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# Alterations of the endocannabinoid system and its therapeutic potential in autism spectrum disorder

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Autism spectrum disorder (ASD) is a group of developmental disabilities, the aetiology of which remains elusive. The endocannabinoid (eCB) system modulates neurotransmission and neuronal plasticity. Evidence points to the involvement of this neuromodulatory system in the pathophysiology of ASD. We investigated whether there is a disruption to the eCB system in ASD and whether pharmacological modulation of the eCB system might offer therapeutic potential. We examined three major components of the eCB system—endogenous cannabinoids, their receptors and associated enzymes—in ASD children as well as in the valproic acid (VPA) induced animal model in autism. Furthermore, we specifically increased 2-arachidonoylglycerol (2-AG) levels by administering JZL184, a selective inhibitor of monoacylglycerol lipase which is the hydrolytic enzyme for 2-AG, to examine ASD-like behaviours in VPA-induced rats. Results showed that autistic children and VPA-induced rats exhibited reduced eCB content, increased degradation of enzymes and upregulation of CBRs. We found that repetitive and stereotypical behaviours, hyperactivity, sociability, social preference and cognitive functioning improved after acute and chronic JZL184 treatment. The major efficacy of JZL184 was observed after administration of a dosage regimen of 3 mg kg<sup>-1</sup>, which affected both the eCB system and ASD-like behaviours. In conclusion, a reduced eCB signalling was observed in autistic children and in the ASD animal model, and boosting 2-AG could ameliorate ASD-like phenotypes in animals. Collectively, the results suggested a novel approach to ASD treatment.

## 1. Introduction

Autism spectrum disorder (ASD) is a collection of heterogeneous neurodevelopmental disorders and it is defined by impairment in communication and social interactions, as well as restricted, repetitive patterns of behaviour [1]. ASD affects approximately 1% of children in mainland China [2], which is comparable to Western countries, and its prevalence seems to be cumulatively increasing. The most recent prevalence estimate of ASD reached 1.85% (one in 54) among children aged 8 years old [3]. Despite its high prevalence and the public health burdens that result, there is a relatively limited understanding of the pathophysiology of ASD, aside from complex interactions between genetic and environmental factors. A multitude of recent publications have suggested that ASD is related to abnormalities in synaptic function. Thus, the endocannabinoid (eCB) system has attracted increasing interest for its potential

in the onset and/or progression of ASD, as this system could modulate different neurotransmitter system, synaptic excitation and inhibition (E/I balance) and plasticity in the brain, and it could also be associated with social interaction, motor control, repetitive behaviours, emotional processing, learning and memory.

The eCB system consists of three major components, i.e. endogenous cannabinoids (eCBs), their receptors and associated enzymes. The most active eCBs are anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which act mainly through cannabinoid type-1 and type-2 receptors (CB1Rs and CB2Rs) that are distributed throughout the central nervous system (CNS). In addition, palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), which are structurally similar to AEA (collectively known as *N*-acylethanolamines), share the same catalyzed enzymes required for their metabolism. *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), which are involved in the synthesis of *N*-acylethanolamines and 2-AG, respectively. Given that eCBs are not stored in any cellular compartment for later use, they are rapidly inactivated by their hydrolytic enzymes, i.e. fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). MAGL, as the predominantly 2-AG degraded enzyme, accounts for up to 85% of 2-AG hydrolysis in the brain [4]. Inhibitors of FAAH and MAGL are the most common tools to manipulate signalling of eCBs. The eCBs are produced on demand from membrane-bound phospholipids in postsynaptic neuronal membranes and act as retrograde messenger on presynaptic CBRs to dampen the release of neurotransmitters (e.g. monoamine, opioids, GABA, glutamate, acetylcholine), thereby affecting a wide range of biological processes [5]. The eCB-mediated retrograde suppression is considered to be a ubiquitous and important form of activity-dependent synaptic modulation.

Indeed, the dysregulation of the eCB system has been documented in relation to several neuropsychological and neurodevelopmental diseases, including ASD. Two case-control studies found decreased circulating levels of AEA, PEA and OEA in ASD children compared with matched healthy children [6,7]. Siniscalco's team reported alterations of the receptors and enzymes in the eCB system in the peripheral blood mononuclear cells (PBMCs) of autistic children [8,9]. It was also suggested that disruption to eCB metabolism was observed in the brain in the case of both genetic and environmental models of ASD. However, these studies appeared to contradict one another. For instance, in *Fmr1* knockout mice, the most common genetic form of autism, MAGL activation was enhanced in the frontal cortex and striatum [10]. In VPA-induced rats, the well-known environmental-based model, there was a reduction in MAGL expression and unaltered levels of AEA, PEA, OEA and 2-AG [11]. Melancia *et al.* [12] showed that CB1R activation decreased, while Zamberletti *et al.* [13] found CB1R up-regulation. BTBR, an inbred mouse strain known to model of idiopathic autism, revealed a higher density of CB1Rs [14]. Furthermore, enhancing AEA signalling partially attenuated social behaviour deficits in these three ASD models [12,15,16]. Although still debated, it is plausible that alternations of the eCB system may contribute to the pathogenesis of ASD.

Generally speaking, 2-AG, which is the most abundant eCB in the brain, is found in much higher concentrations

than *N*-acylethanolamines in the brain (i.e. approx. 1000-fold higher than AEA), and it executes full agonist activity at CBRs with a high efficacy, while AEA is a partial agonist, and PEA and OEA have a lower affinity [5]. Considering that 2-AG plays a broader role in the integrity of the brain's eCB system and CNS development, 2-AG may be a more relevant indicator of eCB tone [17]. However, changes in 2-AG levels have not been reported in individuals with ASD, and evidence of whether enhancing 2-AG tone could cause an improvement in ASD-like behaviours is limited [18,19]. In this study, we examined all three major components of the eCB system, namely, eCBs (AEA, PEA, OEA and 2-AG), CBRs (CB1R and CB2R) and related catalyzed enzymes (NAPE-PLD, FAAH, DAGL and MAGL), in ASD children as well as in the VPA-induced ASD animal model, in order to comprehensively characterize the involvement of the eCB system in the pathogenesis of ASD. In addition, we investigated the effect of altered 2-AG signalling on autistic behaviours, and examined whether behavioural changes exhibited by VPA-induced rats are associated with eCB dysfunction in discrete brain regions, which are known to modulate cognitive and social behaviour, namely the hippocampus and prefrontal cortex (PFC). The present study was designed to better understand the critical role of eCB system in the aetiology of ASD, and provide a novel strategy for the treatment in managing symptoms of ASD.

## 2. Material and methods

### 2.1. Participants

We investigated 70 autistic patients and 70 age- and gender-matched controls (age range 3–12). The 70 autistic patients were recruited from the Child Development and Behaviour Research Center of Harbin Medical University and special education schools, Harbin, China. The inclusion criterion was a diagnosis of ASD, which was made by two independent specialist clinicians according to the *Diagnostic and Statistical Manual of Mental Disorders*, 5th edition (DSM-5) [1]. Exclusion criteria were children with significant sensory and motor impairment, known genetic disorders, seizures at the time of enrolment or other neurological disorders. Seventy unrelated healthy children without a history of developmental delay or other neurological disorders were randomly selected from normal kindergartens and junior schools in Harbin, China as the control group. All procedures are conducted with the written consent of the guardians or parents and approved by the ethics committee of Harbin medical university prior to the study.

The following measures were used as an aid for diagnosis and assessment: Autism Diagnostic Observation Schedule (ADOS), Autism Diagnostic Interview-Revised (ADI-R), Autism Behaviour Checklist (ABC), Childhood Autism Rating Scales (CARS), Vineland Adaptive Behaviour Scale second edition (VABS) and Social Responsiveness Scale (SRS). Sample characteristics are provided in electronic supplementary material, table S1.

### 2.2. Animals and treatments

Adult male and female Wistar rats were purchased from a commercial breeder (YISI, Benxi, China) and housed four per

cage in a controlled environment ( $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ;  $50\% \pm 10\%$  humidity). All rats had free access to water and food and were maintained on a 12 h light/dark cycle. Animals were allowed to acclimatize for one week prior to the experiments. The rat model of ASD was established according to previous studies [20,21]. Briefly, female and male rats were allowed to mate overnight. Pregnancy was determined by the presence of a vaginal plug the next morning, and noon of that day was defined as the embryonic day (E)0.5. Sodium valproic acid (VPA; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.9% saline at a concentration of  $250 \text{ mg ml}^{-1}$ , and pregnant rats received a single intraperitoneal (i.p.) injection of  $600 \text{ mg kg}^{-1}$  VPA or an equal volume of saline (VPA-treated and control groups, respectively) on E12.5. Pregnant rats were individually housed and allowed to raise their own litters. The offspring were weaned on postnatal day (PND) 21 and housed with 4–5 per cage. Experiments were performed on male offspring.

Animals were administered (4-nitrophenyl) 4-[bis(1,3-benzodioxol-5-yl)-hydroxymethyl] piperidine-1-carboxylate (i.p., JZL184, Selleck, Houston, TX, USA), a selective inhibitor of MAGL that enhances the levels of 2-AG. JZL184 was dissolved in dimethyl sulfoxide (DMSO) to prepare a mother liquor at a concentration of  $50 \text{ mg ml}^{-1}$ . It was then diluted with DMSO (5%), PEG400 (40%), Tween 80 (5%) and double distilled water into a clear working solution made up to a volume of  $2.5 \text{ mg ml}^{-1}$ . Doses of JZL184 were selected based on the results of previous studies [22,23]. The male offspring from different dams were randomly divided into six groups as follows (figure 3):

- (i) CON (injection with vehicle solution);
- (ii) VPA (injection with vehicle solution);
- (iii) VPA + 40AJ ( $40 \text{ mg kg}^{-1}$ , i.p., acute JZL184 injection once on PND35);
- (iv) VPA + 1RJ ( $1 \text{ mg kg day}^{-1}$ , i.p., repeated JZL184 injection from PND21–34);
- (v) VPA + 3RJ ( $3 \text{ mg kg day}^{-1}$ , i.p., repeated JZL184 injection from PND21–34);
- (vi) VPA + 10RJ ( $10 \text{ mg kg day}^{-1}$ , i.p., repeated JZL184 injection from PND21–34).

The increase in brain 2-AG levels by JZL184 administration persisted for at least 26 h, indicating that 2-AG could remain elevated throughout the repeated dosing regimen [17]. Twenty-four hours after the last repeated injection and 2 h after the acute injection, animals from each group were anaesthetized with an intraperitoneal injection of 10% chloral hydrate ( $0.3 \text{ ml kg}^{-1}$ ). The animals were sacrificed by decapitation for liquid chromatography-tandem mass spectrometry (LC-MS/MS), quantitative PCR (qPCR) and western blot assay. The brains were removed and the hippocampus and PFC were dissected out, flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Starting from PND35, a series of behavioural tests were performed. In the present study, the biochemical tests were carried out on rats of untested behavioural experiments. The behavioural testing and biochemical testing were conducted on 5–9 rats for separate experiments.

### 2.3. Behavioural testing

Behavioural testing was captured by video cameras and analysed using the SMART (Spontaneous Motor Activity

Recording and Tracking) v. 3.0 software system (Panlab, Barcelona, Spain). The apparatus was cleaned with 0.1% acetic acid between trials to preclude olfactory cues. All behavioural experiments were performed during the light cycle between 09.00 and 17.00, and testing was counterbalanced across treatment groups.

#### 2.3.1. Marble burying test

A clean cage ( $48 \times 35 \times 20 \text{ cm}$ ) was prepared with 5 cm fresh wood chip bedding material. On PND 35, a rat was placed individually in the cage for 15 min for habituation. They were then returned to their home cage. This rat was reintroduced onto the bedding material containing 20 embedded marbles for 30 min, and the number of marbles buried (i.e. covered with wood chip by more than two-thirds volume) was recorded.

#### 2.3.2. Self-grooming test

On PND 35, the rats were placed individually into a white cage ( $48 \times 35 \times 20 \text{ cm}$ ) and allowed to habituate for 5 min. Self-grooming behaviour was recorded for 10 min. A timer was used to assess the cumulative time spent performing self-grooming behaviour, which included paw licking, unilateral and bilateral strokes around the nose, mouth and face, paw movement over the head and behind ears, body fur licking, body scratching with hind paws, tail licking and genital cleaning.

#### 2.3.3. Open field test

On PND 35, the open field was made out of charcoal grey plastic with a top opening. The dimensions of the test box were  $45 \times 45 \times 40 \text{ cm}$ . Before the test, the rats were allowed to adapt to the test box for 5 min. Rats were individually placed in the centre to initiate a 10 min test. The total distance moved and resting time of spontaneous activity, which were used as indicators of anxiety-related behaviours, were analysed.

#### 2.3.4. Three-chamber test

On PND 35, the three-chamber test was used to evaluate the social behaviour of rats. Following acclimation (the rats were placed in the central chamber for 5 min, and the rats were allowed to freely access all chambers), two successive tests (i.e. sociability test and social preference test), which were 10 min in duration, respectively, were carried out. Sociability test: Animals were briefly confined to the central chamber while an unfamiliar rat (labelled as stranger 1) was confined in a small wire cage which was placed in one of the outer chambers. An identical empty wire cage was placed in the other chamber. Social preference test: A novel unfamiliar rat (labelled as stranger 2) was then placed in the empty cage. The sociability index was calculated as the ratio of time spent exploring stranger 1 over the empty cage. The social preference index was calculated as the ratio of the time spent exploring stranger 2 over stranger 1. Familiar and unfamiliar rats originated from different home cages and had never been in physical contact with the subject mice or each other.

#### 2.3.5. Morris water maze test

On PND 36–40, the learning and spatial memory capabilities of rats were evaluated using the Morris water maze test. The

apparatus consisted of a circular black water tank (180 cm in diameter and 58 cm deep) filled with water (about 42 cm deep) at a temperature of 19–21°C. The apparatus was concealed with black curtains, with extra visual cues inside the curtains. A circular platform (10 cm in diameter) was always fixed at 2 cm below the water surface in the centre of the first quadrant of the pool. The test period was divided into two phases. Phase 1: The training trial was carried out continuously for 4 days, twice daily in the same time period. The rats were placed into the water facing the tank wall in a set of semi-randomly selected distal starting positions each day, and the escape latency from the start of swimming to reach the platform was recorded as an index of learning. If the rat failed to reach the platform within 60 s and the latency value was recorded as 60 s, then it was guided to the platform and allowed to remain on the platform for 15 s. Phase 2: On the 5th day, the rats were subjected to a spatial probe trial session during which the platform was removed from the pool. The rats entered the pool from the third quadrant, and the number of times that rats passed through the circular area of the original platform within 60 s was recorded as an index of spatial memory. Given that five consecutive days of tests were conducted, this test was not performed with the rats in the acute injection group.

## 2.4. Biochemical testing

### 2.4.1. Quantitation of eCB levels by LC-MS/MS

Fasting blood samples were collected into EDTA-evacuated tubes in the morning (7.30–8.30) and immediately chilled on ice before centrifuging at 2000 r.p.m. for 20 min at 4°C. Deuterated internal standards AEA-D8, PEA-D4, OEA-D2, 2-AG-D5, arachidonic acid (AA)-D11 (Cayman Chemicals, MI, USA) were used.

A 300 µl volume of methanol (containing internal standards: AEA-D8 at 40 ng ml<sup>-1</sup>, OEA-D2 at 40 ng ml<sup>-1</sup>, PEA-D4 at 40 ng ml<sup>-1</sup> and 2-AG-D5 at 160 ng ml<sup>-1</sup>) was added to a 100 µl aliquot of plasma sample for protein precipitation. The mixture was eddied for 5 min and centrifuged at 16 000 × g for 10 min, and 1 µl of the supernatant was injected into the LC-MS/MS system.

An LC-20ADXR high-performance liquid chromatography (UPLC) system (Shimadzu, Nagoya, Japan) was interfaced with a Sciex Q-trap 5500 mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an electrospray ionization (ESI) source. Data were acquired using Analyst software (v. 1.6.2, Applied Biosystems). The sample vials were maintained at 4°C in a thermostatic autosampler. Chromatographic separation was achieved at 40°C on an Acquity ultra-HPLC HSS T3 column (100 × 2.1 mm, 1.7 µm; Waters, Milford, MA, USA) and VanGuard column (5 mm × 2.1 mm, 1.7 µm; Waters, USA). The mobile phase A was water containing 0.1% formic acid and phase B was acetonitrile. A mobile phase gradient was applied at a flow rate of 0.3 ml min<sup>-1</sup>. The gradient elution was 0–1 min, 5% B; 1–6.0 min, 5–40% B; 6–7 min, 40–100% B; 7–9 min, 100% B. The equilibration time after the gradient was 3 min.

The mass spectrometer was operated in the positive ESI mode with multiple reaction monitoring (MRM) at unit resolution. Nitrogen was used as the nebulizer, heater and curtain gas as well as the collision-activated dissociation gas. The precursor-to-product ion transitions, declustering potential (DP) and collision energy (CE) are listed in electronic supplementary material, table S2. Optimal parameters

were as follows: Nebulizer, heater and curtain gas flow rates of 50, 55 and 40 units, respectively; ion spray needle voltage of 5500 V; heater gas temperature of 550°C; and collision gas (N<sub>2</sub>) medium.

A 50 mg (±0.5 mg) section of brain tissue was weighed in a 2.0 ml Lysis Tube containing 1 mm ceramic beads. The sample was homogenized for 60 s by using a Speed Mill PLUS (ANALYTIKJENA). A 100 µl methanol (containing internal standards: AEA-D8, PEA-D4, OEA-D2 and 2-AG-D5 at 100 ng ml<sup>-1</sup>, AA-D11 at 6 µg ml<sup>-1</sup>) and additional 200 µl methanol was added into the sample. The tube was vortexed for 10 s. Then 1 ml MTBE was added and the mixture was incubated for 1 h at room temperature in a shaker. Phase separation was induced by adding 250 µl of MS-grade water. The sample was incubated at room temperature for 10 min and centrifuged at 16 000 × g for 10 min. A 500-µl upper (organic) phase was collected and dried in a vacuum centrifuge (Savant SPD131DDA SpeedVac, Thermo fisher). Dry residue was re-dissolved in 200 µl of acetonitrile/isopropanol (1 : 1).

Acquity UPLC H-Class (Waters, Milford, MA, USA) was interfaced to a Waters Xevo tq-s micro mass spectrometer (Milford, Massachusetts, USA) with an ESI source. Data were acquired using Masslynx (v. 4.1 package, Waters, Milford, MA, USA). The sample vials were maintained at 4°C in a thermostatic autosampler. Chromatographic separation was achieved at 45°C on an Acquity UPLC BEH C8 column (100 mm × 2.1 mm, 1.7 µm; Waters, Milford, MA, USA). The mobile phase A was acetonitrile/water (60/40) and mobile phase B was isopropanol/acetonitrile (90/10). Both A and B contained 0.1% formic acid and 10 mmol l<sup>-1</sup> ammonium acetate. A mobile phase gradient was applied at a flow rate of 0.3 ml min<sup>-1</sup>. The gradient elution was 0–1 min, 15% B; 1–4.0 min, 15–40% B; 4–8 min, 40–70% B; 8–9 min, 70–100% B. The equilibration time after the gradient was 3 min.

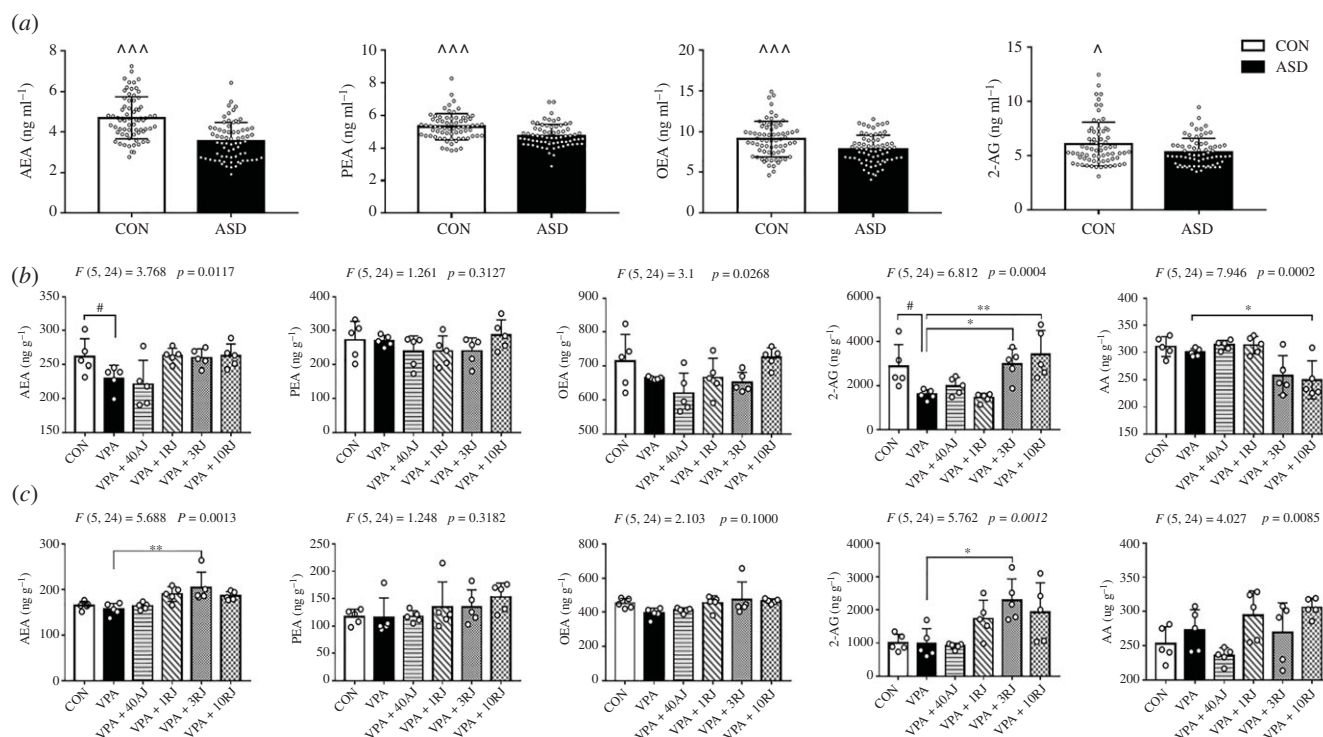
The mass spectrometer was operated in the positive ESI mode with MRM at unit resolution. Nitrogen was used as the desolvation gas. The precursor-to-product ion transitions, cone voltage and CE are listed in electronic supplementary material, table S2. Optimum parameters were as follows: cone gas flow 10 l h<sup>-1</sup>; capillary voltage 3000 V; desolvation temperature 550°C; desolvation gas flow, 1000 l h<sup>-1</sup>.

### 2.4.2. Isolation of peripheral blood mononuclear cells

Human venous blood samples from ASD subjects and control donors were drawn and collected in sterile EDTA tubes. Peripheral PBMCs were isolated by centrifugation over Histopaque 1077 density gradient. Briefly, blood was diluted 1 : 1 in phosphate buffer saline (PBS), overlaid onto lymphocyte separation media, centrifuged at 2000 r.p.m. for 30 min at room temperature and plasma was removed. Mononuclear cell fraction was harvested and washed twice in PBS. The final pellet was resuspended in Trizol Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) or protein lysis buffer for further molecular analysis.

### 2.4.3. RNA extraction, reverse transcription and qPCR

Total RNA was extracted from PBMCs and brain tissue using Trizol Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. RNA quantity was determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and



**Figure 1.** The levels of endogenous cannabinoids. (a) The plasma levels of AEA, PEA, OEA and 2-AG in the participants ( $n = 70$  children per group). Effects of JZL184 treatment on the levels of AEA, PEA, OEA, 2-AG and AA in hippocampus (b) and in the PFC (c) in VPA-exposed offspring ( $n = 5$  pups per group). The error bars represent s.d. (a) Results were analysed by paired Student's *t*-test ( $\wedge p < 0.05$ ,  $\wedge\wedge\wedge p < 0.001$ ). (b,c) Results were analysed by one-way analysis of variance with Dunnett's *post hoc* test ( $\#p < 0.05$ , versus CON group;  $*p < 0.05$ ,  $**p < 0.01$ , versus VPA group). AJ, acute JZL184 treatment; RJ, repeated JZL184 treatment.

purity assessed by A260/A208 ratio. RNA was reverse transcribed to cDNA using high-capacity cDNA reverse transcription kits (Applied Biosystem Inc., Foster City, CA, USA), with the following thermal protocol: 10 min at 25°C, 2 h at 37°C, 5 min for 85°C and for 4°C. The qPCR was performed with SYBR Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA) on a Light cycler 96 system (Roche Applied Science, USA). The thermal cycling conditions were as follows: 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. The corresponding primers were showed on electronic supplementary material, table S3. Each qPCR was repeated at least three times to achieve the best reproducibility data. GAPDH was used as an endogenous control to normalize gene expression data. Amplification of the genes of interest and GAPDH was performed simultaneously. Relative mRNA expression was determined based on the cycle threshold (CT) and calculated using the equation  $2^{-\Delta\Delta CT}$ :  $\Delta CT_{\text{treatment}} = CT_{\text{target}} - CT_{\text{GAPDH}}$ ;  $\Delta CT_{\text{control}} = CT_{\text{target}} - CT_{\text{GAPDH}}$ ;  $\Delta\Delta CT = \Delta CT_{\text{treatment}} - \Delta CT_{\text{control}}$ .

#### 2.4.4. Protein extraction and western blotting

PBMCs and brain tissue lysed on ice for 30 min in RIPA lysis buffer containing phenylmethylsulfonyl fluoride and centrifuged at 12 000 r.p.m. for 15 min at 4°C; the supernatant was immediately transferred to a fresh tube on ice. Protein concentration was measured with the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) with a bovine serum albumin standard concentration curve and absorbance readings at 562 nm on a spectrophotometer. Equivalent amounts of protein (30  $\mu\text{g}$ ) were separated by 10% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidenedifluoride membrane that was blocked with

5% nonfat milk and probed with primary antibodies against CB1R, CB2R, NAPE-PLD, FAAH, DAGL- $\alpha$ , MAGL and GAPDH. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit, and goat anti-mouse secondary antibody (antibodies details seen in electronic supplementary material, table S4). Protein bands were detected with an enhanced chemiluminescence western blotting detection kit (Beyotime Institute of Biotechnology, Shanghai, China). Results were analysed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA) to obtain the optical density ratio of the target protein to GAPDH. Measurements were obtained for triplicate samples.

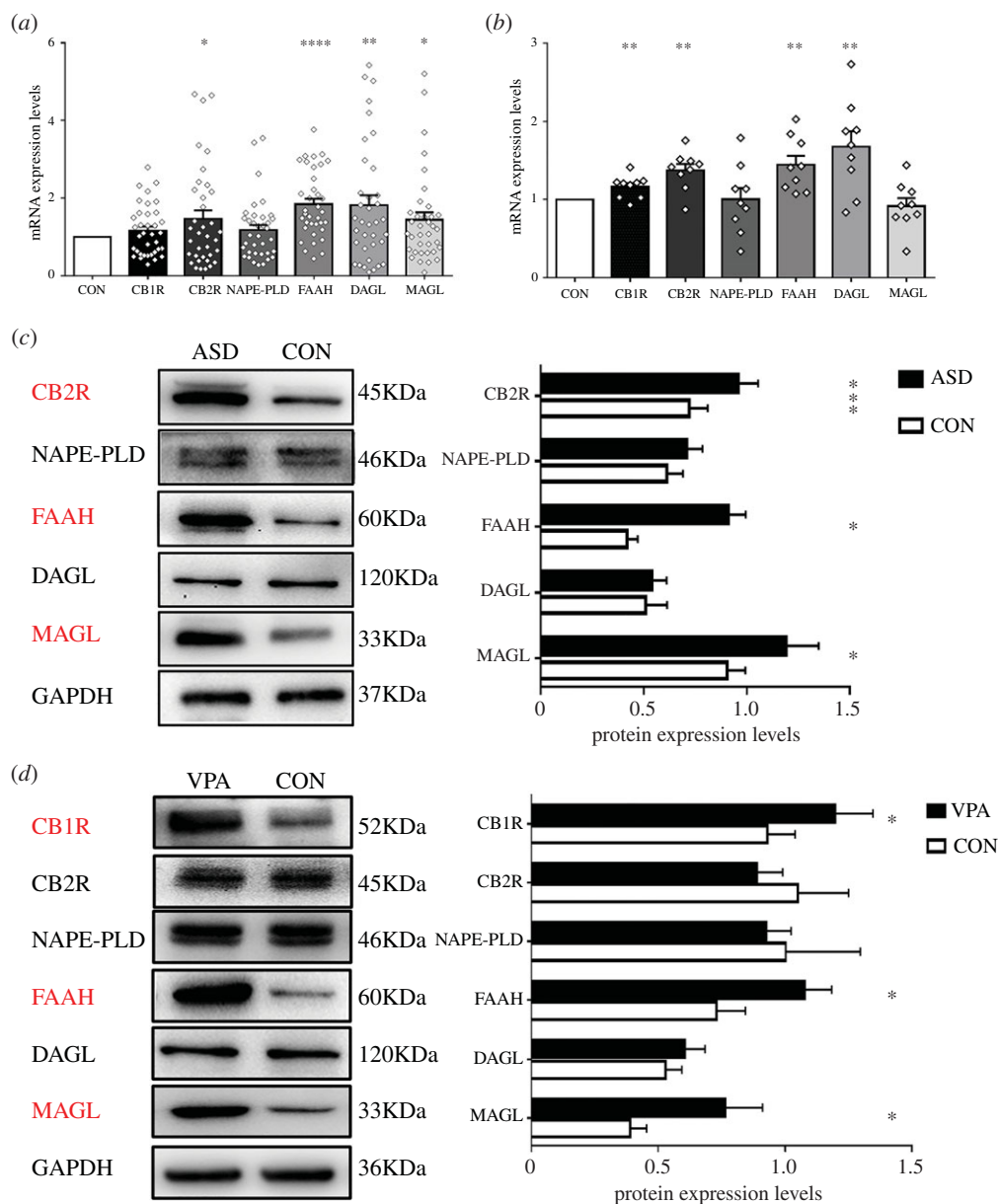
### 2.5. Statistical analyses

The results were presented as means  $\pm$  s.d. or means  $\pm$  s.e.m. which were analysed using GraphPad Prism 7.0 (GraphPad software, CA, USA). The comparison of the data was analysed by carrying out a one-way analysis of variance (ANOVA) or paired Student's *t*-test. A repeated-measures ANOVA was conducted to evaluate the differences in escape latency in the Morris water maze test. Dunnett's *post hoc* test was applied for multiple comparison (comparing all groups to VPA group). All reported *p* values were two-tailed, and the statistical significance was set at the  $\alpha = 0.05$  level.

## 3. Results

### 3.1. Comparison of the components of the eCB system between cases and controls

Autistic children had lower plasma concentrations of AEA, PEA, OEA and 2-AG than healthy controls ( $p < 0.05$ ; figure 1a;



**Figure 2.** The components of eCB system expression levels in cases and controls. (a) Relative mRNA expression levels normalized to GAPDH in PBMCs of participants ( $n = 36$  children per group). (b) Relative mRNA expression levels normalized to GAPDH in hippocampus of rats ( $n = 9$  pups per group). (c) Relative protein expression levels normalized to GAPDH in the PBMCs of participants ( $n = 27$  children per group); while CB1R protein expression was not detected. (d) Relative protein expression levels normalized to GAPDH in the hippocampus of rats ( $n = 9$  pups per group). Specificities of monoclonal antibodies (except for CB1R: polyclonal antibody). Data represented as means  $\pm$  s.e.m. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  from paired student's  $t$ -test.

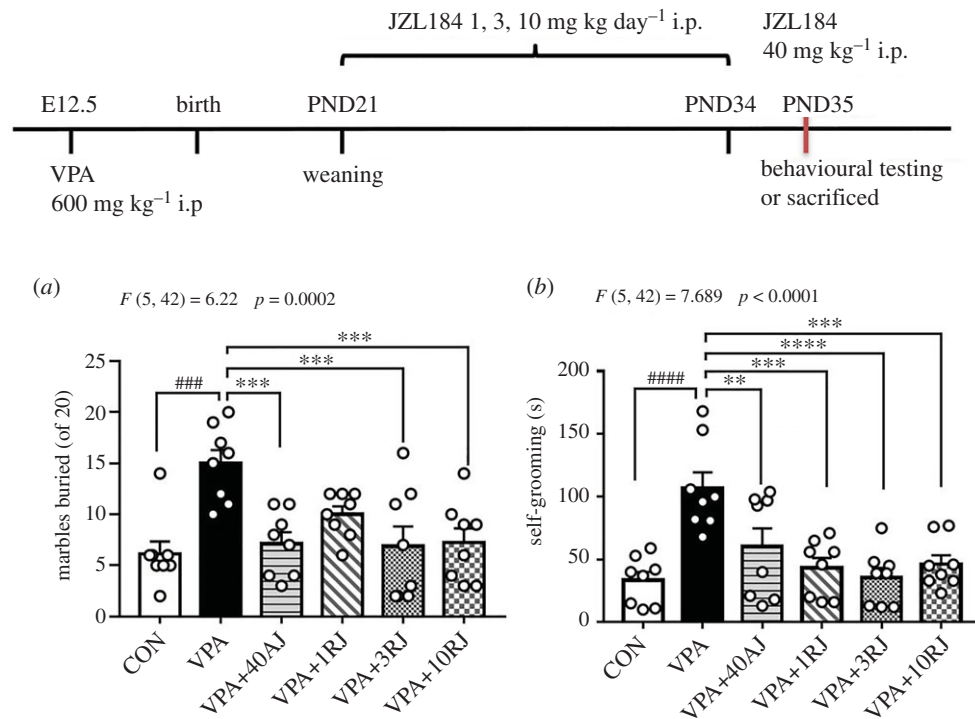
electronic supplementary material, table S5). The levels of PEA in the ASD group were negatively correlated with the total scores of the ABC ( $r = -0.326$ ,  $p = 0.013$ , data not shown). However, only AEA and 2-AG levels in the hippocampus were significantly reduced in VPA-exposed rats compared to controls ( $p < 0.05$ ; figure 1b), and there was no significant difference in the AEA, PEA, OEA and 2-AG levels in the PFC (figure 1c).

CB2R, FAAH, DAGL and MAGL mRNA levels in PBMCs from autistic children were significantly higher than those from the healthy controls, and CB2R, FAAH and MAGL protein levels in PBMCs were also higher ( $p < 0.05$ ; figure 2a,c). However, CB1R protein expression was not detected. Due to no significant differences in eCB levels in the PFC, we then solely investigated hippocampal eCB system expression. Compared with controls, rats prenatally exposed to VPA exhibited differences in mRNA expression of CB1R, CB2R, FAAH and

DAGL in the hippocampus ( $p < 0.05$ ). There was a significant increase in CB1R, FAAH and MAGL protein levels, whereas CB2R and DAGL protein levels did not reach the significance level (figure 2b,d).

### 3.2. Effect of JZL184 treatment on autism-like phenotypes

One-way ANOVA analyses revealed statistically significant result between the groups with respect to marbles buried ( $F_{5,42} = 6.22$ ,  $p = 0.0002$ ). VPA-exposed rats buried more marbles than control rats ( $p = 0.0001$ ), and JZL184-treated rats buried significantly less marbles than VPA-exposed rats (VPA versus VPA + 40AJ:  $p = 0.0007$ , VPA versus VPA + 3RJ:  $p = 0.0005$ , VPA versus VPA + 10RJ:  $p = 0.0009$ ; figure 3a). A significant difference was found with respect to repetitive



**Figure 3.** Flowchart of JZL184 administration in VPA-exposed offspring and effects of JZL184 treatment on repetitive and stereotypical behaviours of VPA-exposed offspring in marble burying test and self-grooming test. (a) The number of marbles buried. (b) The self-grooming time. Data represented as means  $\pm$  s.e.m. ( $n = 8$  pups per group). Results were analysed by one-way ANOVA with Dunnett's *post hoc* test (###  $p < 0.001$ , ####  $p < 0.0001$ , versus CON group; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  versus VPA group). AJ, acute JZL184 treatment; RJ, repeated JZL184 treatment.

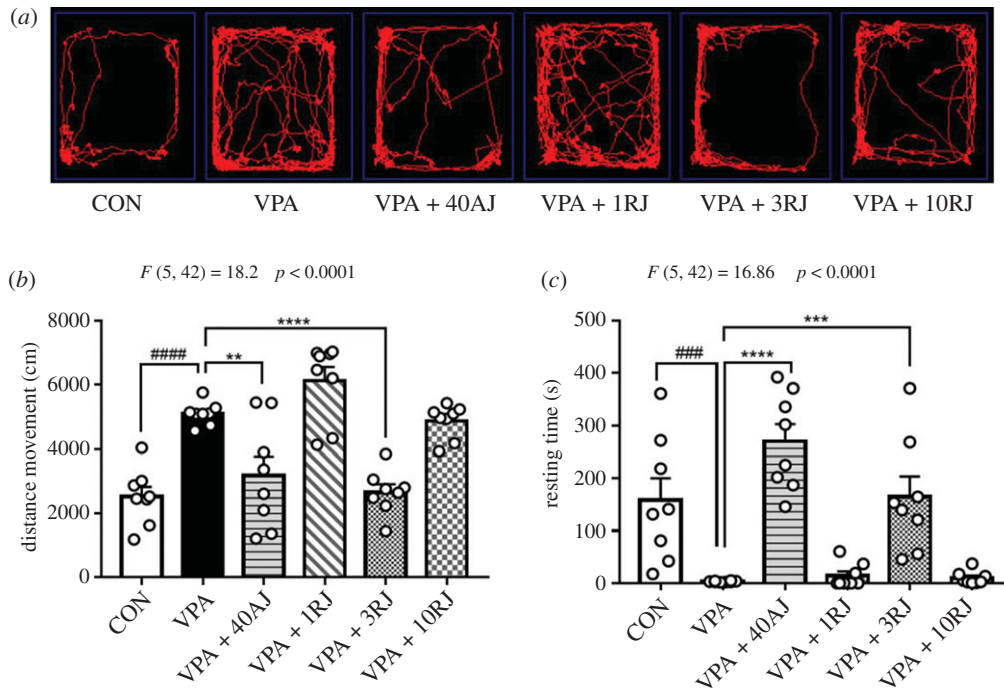
self-grooming behaviour ( $F_{5,42} = 7.689$ ,  $p < 0.0001$ ). The *post hoc* test confirmed that VPA-exposed rats spent more time self-grooming than control rats ( $p < 0.0001$ ), and self-grooming behaviour was significantly reduced by JZL184 treatment (VPA versus VPA + 40AJ:  $p = 0.0087$ , VPA versus VPA + 1RJ:  $p = 0.0002$ , VPA versus VPA + 3RJ:  $p = 0.0001$ , VPA versus VPA + 10RJ:  $p = 0.0005$ ; figure 3b). All in all, JZL184 administration could improve the repetitive and stereotypical behaviours of VPA-exposed rats.

We logged the distance moved and resting time to further gauge anxiety-like behaviour. One-way ANOVA analyses revealed statistically significant result in the vase of locomotor activity among the groups (distance moved:  $F_{5,42} = 18.2$ ,  $p < 0.0001$ , resting time:  $F_{5,42} = 16.86$ ,  $p < 0.0001$ ). Upon exposure to a novel brightly lit aversive open field arena, VPA-exposed rats exhibited hyperlocomotion as demonstrated by an increase in distance moved and a decrease in resting time when compared to the control rats ( $p < 0.0001$ ,  $p = 0.0010$ , respectively; figure 4a–c). JZL184 acute injection and 3 mg kg<sup>-1</sup> repeated injections could reverse the elevated locomotor activity of VPA-exposed rats (distance moved:  $p = 0.0017$ ,  $p = 0.0001$ , respectively; resting time:  $p < 0.0001$ ,  $p = 0.0006$ , respectively; figure 4b,c). By contrast, 1 and 10 mg kg<sup>-1</sup> repeated injections failed to exhibit this effect.

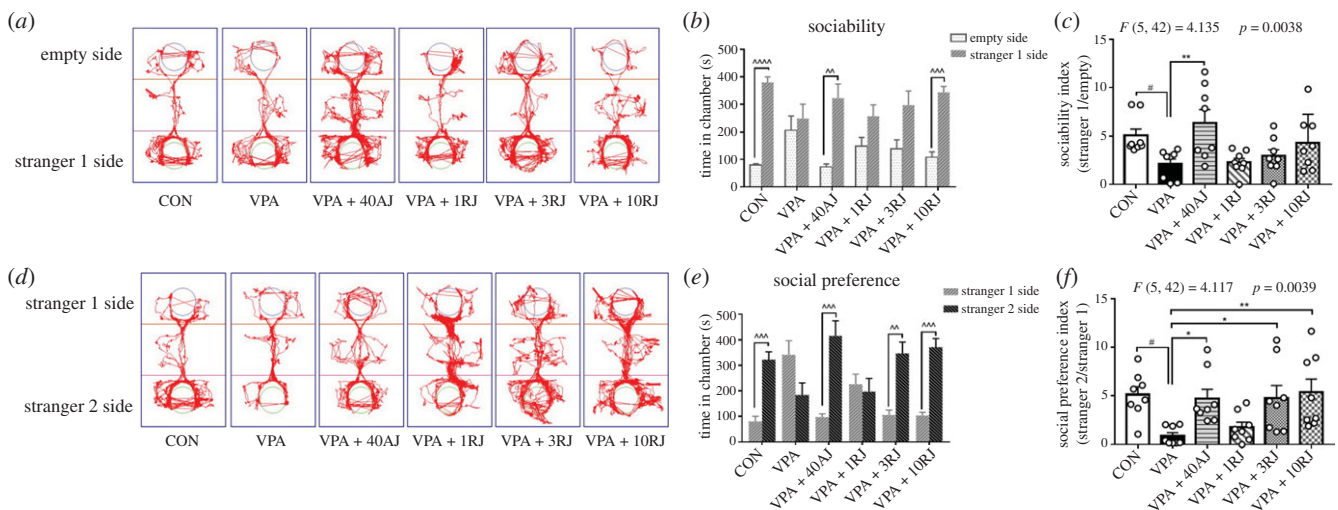
Prenatal VPA exposure impaired social interaction behaviours in offspring as reported previously [20,21]. In the habituation phase, rats from all of the experimental groups did not show any significant chamber preference (data not shown). In the sociability test, VPA-exposed groups exhibited the typical ASD-like phenotype and failed to demonstrate a significant preference for the unfamiliar rat compared with the object. Acute administration of JZL184 and 10 mg kg<sup>-1</sup> administered by repeated injections corrected this aberrant behaviour ( $p = 0.0017$ ,  $p = 0.0006$ , respectively; figure 5a,b).

However, only acute administration significantly enhanced the sociability index ( $p = 0.0035$ ; figure 5c). In the social preference test, which determines whether the experimental rats show a preference for a socially novel rat or familiar one, VPA-exposed rats exhibited reduced social novelty recognition compared to the control rats, which was quantified by the time spent engaging in investigatory behaviour with 'stranger rat 2' and the social preference index ( $p = 0.0006$ ,  $p = 0.0132$ , respectively; figure 5d–f). Acute administration of JZL184, and repeated injections at doses of 3 and 10 mg kg<sup>-1</sup> increased the amount of time spent by 'stranger rat 2' ( $p = 0.0009$ ,  $p = 0.0034$ ,  $p = 0.0004$ , respectively, figure 5e) and the social preference index ( $p = 0.0297$ ,  $p = 0.0258$ ,  $p = 0.0077$ , respectively; figure 5f). By contrast, the influence of 1 mg kg<sup>-1</sup> JZL184 repeated injection on social behaviours could not be observed. These results suggested that JZL184 administration could restore impaired social interaction.

In the Morris water maze test, the rats are required to find a hidden platform in order to escape from swimming in a pool of water. During the training trial, a decrease was observed in the escape latency in all of the groups, and the differences were observed among groups during the same day (repeated-measures ANOVA: group effect:  $F_{4,35} = 14.89$ ,  $p < 0.0001$ ; time effect:  $F_{3,105} = 17.85$ ,  $p < 0.0001$ ; interaction effect between group and time:  $F_{12,105} = 0.93$ ,  $p = 0.52$ ; figure 6a,b). A *post hoc* test showed that compared with the control group, VPA-exposed rats required a longer escape latency, whereas repeated treatment with 3 or 10 mg kg<sup>-1</sup> of JZL184 notably shortened the escape latency during the training period (2nd, 3rd and 4th day,  $p < 0.05$ ; figure 6a,b). On the 5th day, one-way ANOVA analyses revealed statistically significant differences between the groups in the spatial probe test ( $F_{4,35} = 5.101$ ,  $p = 0.0024$ ; figure 6c). The results indicated VPA-exposed rats were not able to



**Figure 4.** Effects of JZL184 treatment on locomotor activity of VPA-exposed offspring in open field test. (a) Representative images of locomotor tracks. (b) Distance movement. (c) Resting time. Data represented as means  $\pm$  s.e.m. ( $n = 8$  pups per group). Results were analysed by one-way ANOVA with Dunnett's *post hoc* test ( $^{###}p < 0.001$ ,  $^{####}p < 0.0001$ , versus CON group;  $^{**}p < 0.01$ ,  $^{***}p < 0.001$ ,  $^{****}p < 0.0001$  versus VPA group). AJ, acute JZL184 treatment; RJ, repeated JZL184 treatment.



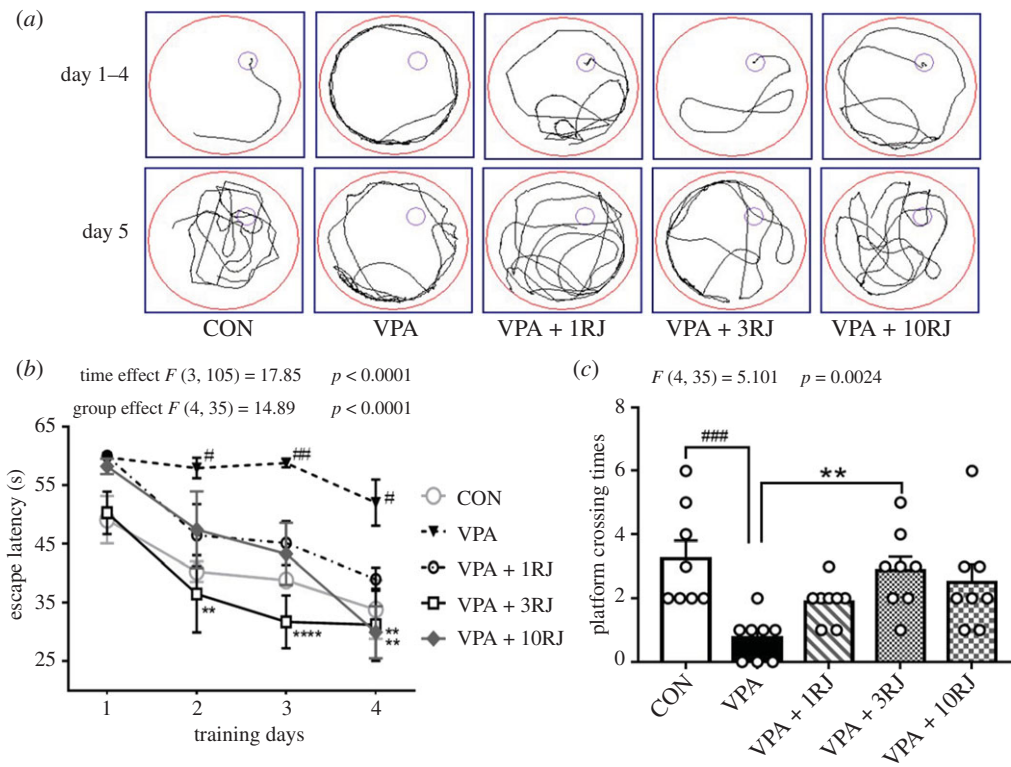
**Figure 5.** Effects of JZL184 treatment on social behaviors of VPA-exposed offspring in three-chamber test. (a–c) Sociability test. (d,e) Social preference test. (a,d) Representative images of track movements. (b) Time spent in chamber of sociability towards stranger rat 1 or empty cage. (c) Sociability index, calculated as time spent in the stranger 1/empty cage. (e) Time spent in chamber of social preference towards stranger 1 or stranger 2. (f) Social preference index, was calculated as time spent in the stranger 2/the stranger 1. Data represented as means  $\pm$  s.e.m. ( $n = 8$  pups per group). (b,e) Results were analysed by paired Student's *t*-test ( $^{\wedge\wedge}p < 0.01$ ,  $^{\wedge\wedge\wedge}p < 0.001$ ,  $^{\wedge\wedge\wedge\wedge}p < 0.0001$ , stranger 1 side versus empty side or stranger 2 side). (c,f) Results were analysed by one-way ANOVA with Dunnett's *post hoc* test ( $^{\#}p < 0.05$  versus CON group;  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  versus VPA group). AJ, acute JZL184 treatment; RJ, repeated JZL184 treatment.

remember the original platform, and the number of crossings of the former platform location (passing time) was less than that observed in the control rats ( $p = 0.0010$ ). Interestingly, VPA-exposed rats administrated with JZL184 repeated injections at doses of  $3 \text{ mg kg}^{-1}$  significantly increased platform crossing times ( $p = 0.0052$ ; figure 3a,c). Taken together, JZL184  $3 \text{ mg kg}^{-1}$  repeated treatment could improve learning and spatial memory deficits in VPA-induced rats.

### 3.3. Effect of JZL184 treatment on the components of the eCB system

Acute administration of JZL184 ( $40 \text{ mg kg}^{-1}$ ) did not alter eCB levels in the hippocampus and the PFC (figure 1b,c). Repeated administration of JZL184 at doses of 3 and  $10 \text{ mg kg}^{-1}$  enhanced the levels of 2-AG in the hippocampus and corresponding reductions in its metabolite, AA. There were no





**Figure 6.** Effects of JZL184 treatment on learning and memory of VPA-exposed offspring in Morris water maze test. (a) Representative images of the escape latency and the passing times through the platform area. (b) Escape latency on different training days. (c) Times of rats passing the site where there had been a platform. Data represented as means  $\pm$  s.e.m. (n = 8 pups per group). (b) Results were analysed by repeated-measures ANOVA. (c) Results were analysed by one-way ANOVA with Dunnett's *post hoc* test ( $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$ ,  $^{\#\#\#}p < 0.001$  versus CON group;  $^{**}p < 0.01$ ,  $^{****}p < 0.001$  versus VPA group). RJ, repeated JZL184 treatment.

differences in the levels of AEA, PEA and OEA (figure 1b). Repeated administration of JZL184 at doses of 3 mg kg<sup>-1</sup> increased the levels of AEA and 2-AG in the PFC without affecting the levels of PEA, OEA and AA (figure 1c).

As shown in figure 7a, CB1R, CB2R and DAGL mRNA expression in the hippocampus were lower in VPA-exposed rats than those in control rats ( $p < 0.05$ ), which was consistent with prior results (figure 2b). Acute administration of JZL184 (40 mg kg<sup>-1</sup>) reduced CB1R and DAGL mRNA levels of VPA-exposed offspring in the hippocampus ( $p < 0.05$ ). Repeated injections of JZL184 at doses of 3 mg kg<sup>-1</sup> influenced DAGL mRNA expression ( $p = 0.0026$ ). Repeated injections of JZL184 at doses of 10 mg kg<sup>-1</sup> decreased CB1R, CB2R and DAGL mRNA expression ( $p = 0.0001$ ,  $p = 0.0001$ ,  $p = 0.0396$ , respectively).

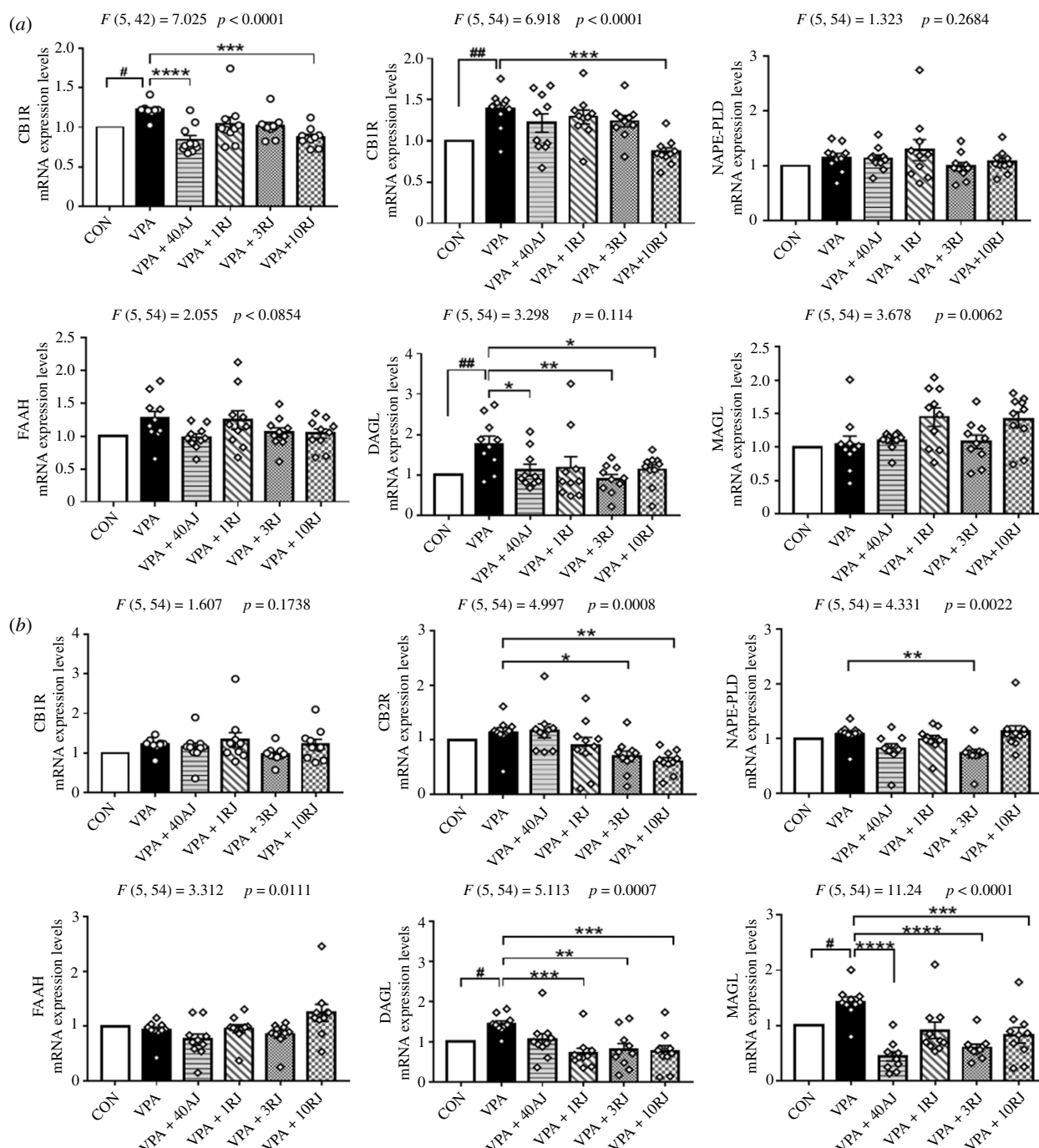
As shown in figure 7b, DAGL and MAGL mRNA expression in the PFC were increased in VPA-exposed rats when compared to controls rats ( $p = 0.0454$ ,  $p = 0.0195$ , respectively), while there were not significant differences in CB1R, CB2R, NAPE-PLD, FAAH mRNA expression. Acute administration of JZL184 (40 mg kg<sup>-1</sup>) reduced MAGL mRNA levels of VPA-exposed offspring ( $p = 0.0001$ ). JZL184 1, 3 and 10 mg kg<sup>-1</sup> repeated injections influenced CB2R, NAPE-PLD, DAGL and MAGL mRNA levels in the PFC ( $p < 0.05$ ).

## 4. Discussion

This study aimed to highlight that ASD children and ASD model rats have been found to exhibit disruption of the eCB system, and that pharmacological modulators of the eCB system may offer therapeutic potential in ASD. Our results of

reduced eCB content, increased degradation of enzymes and compensatory upregulation of CBRs suggested lower eCB signalling in ASD. Moreover, we observed that JZL184 treatment, by enhancing intrinsic 2-AG levels, ameliorated autistic behaviours in VPA-exposed offspring. This finding was characterized by reduced repetitive and stereotypical behaviours in marble burying and self-grooming test, reduced hyperactivity in the open field test, increased sociability and social preference in the three-chamber test and improved cognitive functioning in the Morris water maze test. This research is important to encourage the identification of potential targets for improved therapeutic treatments in ASD.

To date, only two studies with humans provided evidence regarding eCB levels in blood samples of ASD children [6,7], and findings of which demonstrated lower concentrations of AEA, PEA and OEA in autistic children, which are consistent with the findings of the present study. Notably, this is the first time that we found lower circulating 2-AG levels in children with ASD. We also found that children with ASD who have lower PEA levels exhibited more serious ASD symptoms. Interestingly, Kelly *et al.* [24] demonstrated that children with poor communication scores at age 3 years had been found to exhibit dysregulated plasma eCB levels at 1 year of age, which implicated that eCBs may be a potential biomarker for the early diagnosis of ASD. Anecdotally, case reports from Italy first corroborated that PEA, whether alone or combination with other natural supplements, can refine ASD core impairments [25]. Subsequently, an Iranian randomized, double-blind placebo-controlled trial revealed that PEA (600 mg administered twice daily) may augment the therapeutic effects of risperidone on ASD-related irritability and hyperactivity without serious side effects [26]. The non-psychoactive, medical



**Figure 7.** Effects of JZL184 treatment on the relative mRNA expression levels of eCB system normalized to GAPDH in hippocampus (a) and in the PFC (b). Data represented as means  $\pm$  s.e.m. ( $n = 10$  pups per group). Results were analysed by one-way ANOVA with Dunnett's *post hoc* test ( $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$  versus CON group;  $^*p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$ ,  $^{****}p < 0.001$  versus VPA group). AJ, acute JZL184 treatment; RJ, repeated JZL184 treatment.

cannabis (e.g. cannabidiol, cannabidivarin) in ASD patients appears to be well tolerated and safe (low side effects rate), and may be an effective treatment in some countries to relieve autistic symptoms [27,28]. Nevertheless, cannabinoid treatment remains a controversial ethical issue with respect to individuals with ASD, and any kind of cannabinoid consumption is illegal in China. Collectively, whether as a diagnostic biomarker or as a potential therapeutic target, decreased eCB levels were associated with ASD.

Confirming the results found in human studies, we observed that the levels of two major eCBs, namely, AEA and 2-AG, were reduced in the hippocampus of VPA-induced rats. However, Kerr *et al.* [11] found that the levels of AEA, PEA, OEA and 2-AG in the hippocampus did not differ between VPA-exposed and control rats. Intriguingly, they demonstrated that eCB levels

were enhanced in the hippocampus of VPA-exposed rats immediately following the sociability test, i.e. eCB content was susceptible to behavioural testing, which is supported by many other studies [29,30]. To this end, in the present study, the behavioural experiments were paralleled by biochemical measurement, whereas Kerr examined eCB concentration 72 h after the animals underwent behavioural experiments. This might account for the disparity in the two studies. In addition, several lines of work pointed out that eCB changes appeared to be region-specific, so we did not observe a similar profile and magnitude of eCBs in the hippocampus and in the PFC. In addition, changes in the eCB system are more pronounced in the hippocampus with respect to the PFC [13,31].

In the light of PBMCs (i.e. lymphocytes, NK cells and monocytes) which could serve as a tool to investigate eCB

system changes in the CNS in several neuropsychiatric disorders, we also examined eCB-associated receptors and enzymes in PBMCs isolated from the whole blood of autistic children. CB1R is the most abundant G protein-coupled receptor located in the brain, while CB2R is sparsely expressed in the brain and is instead primarily present in immunoregulatory cells, such as microglia and peripheral immune cells [32]. This could explain that the differences in CB1R mRNA expression in PBMCs and CB2R protein expression in the hippocampus were not observed between ASD cases and controls; even CB1R protein expression was not detected in PBMCs in the present study. It is noteworthy that we found that transcription and translation of CB2R in PBMCs and CB1R in the hippocampus were all enhanced. Siniscalco *et al.* [8] found results that are similar to those of the present study, which revealed unchanged CB1R mRNA levels and upregulated CB2R mRNA and protein levels in ASD-PBMCs in comparison to healthy subjects. The Zamberletti study also found that prenatal VPA exposure increased CB1R protein levels [13]. Remarkably, CB1R and CB2R activation exerted diverse consequences across cellular physiology. As the main molecular target of eCBs, CB1R is found high density on presynaptic termini of glutamatergic and GABAergic neurons, and activation of CB1R is implicated in the regulation of excitatory-inhibitory balance, synaptic strength and neurotransmitter release, which ultimately mediates social functioning, learning and memory [33,34]. Furthermore, genetic variants in *CNR1* (encoding CB1R) gene were correlated not only to verbal but to non-verbal social communication in ASD research studies [35,36]. Additionally, via activation of CB2R, the eCB system exerts anti-inflammatory actions and decreases glial activity to prevent excessive inflammation and cell damage [32,37]. We could not obtain brain tissue of patients. Nonetheless, to some extent, the expression of CB2R on peripheral immune cells reflects changes in the CNS [38]. In fact, this study also found an increase in mRNA expression of CB2R in the hippocampus of VPA-exposed offspring. Hence, our findings supported the notion that the eCB system plays a protective role in inflammatory responses in autistic children via increasing CB2R expression [8]. However, further experimental evidence towards neuroinflammation is needed. Because it is impossible that the changes in the components of the eCB system are independent from one another, researchers suggested that there might be negative feedback regulation of eCBs and CBR densities [39,40]. We deduced that upregulation of CBRs reflected a response to the lower levels of eCBs observed in autistic patients and model rats in the current study. Alterations in CBRs levels are transient adaptive reactions which attempt to restore normal homeostasis that is otherwise disrupted by the disease.

Thus far, only Siniscalco's team have previously investigated the eCB system in PBMCs from individuals with ASD, but they incorporated neither 2-AG metabolic enzymes nor protein expression of eCB-related enzymes into their study [8,9]. To our knowledge, this is the first study to explore relatively entire components of the eCB system in PBMCs from humans. The biosynthesis of 2-AG can be catalyzed by two DAGL isoforms, namely DAGL- $\alpha$  and DAGL- $\beta$ . DAGL- $\alpha$ , which are expressed throughout the brain, and 2-AG levels dropped by up to 80–90% in the brain in the DAGL- $\alpha$  null mouse brain [41]. Given that DAGL- $\alpha$  is the main 2-AG synthesizing enzyme in the brain, this study only examined DAGL- $\alpha$  expression (DAGL for short). Surprisingly, we revealed that

DAGL mRNA expression was increased in the human and rat sample, but not protein. Moreover, we found concurrent increases in the expression of FAAH and MAGL, which are responsible for AEA and 2-AG degradation, both in PBMCs and in the hippocampus. The lower eCB levels in the present study may account for the increased degradation of enzymes. The contradictory results that both DAGL and MAGL mRNA expression were increased in the PFC may explain the unchanged levels of eCBs in the PFC. Interestingly, in Siniscalco's study, FAAH mRNA expression did not significantly change, and NAPE-PLD slightly decreased in 17 cases of ASD and 22 cases of healthy controls [8]. Our findings from animals were in keeping with previous reports of upregulated expression of FAAH and MAGL in VPA-induced rats [13,42]. As a whole, our results and those of other studies highlight the presence of decreased eCB signalling in ASD children and in the animal model which might explain the deficits exhibited in the cognitive and social domains.

The animal model studies have shown that prenatal VPA exposure in rodents recapitulates ASD-like pathophysiology at a molecular, cellular and behavioural level. VPA-induced rats have been developed and became a widely used environmental preclinical model of ASD with strong face and construct validity, which also serves as a good platform for testing pharmacological reagents that might be used to treat ASD. On a behavioural level, our findings have confirmed that VPA-exposed rats exhibit the core symptoms of ASD, impaired social interaction and repetitive behaviour, and possibly co-occurring emotional and cognitive problems. Furthermore, we found that prenatal VPA exposure induces a disturbance of the eCB system in offspring rats that is similar that observed in ASD children, which is in accordance with prior research studies [11,13], indicating a reduced eCB tone in ASD. Therefore, we evaluated the efficacy of boosting 2-AG levels by administering the hydrolysis inhibitor JZL184 which attenuates repetitive and stereotypical behaviours, hyperactivity, and deficits in social and cognitive functioning in VPA-induced rats. The results showed that either acute or chronic administration of JZL184 was successful in mitigating ASD-like behaviours, which was in line with previous reports involving *Fmr1* knockout mice and *Shank3B*<sup>-/-</sup> mice [10,18,19].

Rats treated with acute administration 40 mg kg<sup>-1</sup> JZL184 exhibited reduced repetitive marble burying, grooming behaviours and hyperactivity, as well as an improvement in sociability and social preference induced by VPA exposure. We observed that a single injection of JZL184 (40 mg kg<sup>-1</sup>) did not change the levels of eCBs in the hippocampus and PFC, which was consistent with the findings of Kerr *et al.* [43] who did not detect an alternation in the levels of eCBs 2.5 h following injection of JZL184 (10 mg kg<sup>-1</sup>). Nonetheless, JZL184 at a dose of 40 mg kg<sup>-1</sup> could show loss of MAGL activity [44]. Kruk-Slomka *et al.* [45] demonstrated similar results, which revealed that acute injection of JZL184 40 mg kg<sup>-1</sup> significantly decreased locomotion and improved long-term acquisition of memory and learning processes. Although only decreased CB1R and DAGL mRNA expression in the hippocampus and decreased MAGL mRNA expression in the PFC were detected and the eCB levels were not acute JZL184 treatment still exerted a positive effect on behaviours. Repeated treatment with JZL184 at a dose of 3 mg kg<sup>-1</sup> had a restorative effect on repetitive marble-burying and grooming behaviours, locomotor activity, social preference, learning and spatial memory. By contrast, a high dose (10 mg kg<sup>-1</sup>) only partially

affected repetitive behaviours, social preference and learning, and a low dose which was tested ( $1 \text{ mg kg}^{-1}$ ) was ineffective. In fact, the behavioural efficacious dose of  $3 \text{ mg kg}^{-1}$  robustly increased 2-AG levels in both the hippocampus and PFC, whereas concomitantly, a marginal increase in AEA levels was observed. In this regard, *Dagla*<sup>-/-</sup> animals showed an extensive reduction in 2-AG levels and a concomitant decrease in AEA in the hippocampus and cortex. Furthermore, administration of JZL184 to *Dagla*<sup>-/-</sup> mice increased not only 2-AG levels but also the level of AEA [41]. Schlosburg *et al.* [17] also confirmed that chronic dosing also caused a modest elevation in AEA. These data, together with our similar results, suggested a crosstalk of 2-AG and AEA production in the brain; however, the underlying mechanism is not known. We should note that cumulative exposure to JZL184 probably generates a partial effect of blockade on FAAH, rather than MAGL contributing directly to the degradation of AEA [17]. Our findings also indicated that the increase of 2-AG in the hippocampus and PFC might not result from the increase of DAGL, because JZL184 treatment reduced DAGL mRNA expression. Inversely, the increase of 2-AG exerted an inhibitory effect on the DAGL expression. Convergent literature demonstrated that sustained elevation of 2-AG in the brain, caused by either genetic deletion or chronic pharmacological blockade of MAGL, led to CB1R desensitization and tolerance to CB1R agonists, as well as to significant decreases in CB1R number and function, and this effect would limit the therapeutic potential of JZL184 [17]. A chronic JZL184 dose of  $16 \text{ mg kg}^{-1}$  daily (typically one week) reliably produced tolerance [46]. The current study observed that a dose of  $10 \text{ mg kg}^{-1}$  daily (two weeks) reduced CB1R and CB2R expression in the hippocampus, and CB2R expression in the PFC. Thus, repeated administration of a low dose of JZL184 (i.e.  $3 \text{ mg kg}^{-1}$  in the current study) could produce elevated eCB brain levels without behavioural tolerance and CBRs desensitization. Additionally, acute treatment with JZL184 also has an important effect on CBRs [22]. We hypothesized that JZL184 treatment could improve ASD-like behaviours via CBR-dependent and -independent mechanisms: (i) as 2-AG acts via CB1R and generally suppresses synaptic transmission, neuronal excitability and neurogenesis, the improvement of eCB-induced synaptic plasticity could ameliorate ASD-like behaviours [10,18]; and (ii) 2-AG is an important metabolic intermediate in lipid synthesis and it also serves as a major source of AA, which is required for pro-inflammatory prostaglandin synthesis. Pharmacological inactivation of MAGL induced not only elevations in 2-AG, but reductions in the product AA and downstream AA-derived eicosanoids as well. This impairment of eicosanoids production is a direct consequence of the reduction in AA rather than the augmentation of eCB signalling, which is possibly relevant to cyclooxygenase enzymes [44]. Furthermore, inactivation of MAGL could suppress the pro-inflammatory cytokines production and microglial activation induced by LPS [44,47]. Eventually, independent of CBRs, increasing 2-AG provides protection against neuroinflammation, which then ameliorates ASD-like behaviours.

This study had some limitations that must be taken into account in interpreting the results. First, the present study is limited by the use of male offspring only. The eCB system is known to exhibit sexual dimorphism in humans and rodents, particularly in CB1R expression and functionality [31,48]. Second, the study could have benefited from comparisons with plasma eCB levels in VPA-induced rats. Third, this

study may lack some information of value due to not having evaluated enzyme and receptor activity, and additional non-cannabinoid receptor targets which are known to have affinity and activity to eCBs. While the therapeutic use of the eCB system is inviting, extensive research is required to further evaluate this complex regulatory pathway and the safety of pharmacological manipulation.

Current evidence strongly implicates alterations in the eCB system in human patients with ASD and in animal models. The reduced eCB content, elevated degradation of enzymes and compensatory upregulation of CBRs indicates reduced eCB signalling in ASD. In addition, augmentation of 2-AG levels by pharmacological inhibition of MAGL resulted in the normalization of ASD-related behavioural abnormalities in VPA-exposed offspring. The improvement of behavioural phenotypes was consistent with the observed increase in 2-AG in the hippocampus and PFC following administration of JZL184 at a dose of  $3 \text{ mg kg}^{-1}$ . These data provide preclinical evidence which supports the ability of JZL184 to ameliorate behavioural abnormalities resembling core and associated symptoms of ASD. The high heterogeneity in the phenotypic presentation of ASD poses investigative and clinical challenges for treatment, and subgroups of ASD individuals may benefit more from drugs that increase cannabinoid levels.

## Abbreviations

2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ABC, Autism Behaviour Checklist; ADI-R, Autism Diagnostic Interview-Revised; ADOS, Autism Diagnostic Observation Schedule; AEA, anandamide; ANOVA, one-way analysis of variance; ASD, autism spectrum disorder; BMI, body mass index; CARS, Childhood Autism Rating Scales; CB1R, type 1 cannabinoid receptors; CB2R, type 2 cannabinoid receptors; CNS, central nervous system; CSS, calibrated severity scores; DAGL, diacylglycerol lipase; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acylphosphatidylethanolamine-specific phospholipase D; OEA, oleoylethanolamide; PBMC, peripheral blood mononuclear cell; PEA, palmitoylethanolamide; PND, postnatal day; qPCR, quantitative PCR; SRS, Social Responsiveness Scale; VABS, Vineland Adaptive Behaviour Scale; VPA, valproic acid.

**Ethics.** The study protocol was approved by the Institutional Review Board of Harbin Medical University for Medical Sciences (HMUIRB20200007). All experiments on human subjects were conducted in accordance with the Declaration of Helsinki. All procedures were carried out with the adequate understanding and written informed consent from the guardian of each participant prior to the study. Animal experiments were carried out in accordance with the National Institutes of Health (NIH) guidelines for the care and use of experimental animals (NIH publication no. 8023, revised 1978), and the protocol was reviewed and approved by the Animal Care Committee of Harbin Medical University.

**Data accessibility.** The dataset used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions.** L.Wu. and C.S. conceived and designed the research; Y.L., S.X., D.L., L.L., F.W., Y.Z. and M.Z. performed the experiments; Y.L. and M.Z. analysed the data; M.Z., C.S. and L.Wu. contributed reagents/materials/analysis tools; S.X., L.Wa. and W.X. performed clinical assessment; M.Z. wrote the paper. All authors contributed to and have approved the final manuscript.

**Competing interests.** The authors disclose no potential conflicts of interest.

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