examined at 5–6 weeks post birth. Anxiety-like behaviors were tested at 6–7 weeks post birth. We first validated the ASD-inducing effects of traditional VPA protocol (Supplementary Fig. 2A). The results showed that, in comparison with saline controls, VPA-pretreated mice emitted less numbers of ultrasonic calls, exhibited less interest in novel mice, and spent longer time grooming and burying marbles, showing the core syndromes of ASD (Supplementary Figs. 2B–I). However, we noticed that although VPA-pretreated mice displayed obvious social dysfunction, these mice only showed mild stereotype repetitive behavior. Only 9%–16 % VPA-pretreated mice exhibited longer grooming time or burying more marbles than saline control mice. Thus, this model requires large numbers of pregnant mice to obtain sufficient ASD offsprings for experimental studies.

Then, we compared the social behaviors and stereotype repetitive behaviors among four single-hit models (E-VPA, E-Poly:IC, P-

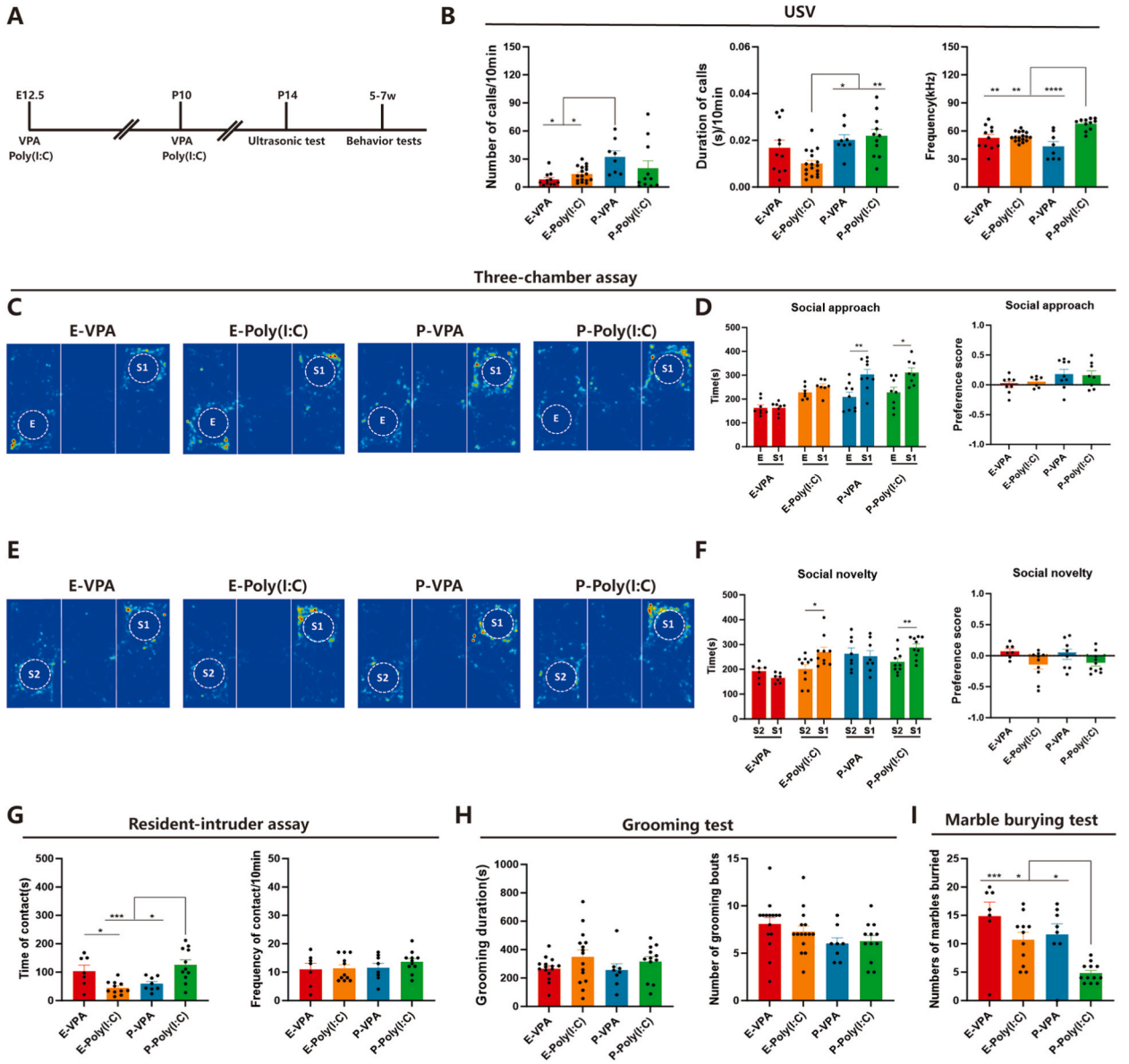

Fig-2. ASD phenotypes of single-hit of VPA/poly:IC. (A) Experimental design. (B) USV recording and quantification in four single-hit groups of mice. (C–F) Three-chamber assay of four single-hit groups of mice. (G) Resident-intruder assay. (H) Grooming test. (I) Marble-burying test. One way ANONA. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.N = 7–17 mice per group. E, empty. S1, social mice #1. S1, social mice #2. USV, ultrasonic vocalization.

VPA and P-Poly:IC, Fig. 2A). In comparison with E-VPA group, all other 3 groups did not show significant reduction of USV number, call duration and frequency (Fig. 2B). In three-chamber assay, no significant difference of social approach and social novelty was found among all four single-hit groups (Fig. 2C–F). In resident-intruder assay, the contacting time in E-Poly:IC pretreated mice was significantly shorter than that in E-VPA pretreated mice (Fig. 2G). In grooming test, all four groups of mice spent similar time self-grooming (Fig. 2H). In marble-burying test, P-Poly:IC pretreated mice showed significant decrease of marble burying, while other groups of mice not, in relative to that of E-VPA pretreated mice (Fig. 2I). Overall, these data demonstrated that single exposure of VPA or Poly:IC,

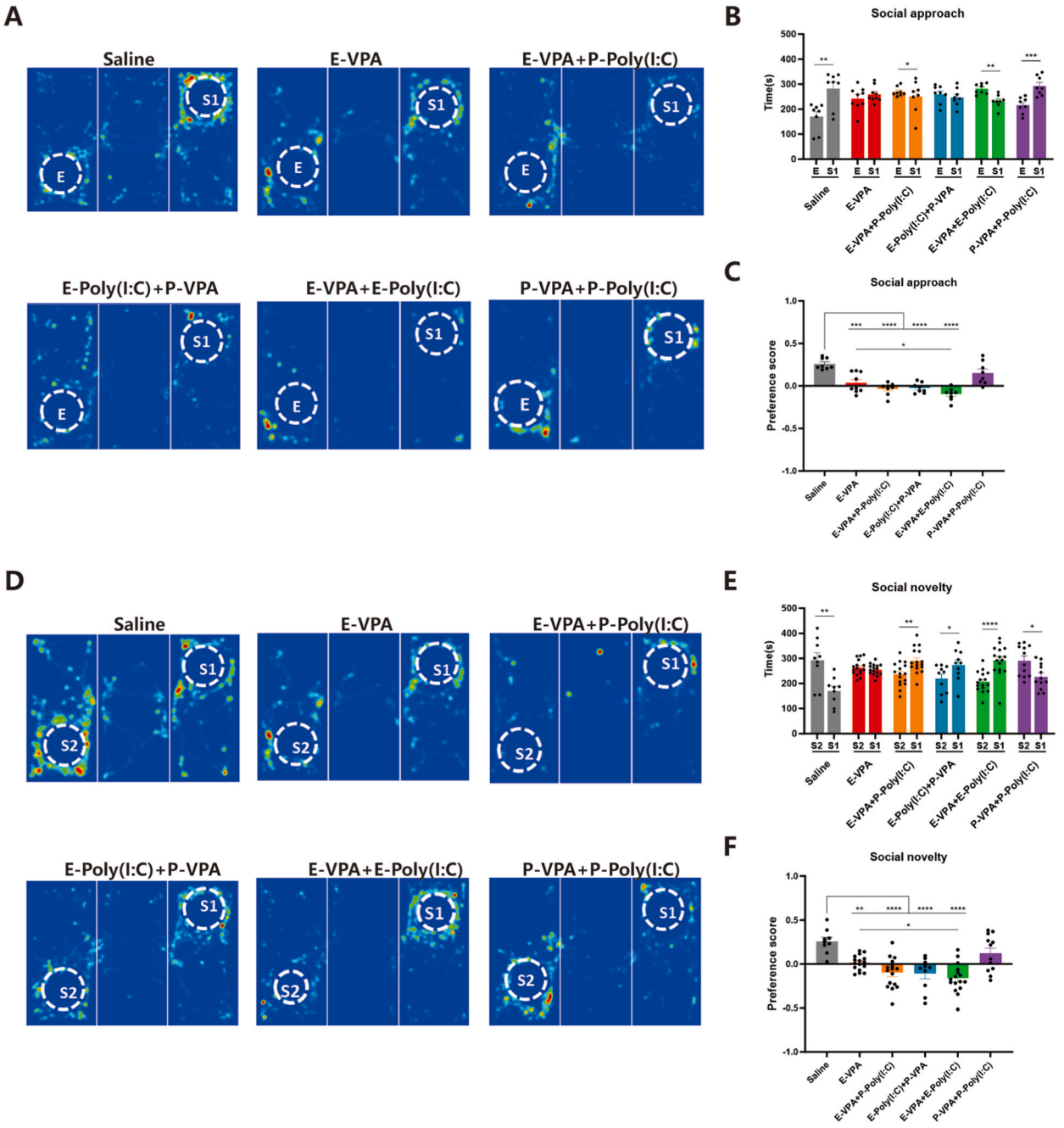

Fig-3. Three-chamber assay of ASD mice exposed to two-hit of VPA/poly:IC. (A–C) Social preference test of saline control, E-VPA, E-VPA/E-poly:IC, E-VPA/P-poly:IC, E-Poly:IC/P-VPA, P-Poly:IC/P-VPA. (D–F) Social novelty test of saline control, E-VPA, E-VPA/E-poly:IC, E-VPA/P-poly:IC, E-Poly:IC/P-VPA, P-Poly:IC/P-VPA. Notice the significant decrease of social preference and social novelty in E-VPA/E-poly:IC pretreated mice, as compared with E-VPA pretreated mice. One way ANONA. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$. N = 8–12 mice per group. E, empty. S1, social mice #1. S1, social mice #2.

either at embryonic stage or postnatal stage, are similar in their efficiencies of inducing ASD-like behaviors in mice.

3.3. Social behaviors of mice exposed to two-hit of VPA/poly:IC

To examine the effectiveness of two-hit procedures, we first evaluated sociability by three-chamber assay. In comparison with saline control, three groups (E-VPA/E-Poly:IC, E-VPA/P-Poly:IC, E-Poly:IC/P-VPA) showed impairment of both social approach and social novelty (Fig. 3A, B, 3D, 3E). Among these three groups, only E-VPA/E-Poly:IC pretreated mice showed significant reduction of social approach as compared with E-VPA pretreated mice (Preference score of E-VPA/E-Poly:IC pretreated mice was -0.09771 ± 0.02612, while that of E-VPA pretreated mice was 0.03594 ± 0.03884, $P = 0.0498$, Fig. 3C). In terms of social novelty, only E-VPA/E-Poly:IC pretreated mice showed remarkable reduction of social novelty, in comparison with that of E-VPA pretreated mice (Preference score of E-VPA/E-Poly:IC pretreated mice was -0.1624 ± 0.04143, while that of E-VPA mice was 0.01321 ± 0.02155, $P = 0.0348$, Fig. 3F).

In resident-intruder assay which assesses the social interaction of testing mice (Fig. 4A), all four two-hit groups showed significant reduction of contacting time with intruder mice as compared with saline control. In comparison with E-VPA pretreated mice, the time of social contacts of E-VPA/E-Poly:IC pretreated mice reduced by approximately 43 % ($P = 0.0348$). The time of social contacts in other 3 two-hit groups were similar as that of E-VPA pretreated group (Fig. 4B).

In maternal separation induced USV assay which reflects social communication ability, all 4 two-hit groups emitted less numbers of calls during the period of recording as compared with saline control (Fig. 5A and B). Similarly, only E-VPA/E-Poly:IC pretreated mice exhibited significant reduction of USV calls (4.2 ± 0.7051 calls/10 min), as compared with E-VPA pretreated mice (21.5 ± 3.471 calls/10 min, $P = 0.0413$, Fig. 5B). In terms of call duration, three groups (E-VPA/E-Poly:IC, E-VPA/P-Poly:IC, E-Poly:IC/P-VPA) showed significant decrease of average call duration as compared with saline control (Fig. 5C). No significant difference of call duration was found among these 3 groups. As to the frequency of USV calls which usually remain unchanged even in many ASD mice models, two groups (E-VPA/E-Poly:IC, E-VPA/P-Poly:IC) showed significant reduction of main frequency, as compared with saline control (Fig. 5D).

In together, these data indicated that two-hit of E-VPA/E-Poly:IC leads to more severe social dysfunction than traditional VPA protocol does.

3.4. Repetitive behaviors of mice exposed to two-hit of VPA/poly:IC

To evaluate stereotype behaviors, we first conducted grooming assay (Fig. 6A). The results showed that, 3 groups of mice (E-VPA/E-Poly:IC, E-VPA/P-Poly:IC, E-Poly:IC/P-VPA) spent significantly longer time grooming their hair than saline control. Among these 3 groups, E-VPA/E-Poly:IC pretreated mice spent about 50 % more time than that of E-VPA pretreated group in self-grooming ($P = 0.0036$, Fig. 6B). In marble burying assay which also reflects repetitive behavior, E-VPA/E-Poly:IC pretreated mice buried approximately 1.5 times of marbles as E-VPA pretreated mice did (Fig. 6C and D). These data indicated that two-hit of E-VPA/E-Poly:IC are more effective in inducing stereotype repetitive behaviors than traditional VPA protocol.

3.5. Anxiety-like behaviors of mice exposed to two-hit of VPA/poly:IC

Considering that anxiety is one of the most frequently observed concomitant symptoms of ASD patients [19], we examined the anxiety-like behaviors of these two-hit mice. In open field assay, all four two-hit groups of mice spent significant less time in the center

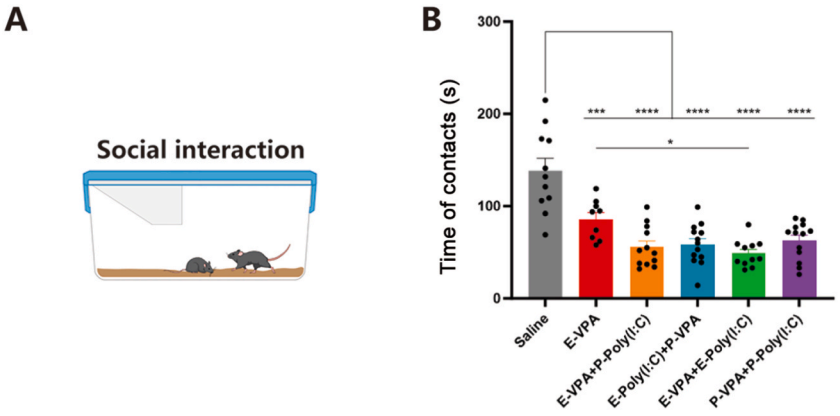

Fig-4. Resident-intruder assay of ASD mice exposed to two-hit of VPA/poly:IC. (A) Experimental diagram. Unfamiliar juvenile mouse was put into the home cage of ASD mice for testing social interaction. (B) Quantification of interacting time of saline control, E-VPA, E-VPA/E-poly:IC, E-VPA/P-poly:IC, E-Poly:IC/P-VPA, P-Poly:IC/P-VPA mice. Notice the significant decrease of interacting time of E-VPA/E-poly:IC pretreated mice, as compared with E-VPA pretreated mice. One way ANONA. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$. N = 9–13 mice per group.

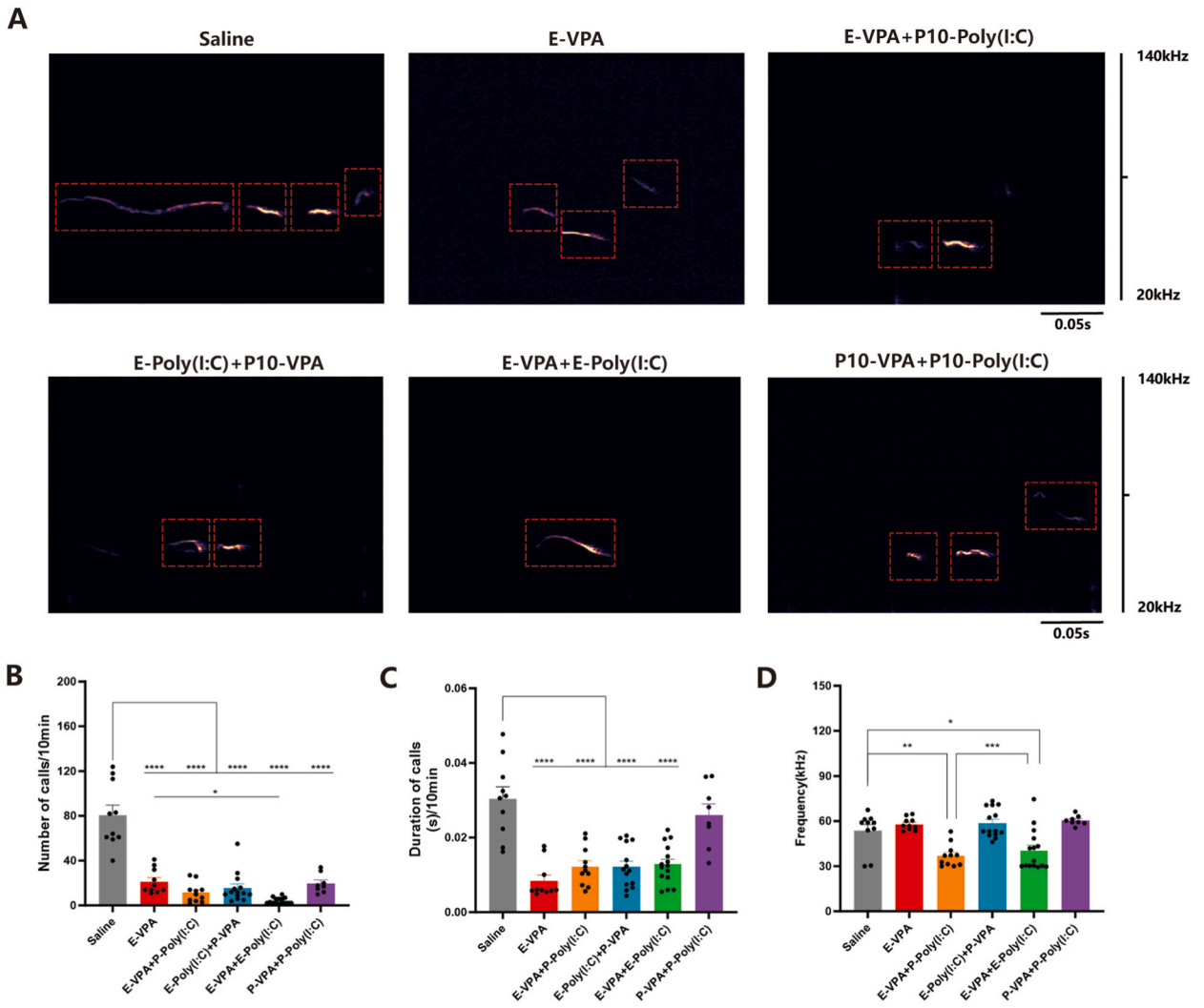

Fig-5. Ultrasonic vocalization of ASD mice exposed to two-hit of VPA/poly:IC. (A) Representative USV recordings of saline control, E-VPA, E-VPA/E-poly:IC, E-VPA/P-poly:IC, E-Poly:IC/P-VPA, P-Poly:IC/P-VPA mice. (B–D) Quantifications of call numbers, call duration and frequency. Notice the significant reduction of cell number of E-VPA/E-poly:IC pretreated mice, as compared with E-VPA pretreated mice. One way ANONA. $^{*}P < 0.05$. $^{***}P < 0.001$. $^{****}P < 0.0001$. N = 8–15 mice per group.

area of open field than saline control mice did (Supplementary Figs. 3A and 3B). As to the moving distance and entries in the center area, three groups of mice (E-VPA/E-Poly:IC, E-VPA/P-Poly:IC, E-Poly:IC/P-VPA) showed significantly less moving distance and entries, as compared with saline control (Supplementary Figs. 3C and 3D).

In elevated plus maze test, two groups of mice (E-VPA/E-Poly:IC, E-VPA/P-Poly:IC) spent less time and showed less moving distance in the open arm, relative to saline control. In comparison with E-VPA pretreated mice, only E-VPA/E-Poly:IC pretreated mice spent less time (35.7 ± 4.632 s vs 62.9 ± 7.043 s) and moved less distance (137.2 ± 26.02 cm vs 326.1 ± 27.24 cm) in open arm (Supplementary Figs. 4A–C). These data indicated that E-VPA/E-Poly:IC induced ASD mice are prone to concomitant anxiety.

4. Discussion

In the present study, by combining VPA with Poly:IC at embryonic and postnatal stage, we compared the ASD-inducing effects of four two-hit exposure procedures with traditional VPA-induced ASD model. Given that ASD-like behaviors vary substantially between different researches, we adopted a battery of recommended behavioral series [20], which include three chamber assay, resident-intruder assay, USV recording, open-field grooming, marble-burying, open-field movement assay and elevated plus maze test, to evaluate both the core and concomitant syndromes of ASD. Our data demonstrated that embryonic two-hit of VPA/Poly:IC is highly effective in inducing typical ASD phenotypes in the offsprings.

Relative to genetic factors, how environmental factors induce ASD has been poorly illustrated. Maternal and paternal age, fetal

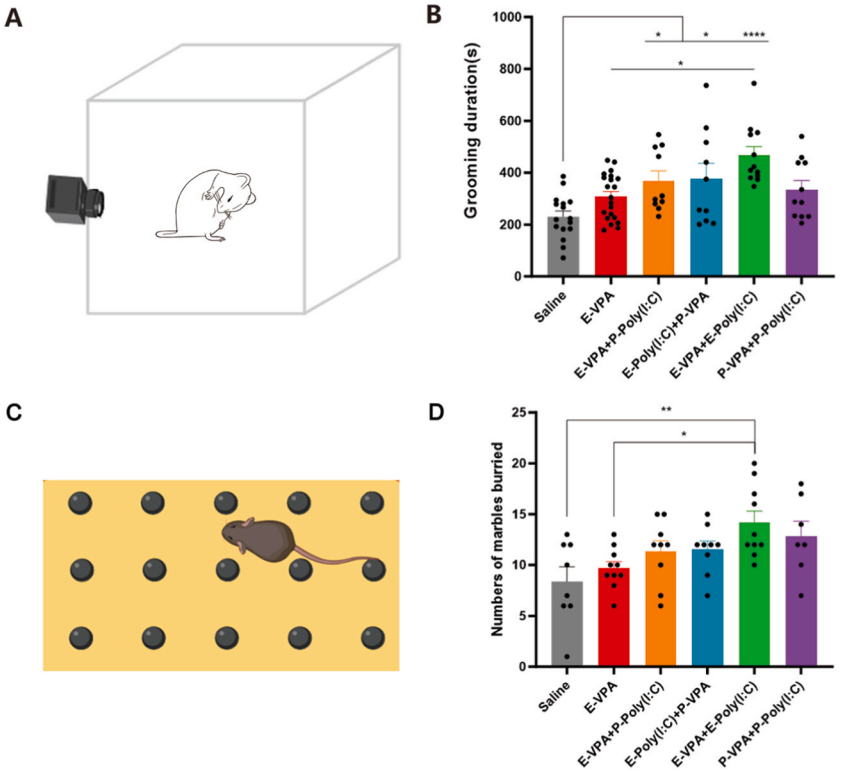

Fig-6. Grooming and Marble-burying assay of ASD mice exposed to two-hit of VPA/poly:IC. (A, B) Grooming assay of saline control, E-VPA, E-VPA/E-poly:IC, E-VPA/P-poly:IC, E-Poly:IC/P-VPA, P-Poly:IC/P-VPA mice. (C, D) Marble-burying assay of saline control, E-VPA, E-VPA/E-poly:IC, E-VPA/P-poly:IC, E-Poly:IC/P-VPA, P-Poly:IC/P-VPA mice. Notice the significant increase repetitive behavior of E-VPA/E-poly:IC pretreated mice, as compared with E-VPA pretreated mice. One way ANONA. *$P < 0.05$. ***$P < 0.001$. ****$P < 0.0001$. N = 7–21 mice per group.

environment, perinatal and obstetric events, medication, smoking and alcohol use, nutrition, vaccination, toxic exposures, inflammation and psychiatric stress are thought to cause atypical neural development [5]. Given the complexity of etiology, researchers started to pay attention to the "two-hit" or "multi-hit" hypothesis in the development of ASD. Several research groups have reported synergistical effects of gene-environment interactions in producing the core syndromes of ASD [21–23]. In terms of environmental factors, two models, namely simultaneous or cumulative multi-hit have been proposed [5,24,25]. Kulesza's group reported that the two-hit of repeated VPA exposure at E10.5 and E12.5 could result in severe cerebellar hypoplasia [26], which supports the cumulative multi-hit model. Our data that combined exposure of VPA and Poly:IC at E12.5 were more efficient than embryonic-postnatal sequential exposure of VPA/Poly:IC in inducing ASD-like phenotypes added weight to the simultaneous multi-hit model, at least in cases of chemical toxic exposure and virus-induced maternal immune activation. As both models are possible in real world, further works are needed to elucidate the most harmful combination of other environmental risk factors. On the other hand, our data was in line with the previously identified time window of E9.5-E14.5 for both VPA and Poly:IC [27], indicating E12.5 as a crucial time window for environmental factor attack.

Mechanistically, previous studies have revealed that VPA acts mainly on neurons or neural progenitor cells by hyperacetylation of histones, activation of Wnt signaling and inhibition of GABA transaminases [11,28,29]. Poly:IC mainly activates microglia and induces long-term elevation of pro-inflammatory cytokines in multiple brain regions [30]. Although neural developmental deficits have been reported for both models, researchers noticed significant variations in the behavioral abnormalities among the animals pretreated by VPA/Poly:IC single-hit [20]. Recent studies revealed that maternal exposure to VPA or Poly:IC caused sustained dysregulation of gene expression [31] and embryonic microglia maintain structural integrity during brain morphogenesis [32]. Thus, the neuron-microglia interaction may underlie the synergistic effects we observed. Detailed molecular mechanisms are worthy to be investigated in future.

5. Conclusion

Embryonic two-hit of VPA with Poly I:C at E12.5 is efficient in inducing ASD-like phenotypes in mice.

Ethics approval and consent to participate

All the animal experiments have been approved by The Fourth Military Medical University's Animal Care and Use Committee (File

No. 20211024).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Wei'an Zheng: Investigation, Methodology. **Mengmeng Wang:** Investigation, Methodology. **Yi Cui:** Investigation, Methodology. **Qing Xu:** Investigation. **Yujiang Chen:** Investigation. **Panpan Xian:** Investigation. **Qinghu Yang:** Conceptualization, Data curation, Project administration, Resources, Supervision, Validation, Visualization. **Shengxi Wu:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Yazhou Wang:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing interests which influence the work reported in this paper.

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Appendix ASupplementary data

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