

The Alternations of Endocannabinoid System and its Therapeutic Potential in Autism Spectrum Disorder

Mingyang Zou

Harbin Medical University

Yu Liu

Harbin Medical University

Shu Xie

Harbin Medical University

Luxi Wang

Harbin Medical University

Dexin Li

Harbin Medical University

Ling Li

Harbin Medical University

Wei Xia

Harbin Medical University

Caihong Sun

Harbin Medical University

Lijie Wu (✉ wulijiehyd@126.com)

Public Health College of Harbin Medical University <https://orcid.org/0000-0002-7521-2388>

Research

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Abstract

Background: Autism spectrum disorder (ASD) is a group of developmental disabilities whose etiology remains elusive. The endocannabinoid (eCB) system modulated neurotransmission and neuronal plasticity. Evidence points to the involvement of this neuromodulatory system in the pathophysiology of ASD. Herein we investigated if there was disruption of eCB system in ASD and if pharmacological modulators of eCB system might have a therapeutic potential.

Methods: We examined three major components of eCB system, namely, endogenous cannabinoids, their receptors and associated enzymes, in ASD children as well as in VPA-induced ASD animal model. Meanwhile we specially increased 2-arachidonylglycerol (2-AG) by administering JZL184, a selective inhibitor of monoacylglycerol lipase, the hydrolytic enzyme for 2-AG, and to examine ASD-like behaviors in VPA-induced rats.

Results: Autistic children and VPA-induced rats exhibited the reduced eCBs content, elevated degrading enzymes, and up-regulation of CBRs. We found that repetitive and stereotypical behaviors, hyperactivity, sociability, social preference, and cognitive functioning were improved after acute and chronic JZL184 treatment. The major efficacy of JZL184 was observed at the dose of 3mg/kg for both affecting eCB system and ASD-like behaviors.

Conclusions: There were a lower eCB signaling in autistic children and ASD animal model, and boosting 2-AG could ameliorate ASD-like phenotypes in animals. Collectively, the results suggest a novel approach to ASD treatment.

Background

Autism spectrum disorder (ASD) is a collection of heterogeneous neurodevelopmental disorders defined by impairment in communication and social interactions, and restricted, repetitive patterns of behavior [1]. ASD affects around 1% children in mainland China[2], which is comparable to western countries, and the prevalence seems to be cumulatively increasing. The latest prevalence of ASD has reached to 1.85% (one in 54) children aged 8 years[3]. Despite its high prevalence and the public health burdens it imparts, there is relatively limited understanding of the pathophysiology of ASD aside from complex interactions between genetic and environmental factors. A multitude of recent literatures have suggested that ASD is linked to abnormalities in synaptic function. Thus, the endocannabinoid (eCB) system, which could modulate different neurotransmitter system, synaptic excitation/inhibition balance and plasticity in the brain, and also be associated with social interaction, motor control, repetitive behaviors, emotional processing, learning and memory, has attracted increasing interest for its potential in the onset and/or progression of ASD.

The eCB system is a retrograde feedback signaling system that plays a key, pro-homeostatic role in the central nervous system (CNS). It consists of three major components: endogenous cannabinoids (eCBs), their receptors and associated enzymes. The most active eCBs are anandamide (AEA) and 2-

arachidonylglycerol (2-AG), which act mainly through type 1 and type 2 cannabinoid receptors (CB1R and CB2R) that are distributed throughout the CNS. In addition, Palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), structurally similar to AEA (collectively as N-acylethanolamines), share the same catalyzed enzymes in 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: 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 eCBs signaling.

The eCBs are produced on demand from polyunsaturated fatty acids (PUFAs) in presynaptic neurons membranes and act as retrograde messenger on presynaptic CBRs to dampen the release of neurotransmitters (for example, monoamine, opioids, GABA, glutamate, acetylcholine), thereby affecting a wide range of biological processes[5]. eCB-mediated retrograde suppression is considered to be a ubiquitous and important form of activity-dependent synaptic modulation. At first, some researchers have revealed that lacking n-3 PUFAs inhibited eCB-associated synaptic plasticity. In mice on a n-3 PUFAs-deficient diet, there was a complete absence of learn-related synaptic plasticity (long-term potentiation: LTD) triggered by eCB in different brain areas, whereas synaptic plasticity induced by other neurotransmitters was unaffected[6]. Moreover, the enhancement of eCB signal could promote the formation of LTD, and rescue the lack of eCB-mediated LTD in two types of monogenetic model of ASD mice[7]. Intriguingly, studies from our laboratory previously reported lower n-3 PUFAs levels were in autistic children, as well as in valproic acid (VPA)-induced rats[8, 9], and the expressions of rate-limiting PUFA metabolic enzymes were downregulated in the VPA rats[8]. Afterwards, both prenatal and postnatal n-3 PUFA supplementation could markedly improve the learning and memory deficits of offspring[10]. In this scenario, we could draw the conclusion that eCB system, as intermedium of n-3 PUFA, plays an important role in the development of ASD, which stimulates our team to carry out continued studies to elucidate the relationship between eCB system and ASD pathophysiology.

Indeed, the dysregulation of eCB system has been documented in 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 their matched healthy children [11, 12]. Siniscalco's team reported altered receptors and enzymes of eCB system in peripheral blood mononuclear cells (PBMCs) of autistic children[13, 14]. The disruption in the metabolism of eCB system was suggested to be also present in the brain of both genetic and environmental ASD models. 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[15]. In VPA-induced rats, well-known environmental-based model, there was a reduction in MAGL expression and unaltered levels of AEA, PEA, OEA and 2-AG [16]. Melancia[17] showed CB1R activation decreased, while Zamberletti[18] found CB1R upregulated. BTBR, an inbred mouse strain known to model of idiopathic autism, showed higher CB1R density[19]. What's more, enhancing AEA signaling partially attenuated social behavior

deficits in these three ASD models[17, 20, 21]. Although still debated, it is plausible that alternations of eCB system may contribute to the pathogenesis of ASD.

Generally speaking, 2-AG, the most abundant eCB in the brain, is found much higher concentrations than N-acylethanolamines in the brain (i.e., approximately 1000-fold higher than AEA), and executes full agonist activity at CBRs with a high efficacy, while AEA is a partial agonist, and PEA and OEA with lower affinity[5]. Considering that 2-AG plays a broader role in the integrity of brain eCB system and CNS development, 2-AG may be a more relevant indicator of eCB tone[22]. However, changes in 2-AG levels have not been reported in individuals with ASD, and evidence regarding whether enhancing 2-AG tone could cause improvement of ASD-like behaviors is limited. Herein, for one thing, we examined all three major components of 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 VPA-induced ASD animal model, to characterize comprehensively the involvement of eCB system in the pathogenesis of ASD. For another, we investigated the effect of altered 2-AG signaling on the autistic behaviors, and if behavioral changes exhibited by VPA-induced rats are associated with eCB dysfunction in discrete brain regions known to module cognitive and social behavior, including the hippocampus and prefrontal cortex (PFC). The present study was designed to better understand the critical role of eCB system in the etiology of ASD, and provide a novel strategy for treatment in managing symptoms of ASD.

Materials And Methods

Participants

We investigate 70 autistic patients and 70 age- and gender-matched controls (age ranging 3–12). Seventy autistic patients were recruited from the Child Development and Behavior 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 enrollment, 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 methods were used as an aid for diagnosis and assessment: Autism Diagnostic Observation Schedule (ADOS), Autism Diagnostic Interview-Revised (ADI-R), Autism Behavior Checklist (ABC), Childhood Autism Rating Scales (CARS), Vineland Adaptive Behavior Scale second edition (VABS) and Social Responsiveness Scale (SRS). Sample characteristics were provided in Table 1.

Table 1
Participant characteristics (means \pm SD)

Items	Case (n = 70)	Control (n = 70)
Gender (male/female)	62/8	62/8
Age (years)	5.44 \pm 1.36	5.12 \pm 0.79
BMI	17.21 \pm 3.00	16.33 \pm 2.52
Ethnicity (Chinese Han)	70	70
ADOS CSS score	6.82 \pm 1.22 (n = 56)	-
ADI-R total	48.03 \pm 9.29 (n = 62)	-
ABC total	63.47 \pm 29.73 (n = 57)	-
CARS total	31.02 \pm 3.19 (n = 58)	-
VABS standard score	66.77 \pm 14.19 (n = 61)	-
SRS raw score	90.93 \pm 20.88 (n = 57)	-
There were no differences in terms of gender, age and BMI between case and control groups. BMI: body mass index; ADOS: Autism Diagnostic Observation Schedule; CSS: calibrated severity scores; ADI-R: Autism Diagnostic Interview-Revised; ABC: Autism Behavior Checklist; CARS: Childhood Autism Rating Scales; VABS: Vineland Adaptive Behavior Scale; SRS: Social Responsiveness Scale.		

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 °C \pm 2 °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 1 week prior to experiments. The rat model of ASD was established according to previous studies [23, 24]. 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 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 mg/mL, and pregnant rats received a single intraperitoneal (i.p.) injection of 600 mg/kg 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 kept 4–5 to a cage. Experiments were performed on male offspring.

Animals were administered with 4-nitrophenyl-4-[bis(1,3-benzodioxol-5-yl)(hydroxy) methyl] piperidine-1-carboxylate (i.p., JZL184, Selleck, USA), a selective inhibitor of MAGL that enhances the levels of 2-AG. JZL184 was dissolved in DMSO to prepare a mother liquor at a concentration of 50 mg/mL. Then it was diluted with DMSO (5%), PEG400 (40%), Tween 80 (5%) and double distilled water into a clear working solution in a volume of 2.5 mg/mL. Doses of JZL184 were selected based on the results of previous

studies[25, 26]. The male offspring from different dams were randomly divided into six groups as follows (Fig. 1):

- (1) CON (injection with vehicle solution);
- (2) VPA (injection with vehicle solution);
- (3) VPA + 40AJ (40 mg/kg, i.p., Acute JZL184 injection once on PND35);
- (4) VPA + 1RJ (1 mg/kg/day, i.p., Repeated JZL184 injection from PND21-34);
- (5) VPA + 3RJ (3 mg/kg/day, i.p., Repeated JZL184 injection from PND21-34);
- (6) VPA + 10RJ (10 mg/kg/day, 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 [22]. 24 h after the last repeated injection and 2 h after the acute injection, animals from each group were anesthetized through an intraperitoneal injection of 10% chloral hydrate (0.3 mL/kg). The animals were sacrificed by decapitation for western blotting, quantitative PCR (qPCR), **liquid chromatography-tandem mass spectrometry (LC-MS/MS)**. The brain removed hippocampus and PFC dissected out and flash frozen in liquid nitrogen and stored at -80 °C. Starting from PND35, a series of behavioral tests were performed. The biochemical testing in the present study were applied in the rats of untested behavioral experiments. The behavioral testing and biochemical testing were carried out in 5–9 rats for separate experiments.

Behavioral testing

Behavioral testing was captured by video cameras and analyzed with the SMART (Spontaneous Motor Activity Recording and Tracking) v3.0 software system (Panlab, Barcelona, Spain). The apparatus were cleaned with 0.1% acetic acid between trials to preclude olfactory cues. All behavioral experiments were performed during the light cycle between 09:00 and 17:00, and testing was counterbalanced across treatment groups.

marble burying test

A clean cage (48 × 35 × 20 cm) was prepared with 5 cm fresh wood chip bedding material. On PND 35, a rat was placed individually to habituate the cage for 15 min. then they were 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 was recorded, which were covered with wood chip by more than 2/3 vol.

Self-grooming test

On PND 35, the rats were placed individually into a white cage (48 × 35 × 20 cm) and allowed to habituate for 5 min. Self-grooming behavior was recorded for 10 min. A timer was used to assess the cumulative time spent in self-grooming behavior, 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..

Open field test

On PND 35, the open-field was made out of charcoal grey plastic with a top opening. The dimensions of test box were 45 × 45 × 40 cm. Before the test, the rats were allowed to adapt to the test box for 5 minutes. Rats were individually placed in the center to initiate a 10-min test. The total distance moved and resting time of spontaneous activity, as indication of anxiety-related behaviors, were analyzed.

Three-chamber test

On PND 35, the three-chamber test was used to evaluate the social behavior of rats. Following acclimatization period (the rats were placed in the central chamber for 5 min, and the rats were allowed freely access to all chambers), two successive tests, including sociability test and social preference test, were lasting 10 minutes, respectively. Sociability test: animals were briefly confined to the central chamber while an unfamiliar rat (labeled as stranger 1) confined in a small wire cage 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 (labeled as stranger 2) was then placed in the empty cage. Sociability index was calculated as the ratio of time spent exploring stranger 1 over the empty cage. 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.

Morris water maze test

On PND36-40, the learning and spatial memory capabilities of rats were evaluated with 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 closed by 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 center of the first quadrant of the pool. The test period was divided into two phases. Phase 1: 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 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 guiding it to the platform and allowing it to remain on the platform for 15 s. Phase 2: On the 5th day, the rats were subjected to a spatial probe trial session in 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. Due to the consecutive 5 days' test, this test was not performed in the rats of acute injection group.

Biochemical testing

Quantitation of eCBs levels by LC-MS/MS

Fasting blood samples were collected into EDTA-evacuated tubes in the morning (7:30–8:30 a.m.) and immediately chilled on ice before centrifuging at 2000 rpm 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).

A 300- μ L volume of methanol (containing internal standards: AEA-D8 at 40 ng/mL, OEA-D2 at 40 ng/mL, PEA-D4 at 40 ng/mL and 2-AG-D5 at 160 ng/mL) was added to a 100- μ L aliquot of plasma sample for protein precipitation. The mixture was eddied for 5 min and centrifuged at 16,000 \times g for 10 min, and 1 μ L 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 v.1.6.2 software (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 \times 2.1 mm, 1.7 μ m; Waters, Milford, MA, USA) and VanGuard column (5 mm \times 2.1 mm, 1.7 μ m; Waters, USA). 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. 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 Table S1. 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 (N₂) medium.

A 50 mg (\pm 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 μ L methanol (containing internal standards: AEA-D8, PEA-D4, OEA-D2 and 2-AG-D5 at 100 ng/m) and additional 200 μ L 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 μ L of MS-grade water. The sample was incubated at room temperature for 10 min and centrifuged at 16,000 \times g for 10 min. A 500- μ L upper (organic) phase was collected and dried in a vacuum centrifuge (Savant™ SPD131DDA SpeedVac™, Thermo fisher). Dry residue was re-dissolved in 200 μ L 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 version 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). 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 ammonium acetate. A mobile phase gradient was applied at a flow rate of 0.3 mL/min. 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 Table S1. Optimum parameters were as follows: Cone Gas Flow 10 l/h; Capillary voltage 3000 V; desolvation Temperature 550°C; desolvation gas flow, 1000 l/h.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Human venous blood samples from ASD subjects and control donors were drawn and collected in sterile EDTA tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Histopaque 1,077 density gradient. Briefly, blood was diluted 1:1 in phosphate buffer saline (PBS), overlaid onto lymphocyte separation media, centrifuged at 2,000 rpm 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, Inc., Waltham, MA, USA) or protein lysis buffer for further molecular analysis.

RNA Extraction, reverse transcription and qPCR

Total RNA was extracted from PBMCs and brain tissue using Trizol Reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RNA quantity was determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 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 quantitative PCR (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 Table S2. 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 compared using the $2^{-\Delta\Delta CT}$ method.

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 rpm 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 µg) 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- α , MAGL and GAPDH. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit, and goat anti-mouse secondary antibody (antibodies details seen Table S3). Protein bands were detected with an enhanced chemiluminescence western blotting detection kit (Beyotime Institute of Biotechnology, Shanghai, China). Results were analyzed 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.

Statistical analyses

The results were presented as means \pm SD or means \pm SEM and were analyzed with GraphPad Prism 7.0 (GraphPad software, CA, USA). The comparison of the data was analyzed by one-way analysis of variance (ANOVA) or paired student's *t* test. Repeated-measures ANOVA was used to evaluate the differences in escape latency in Morris water test. Tukey's *post hoc* test was applied in multiple comparison. All reported *p* values were two-tailed, and statistical significance was defined at the $\alpha = 0.05$ level.

Results

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 ($P < 0.05$, Fig. 2A). The levels of PEA in the ASD group were negatively correlated with the total scores of the Autism Behavior Checklist (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 rats compared to CON rats ($P < 0.05$, Fig. 2B), and there was no significant difference in the AEA, PEA, OEA and 2-AG levels in the PFC (Fig. 2C).

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 also higher ($P < 0.05$, Fig. 3A, 3C). Due to no significant differences in eCBs levels in the PFC, we next solely investigated hippocampal eCB system expression. Prenatal VPA exposure differed the mRNA expression of CB1R, CB2R, FAAH and DAGL in the hippocampus compared to CON rats ($P < 0.05$). There was significant increase in CB1R, FAAH and MAGL protein levels, whereas CB2R and DAGL protein levels did not reach the significance (Fig. 3B, 3D).

Effect of JZL 184 treatment on autism-like phenotypes

One-way ANOVA analyses revealed statistical significance in marbles buried among groups ($F_{(5, 42)} = 6.22$, $P = 0.0002$). Marble-burying in VPA rats was greater than in CON rats ($P = 0.0004$), and a trend towards JZL184 treated rats burying less than VPA rats was obvious (VPA vs. VPA + 40AJ: $P = 0.0020$, VPA vs. VPA + 3RJ: $P = 0.0014$, VPA vs. VPA + 10RJ: $P = 0.0025$, Fig. 4A). There was also significant difference in the repetitive self-grooming behavior ($F_{(5, 42)} = 7.689$, $P < 0.0001$). The *Post Hoc* test confirmed that VPA rats self-groomed more excessively than CON rats ($P < 0.0001$), and a decreased trend after JZL184 treated (VPA vs. VPA + 40AJ: $P = 0.0225$, VPA vs. VPA + 1RJ: $P = 0.0006$, VPA vs. VPA + 3RJ: $P = 0.0001$, VPA vs. VPA + 10RJ: $P = 0.0013$, Fig. 4B). All in all, JZL184 administration could normalize the repetitive and stereotypical behaviors of VPA rats.

We logged the distance movement and resting time to further gauge anxiety-like behavior. One-way ANOVA analyses revealed statistical significance in locomotor activity among groups (distance movement: $F_{(5, 42)} = 18.2$, $P < 0.0001$, resting time: $F_{(5, 42)} = 16.86$, $P < 0.0001$). On exposure to a novel brightly lit aversive open field arena, VPA rats exhibited hyperlocomotion as demonstrated by an increase in distance movement and decrease in resting time when compared to CON rats ($P < 0.0001$, $P = 0.0027$, respectively; Fig. 4C-E). JZL184 acute injection and 3 mg/kg repeated injection could reverse the elevated locomotor activity of VPA rats (distance movement: $P = 0.0045$, $P = 0.0002$, respectively; resting time: $P < 0.0001$, $P = 0.0016$, respectively; Fig. 4D and E). In contrast, 1 mg/kg and 10 mg/kg repeated injection failed to exhibit this effect, and VPA rats with 1 mg/kg and 10 mg/kg repeated injection still showed increased locomotor activity with respect to CON rats (distance movement: $P < 0.0001$, $P = 0.0003$, respectively; resting time: $P = 0.0064$, $P = 0.0044$, respectively; Fig. 4D and E).

Prenatal exposure to VPA impaired social interaction behaviors in offspring as reported previously. In habituation phase, rats from all the experimental groups did not show any significant chamber preference (data not shown). In sociability test, VPA groups displayed the typical autism-like phenotype and failed to present a significant preference toward the unfamiliar rat compared with the object, JZL184 acute injection and 10 mg/kg repeated injection corrected this aberrant behavior ($P = 0.0017$, $P = 0.0006$, respectively, Fig. 4F and G). However, only acute injection significantly enhanced sociability index ($P = 0.0095$, Fig. 4H). In social preference test, which determines whether the experiment rats prefer socially novel rat or familiar one, VPA rats showed a reduced social novelty recognition compared to CON rats which quantified by time spent in investigatory behavior with stranger rat 2 and social preference index ($P = 0.0006$, $P = 0.0377$, respectively, Fig. 4I-K). JZL184 acute injection, and repeated injection at doses of 3 mg/kg and 10 mg/kg increased time spend in stranger 2 side ($P = 0.0009$, $P = 0.0034$, $P = 0.0004$, respectively, Fig. 4J) and social preference index ($P = 0.0297$, $P = 0.0258$, $P = 0.0077$, respectively, Fig. 4K). In contrast, the influence of 1 mg/kg JZL184 repeated injection on social behaviors could not be observed. These results suggested JZL184 administration could restore impaired social interaction.

In the Morris water test, the rats are required to find a hidden platform to escape from swimming in a pool of water. During the training trial, escape latency decreased in all 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: $F_{(12, 105)} = 0.93$, $P = 0.52$; Fig. 4L and M). A *Post Hoc* test showed that compared with CON group, VPA rats required longer escape latency, whereas repeated treatment with 3 or 10 mg/kg JZL184 notably shortened the escape latency during the training days (2nd, 3rd, and 4th day, $P < 0.05$, Fig. 4L and M). On the 5th day, one-way ANOVA analyses revealed that there was statistical significance among groups in the spatial probe test ($F_{(4, 35)} = 5.101$, $P = 0.0024$, Fig. 4N). The results indicated VPA rats were not able to remember the original platform, and passing less times with respect to CON rats ($P = 0.0022$). Interestingly, VPA rats with JZL184 repeated treatment at dose of 3 mg/kg significantly increased platform crossing times ($P = 0.0117$; Fig. 4L and N). Taken together, JZL184 3 mg/kg repeated treatment could improve learning and spatial memory deficits of VPA-induced rats.

Effect of JZL184 treatment on the components of the eCB system

Acute administration of JZL184 (40 mg/kg) did not alter eCBs levels in the hippocampus and the PFC (Fig. 5A-J). Repeated administration of JZL184 at doses of 3 mg/kg and 10 mg/kg enhanced the levels of 2-AG in the hippocampus and corresponding reductions in its metabolite, AA. There were no differences in the levels of AEA, PEA and OEA (Fig. 5A-E). Repeated administration of JZL184 at doses of 3 mg/kg increased the levels of AEA and 2-AG in the PFC without affecting the levels of PEA, OEA and AA (Fig. 5F-J).

Acute administration of JZL184 (40 mg/kg) reduced CB1R mRNA levels of VPA-exposed offspring in the hippocampus ($P < 0.0001$). Repeated injection JZL184 at dose of 10 mg/kg influenced CB1R, CB2R and DAGL mRNA expression (Fig. 6).

Discussion

This study aimed at illuminating that ASD children and ASD model rats exhibited disruption of eCB system and pharmacological modulators of eCB system may have a therapeutic potential in ASD. Our results of reduced eCBs content, elevated degrading enzymes together with a compensatory up-regulation of CBRs suggested lower eCB signaling in ASD. In the meantime, we observed that JZL184 treatment, through enhancing intrinsic 2-AG levels, ameliorated autistic behaviors in VPA-exposed offspring. This is characterized by a reduced repetitive and stereotypical behaviors in marble burying and self-grooming test, reduced hyperactivity in open field test, increased sociability and social preference in three-chamber test and improved cognitive functioning in Morris water maze test. This research is important in driving the identification of potential targets for improved therapeutic treatments in ASD.

To date, there were only two human evidence about the eCBs levels in ASD children's blood sample[11, 12], and their findings that lower concentrations of AEA, PEA and OEA in autistic children were consistent with ours. Noteworthy, this is the first time that we found circulating 2-AG levels were reduced in autistic children. We also found the ASD children with lower PEA level represented more serious ASD traits.

Interestingly, Kelly[27] demonstrated that plasma eCBs levels were dysregulated at age one in children with poor communication scores at age three, which implicating eCBs may be potential biomarker for early diagnosis of ASD. Anecdotally, case reports from Italy firstly have corroborated that PEA, alone or combination with other natural supplements, can refine ASD core impairments [28]. Subsequently, an Iranian randomized, double-blind placebo-controlled trial revealed that PEA (600 mg twice daily) may augment therapeutic effects of risperidone on ASD-related irritability and hyperactivity without serious side effects [29]. The non-psychoactive, medical cannabis (e.g. cannabidiol, cannabidivarin) in ASD patients appears to be well tolerated, safe (a low side effects rate) and effective option to relieve autistic symptoms in some countries [30, 31], nevertheless, the cannabinoid treatment remains a controversial ethical issue in individuals with ASD, and any kind of cannabinoid consumption is illegal in China. Collectively, eCBs, either as diagnostic biomarker or as potential therapeutic target, their decreased levels were associated with ASD.

Confirming the human results, we found levels of two major eCBs, AEA and 2-AG, were reduced in the hippocampus of VPA-induced rats. However, Kerr and colleagues[16] found levels of AEA, PEA, OEA and 2-AG in hippocampus did not differ between VPA and CON rats. Intriguingly, they demonstrated that eCBs levels were enhanced in the hippocampus of VPA-exposed rats immediately following sociability test, that is, the contents of eCBs were susceptible to the behavioral testing which is supported by many other studies[32, 33]. To this end, in the present study behavioral experiments were paralleled by biochemical measurement, whereas Kerr examined eCBs concentration 72 h after animals underwent behavioral 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 similar profile and magnitude of eCBs in the hippocampus and in the PFC. In line with that changes of eCB system are more pronounced in the hippocampus with respect to the PFC[18].

In light of PBMCs (lymphocytes, NK cells and monocytes) could serve as a tool to investigate eCB system changes in CNS in several neuropsychiatric disorders, we also examined eCB-associated receptors and enzymes in PBMCs from autistic children. CB1R is the most abundant G protein-coupled receptor located in the brain, while CB2R is sparsely expressed in the brain and instead principally in immunoregulatory cells, like microglia and peripheral immune cells[34]. 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. Of note, we found that transcription and translation of CB2R in the PBMCs and CB1R in the hippocampus were all enhanced. Siniscalco[13] showed similar results to ours, they found unchanged CB1 mRNA levels and upregulated CB2R mRNA and protein levels in ASD-PBMCs as compared to healthy subjects. That prenatal VPA exposure increased CB1R protein levels were also observed in Zamberletti study[18]. Remarkably, CB1R and CB2R activation exert diverse consequences across cellular physiology. As the main molecular target of eCBs, CB1R is found high densities on presynaptic termini of glutamatergic and GABAergic neuron, thereby, the activation of CB1R is implicated in regulation of excitatory-inhibitory balance, synaptic strength and neurotransmitter release, ultimately mediating social functioning, learning and memory[35, 36]. In conjunction, genetic variants in *CNR1* (encoding CB1R) gene

were correlated to not only verbal but non-verbal social communication as well in ASD researches[37, 38]. Additionally, via activation of CB2R, eCB system exerts anti-inflammatory actions and decrease glial activity to prevent excessive inflammation and cell damage [34, 39]. We could not obtain brain tissue of patients, nonetheless, to some degree, the expression of CB2R on peripheral immune cells reflects changes in the CNS[40]. Actually, there was also an increase in mRNA expression of CB2R in the hippocampus of VPA-exposed offspring in this study. Hence, our findings support the notion that eCB system played the protective role of inflammatory response in autistic children via elevating CB2R expression[13], however further experimental evidence towards neuroinflammation is needed. Since it is impossible that the changes in components of eCB system are independent from one another, researchers suggested there might be a negative feedback regulation between eCBs and CBRs densities [41, 42]. We deduced that upregulation of CBRs was in response to the lower levels of eCBs in autistic patients and model rats in the current study. Alterations in CBRs levels are transient adaptive reactions which attempt to re-establish normal homeostasis disrupted by the disease.

So far, only Siniscalco's team previously investigated eCB system in PBMCs from individual with ASD, but they incorporated neither 2-AG metabolic enzymes nor protein expression of eCB-related enzymes into their study[13, 14]. To our knowledge, this is the first study to explore relatively entire components of eCB system in PBMCs from human. The biosynthesis of 2-AG can be catalyzed by two diacylglycerol lipase isoforms, namely DAGL- α and DAGL- β . DAGL- α is expressed throughout the brain and 2-AG levels dropped by up to 80–90% in the brain in the DAGL- α null mouse brain[43]. Given that DAGL- α is the main 2-AG synthesizing enzyme in the brain, in this study DAGL- α expression was solely examined (DAGL for short). We surprisingly revealed DAGL mRNA expression was increased in the human and rat sample, but not protein. Meanwhile, we found concurrent increases in expression of FAAH and MAGL, responsible for AEA and 2-AG degradation, both in the PBMCs and in the hippocampus. The lower eCB levels in the present study may account for increased degraded enzymes. Interestingly, in Siniscalco's study, FAAH mRNA expression did not significantly change and NAPE-PLD slightly decreased in 17 ASD cases and 22 healthy controls[13]. Our findings from animals were in keeping with previous reports that upregulated expression of FAAH and MAGL in VPA-induced rats[18, 44]. As a whole, our results and other studies highlighted the presence of a reduced eCB signaling in ASD children and animal model that might be responsible for the deficits in the cognitive and social domain.

The animal model studies have shown that prenatal VPA exposure in rodents recapitulates ASD-like pathophysiology at a molecular, cellular and behavioral level. VPA-induced rats have been developed and became a widely used environmental preclinical model of ASD with strong face and construct validity, and also serve as a good platform for testing pharmacological reagents that might be use treating ASD. On behavioral level, our findings have confirmed that VPA-exposed rats exhibited the core symptoms of ASD, impaired social interaction and repetitive behavior, possibly co-occurred emotional and cognitive problems. Meanwhile, what we found that prenatal VPA exposure inducing similar disturbance of eCB system in offspring rats to ASD children was in accordance with prior researches[16, 18], which indicating a reduced eCB tone in ASD. Therefore, we evaluated efficacy of boosting 2-AG levels by hydrolysis inhibitor JZL184 toward repetitive and stereotypical behaviors, hyperactivity, deficits in social and

cognitive functioning in VPA-induced rats. Results here presented showed either acute or chronic JZL184 administration was able to relieve ASD-like behaviors, which were in line with previous reports in *Fmr1* knock-out mice and *Shank3B*^{-/-} mice [15, 45, 46].

Rats treated with acute administration 40 mg/kg JZL184 exhibited reduced repetitive marble-burying and grooming behaviors and hyperactivity, and improvement in sociability and social preference induced by VPA exposure. We observed that single injection of JZL184 (40 mg/kg) did not change the levels of eCBs in the hippocampus and PFC, which was in consistence with the findings from Kerr [47] who did not detect the alternation in the levels of eCBs 2.5 h after injection JZL184 (10 mg/kg). Nonetheless, JZL184 at dose of 40 mg/kg could show loss of MAGL activity[48]. Kruk-Slomka [49] showed the similar results that acute injection of JZL184 40 mg/kg significantly decreases locomotion and improved long-term acquisition of memory and learning process. Although the alternations of eCB system were not yet appeared, acute JZL184 treatment still exhibited its positive effect on behaviors. Repeated treatment with JZL184 at dose of 3 mg/kg displayed a restored effect on repetitive marble-burying and grooming behaviors, locomotor activity, social preference, learning and spatial memory. In contrast, high dose (10 mg/kg) only partially affected repetitive behaviors, social preference and learning, and low dose tested (1 mg/kg) was instead ineffective. In fact, the behavioral efficacious dose of 3 mg/kg robustly increased 2-AG in both hippocampus and PFC as well, whereas concomitantly with a little increase in AEA levels. In this regard, *Dagla*^{-/-} animals showed an extensive reduction in 2-AG levels and concomitant decrease in AEA in the hippocampus and cortex, furthermore, administration of JZL184 to *Dagla*^{-/-} mice increased not only 2-AG levels but also the level of AEA[43]. Schlosburg also confirmed that chronic dosing also caused a modest elevation in AEA[22]. These data, together with our similar results, suggests 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 likely generates a partial effect of blockade on FAAH, rather than MAGL contributes directly to the degradation of AEA[22]. Convergent literature demonstrated that sustained elevations in brain 2-AG caused by either genetic deletion or chronic pharmacological blockade of MAGL led to CB1R desensitization and tolerance to CB1R agonists, significant decreases in CB1R number and function, this effect would limit therapeutic potential of JZL184[22]. A chronic JZL184 dose of 16 mg/kg daily (typically 1 week) reliably produced tolerance[50]. The current study observed the dose of 10 mg/kg daily (2 weeks) has induced reduced the CB1R and CB2R expression in the hippocampus. Thus, repeated administration of a low dose JZL184, 3 mg/kg in the current study, could produce elevated eCB brain levels without behavioral tolerance and CB1R desensitization. Additionally, acute treatment with JZL184 also has an important effect on CBRs[25]. We hypothesized that JZL184 treatment could improve the ASD-like behaviors via CBRs-dependent and -independent way: (1) As 2-AG acting via CB1R generally suppresses synaptic transmission, neuronal excitability, and neurogenesis, the improvement of eCB-induced synaptic plasticity could ameliorate ASD-like behaviors. (2) 2-AG is an important metabolic intermediate in lipid synthesis and also serves as a major source of AA, which is required for pro-inflammatory prostaglandin synthesis. Pharmacological inactivation of MAGL induced not only elevations of 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 reduction in AA rather than the augmentation of eCB signaling, possibly relevant to cyclooxygenase enzymes[48]. Furthermore, inactivation of MAGL could suppress the pro-inflammatory cytokines production and microglial activation induced by LPS[48, 51]. Eventually, independent of CBRs, increasing 2-AG generates protective function against neuroinflammation, and then ameliorates ASD-like behaviors.

Limitations

This study had some limitations that must be taken into account in the interpretation of the results. First, the present study is limited by the use male offspring only. The eCB system is known to exhibit sexual dimorphism in human and rodents, particularly in CB1R expression and functionality. Second, this study might lose some information of value because of not detecting the activity of enzymes and receptors, and additional non-cannabinoid receptors targets which are known to have affinity and activity at. While the therapeutic use of eCB system is inviting, extensive research will be required to further evaluate this complex regulatory pathway and the safety of pharmacological manipulation.

Conclusions

The current evidence strongly implicated alterations in the eCB system in human patients of ASD and animal models. The reduced eCBs content, elevated degrading enzymes together with a compensatory up-regulation of CBRs indicated a lower eCB signaling in ASD. Subsequently, augmentation of 2-AG levels by pharmacological inhibition of MAGL resulted in the normalization of ASD-related behavioral abnormalities in the VPA-exposed offspring. The improvement of behavioral phenotypes was consistent with the observed increase in 2-AG in the hippocampus and PFC when administration JZL184 with 3 mg/kg. These data provide preclinical evidence in support of the ability of JZL184 to ameliorate behavioral abnormalities resembling core and associated symptoms of ASD. The high heterogeneity in phenotypic presentation of ASD poses investigative and clinical challenges for treatment, subgroups of ASD individuals may benefit more from drugs that increase cannabinoid levels.

Abbreviations

2-AG
2-arachidonylglycerol; AA:arachidonic acid; ABC:Autism Behavior 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; LTD:monoacylglycerol lipase; MAGL:monoacylglycerol lipase; NAPE-PLD:N-acylphosphatidylethanolamine-specific phospholipase D; OEA:oleoylethanolamide; PBMC:peripheral blood mononuclear cell; PEA:Palmitoylethanolamide; PND:postnatal day; PUFA:polyunsaturated fatty

acid; qPCR:quantitative PCR; SRS:Social Responsiveness Scale; VABS:Vineland Adaptive Behavior Scale; VPA:valproic acid.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board of Harbin Medical University for Medical Sciences. 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.

Consent to publication

Not applicable.

Availability of data and materials

The dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors disclose no potential conflicts of interest.

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Authors' contributions

Lijie Wu and Caihong Sun conceived and designed the research; Yu Liu, Shu Xie, Dexin Li, Ling Li, and Mingyang Zou performed the experiments; Yu Liu and Mingyang Zou analyzed the data; Mingyang Zou, Caihong Sun and Lijie Wu contributed reagents/materials/analysis tools; Shu Xie, Luxi Wang and Wei Xia performed clinical assessment; Mingyang Zou wrote the paper. All authors contributed and have approved the final manuscript.

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Figures

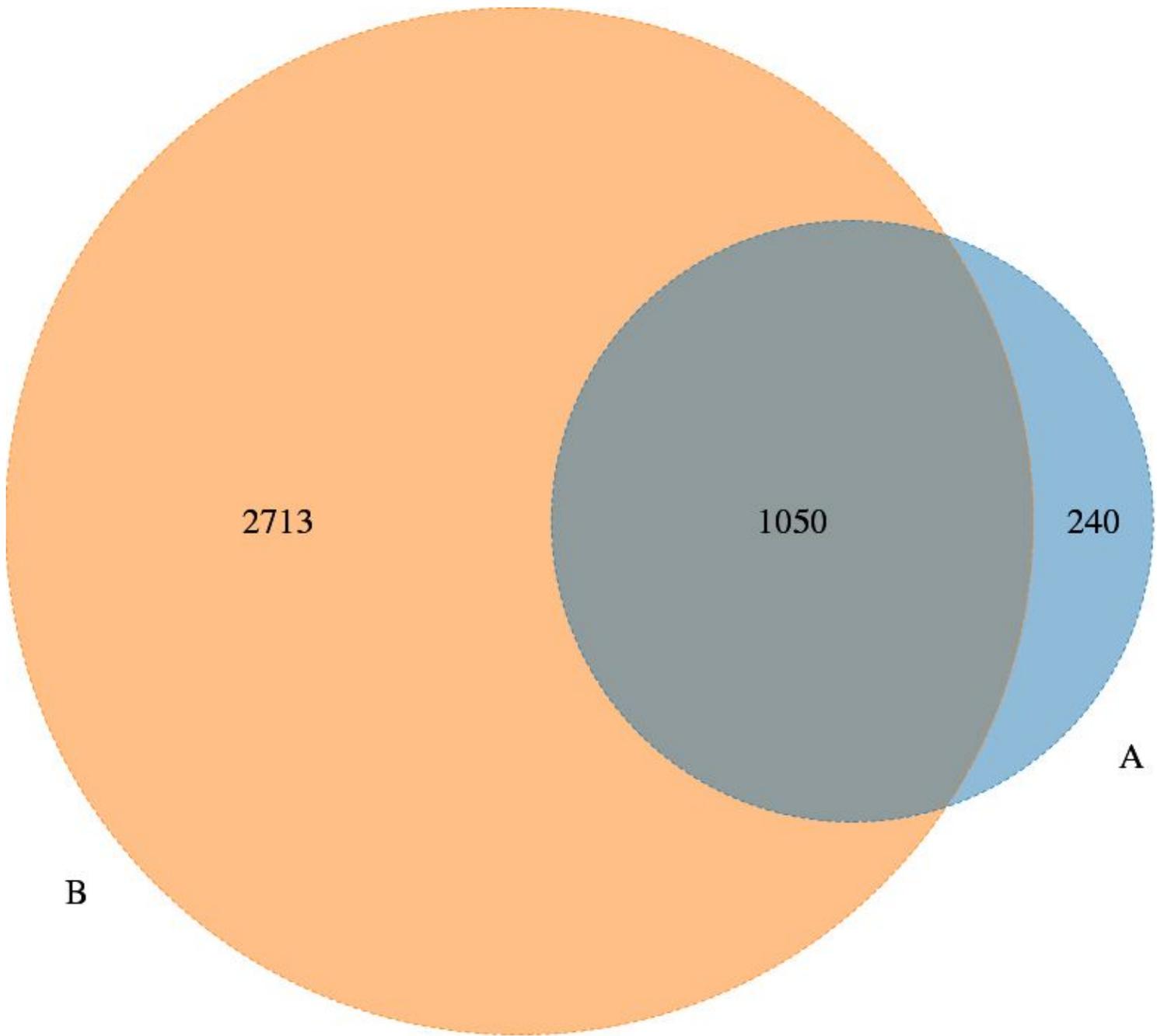


Figure 1

flowchart of JZL184 administration in VPA-exposed offspring.

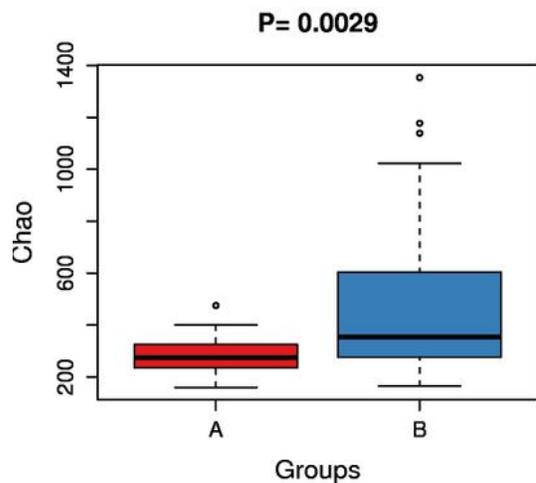
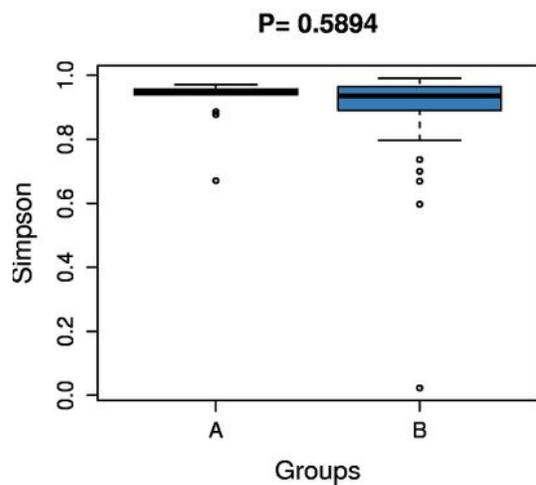
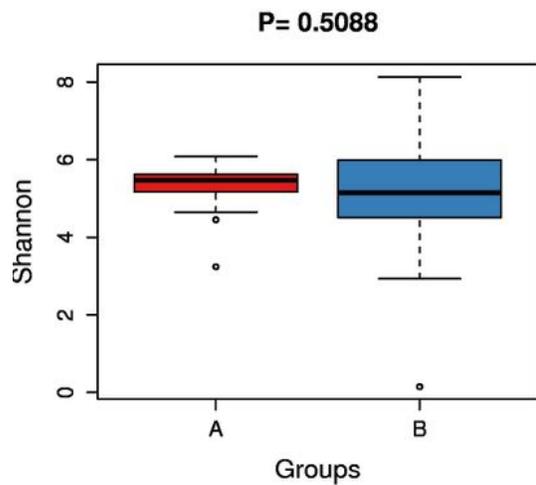


Figure 2

The levels of AEA, PEA, OEA and 2-AG in cases and controls. (A) Plasma levels in participants (n=70 children/group); (B) Hippocampal levels in rats (n=5 pups/group); (C) Prefrontal cortical levels in rats (n=5 pups/group). The error bars represent SD, * P < 0.05, *** P < 0.001 from paired student's t test.

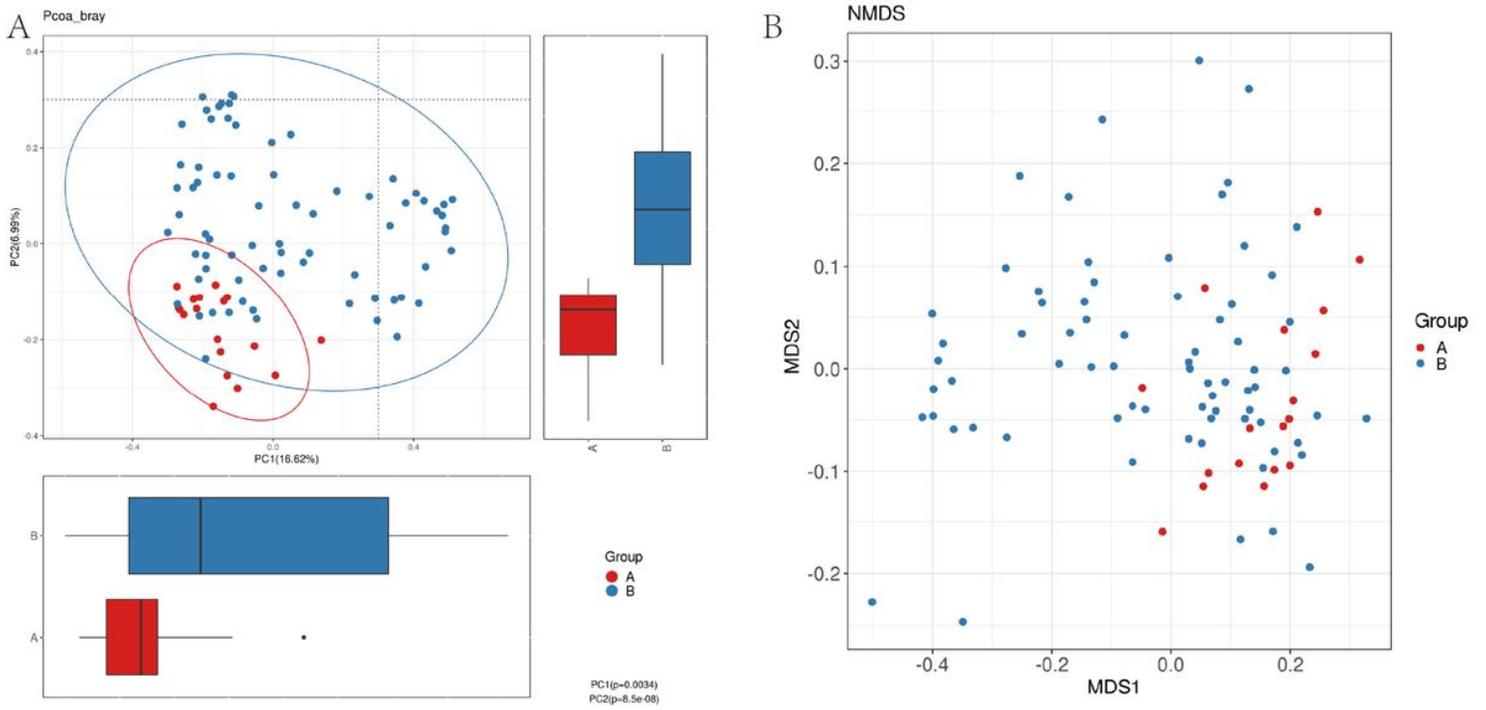


Figure 3

The components of eCB system expression levels in cases and controls. (A) relative mRNA expression levels in PBMCs of participants (n=36 children/group); (B) relative mRNA expression levels in hippocampus of rats (n=9 pups/group). (C) relative protein expression levels in the PBMCs of participants (n=27 children/group); (D) relative protein expression levels in the hippocampus of rats (n=9 pups/group). Data represented as means \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 from paired student's t test.

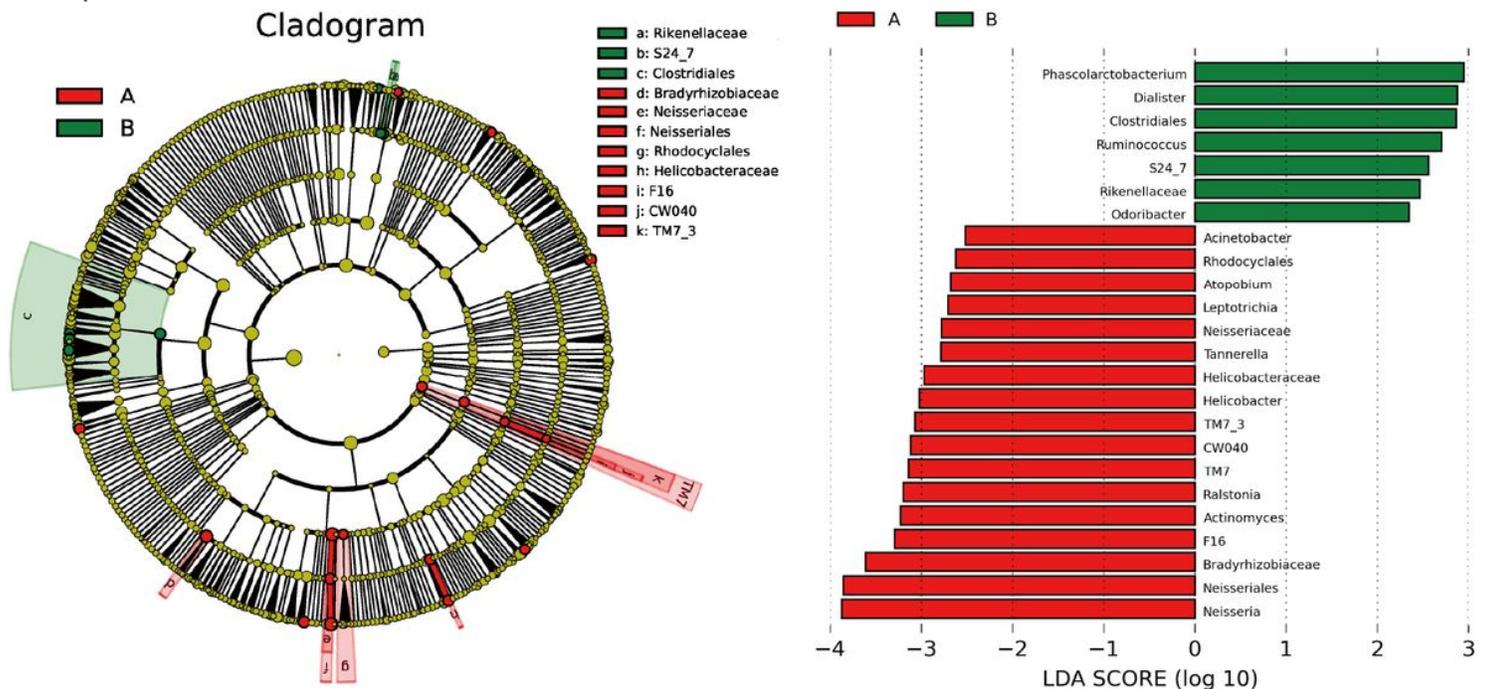


Figure 4

Effects of JZL184 treatment on behaviors of VPA-exposed offspring. (A-B) repetitive and stereotypical behaviors in marble burying test and self-grooming test: (A) The number of marbles buried. (B) the self-grooming time. (C-E) locomotor activity in open field test: (C) Representative images of locomotor tracks. (D) Distance movement. (E) Resting time. (F-K) social behavior in three-chamber test: (F-H) Sociability test. (I-K) Social preference test. (F and I) Representative images of track movements. (G) time spent in chamber of sociability towards stranger rat 1 or empty cage (H) Sociability index, calculated as time spent in the stranger 1 / empty cage. (J) time spent in chamber of social preference towards stranger 1 or stranger 2 (K) Social preference index, was calculated as time spent in the stranger 2 / the stranger 1. (L-N) learning and memory in Morris water maze test: (L) Representative images of the escape latency and the passing times through the platform area. (M) Escape latency on different training days. (N) Times of rats passing the site where there had been a platform. Data represented as means \pm SEM (n =8 pups/group). (G and J) Results were analyzed by Paired student's t test ($^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, stranger 1 side vs. empty side or stranger 2 side). (M) Results were analyzed by repeated-measures ANOVA. Remaining results were analyzed by one-way ANOVA with Tukey's post hoc test ($\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, $\#\#\#\#P < 0.0001$, vs. CON group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, vs. VPA group). AJ, acute JZL184 treatment; RJ, repeated JZL184 treatment.

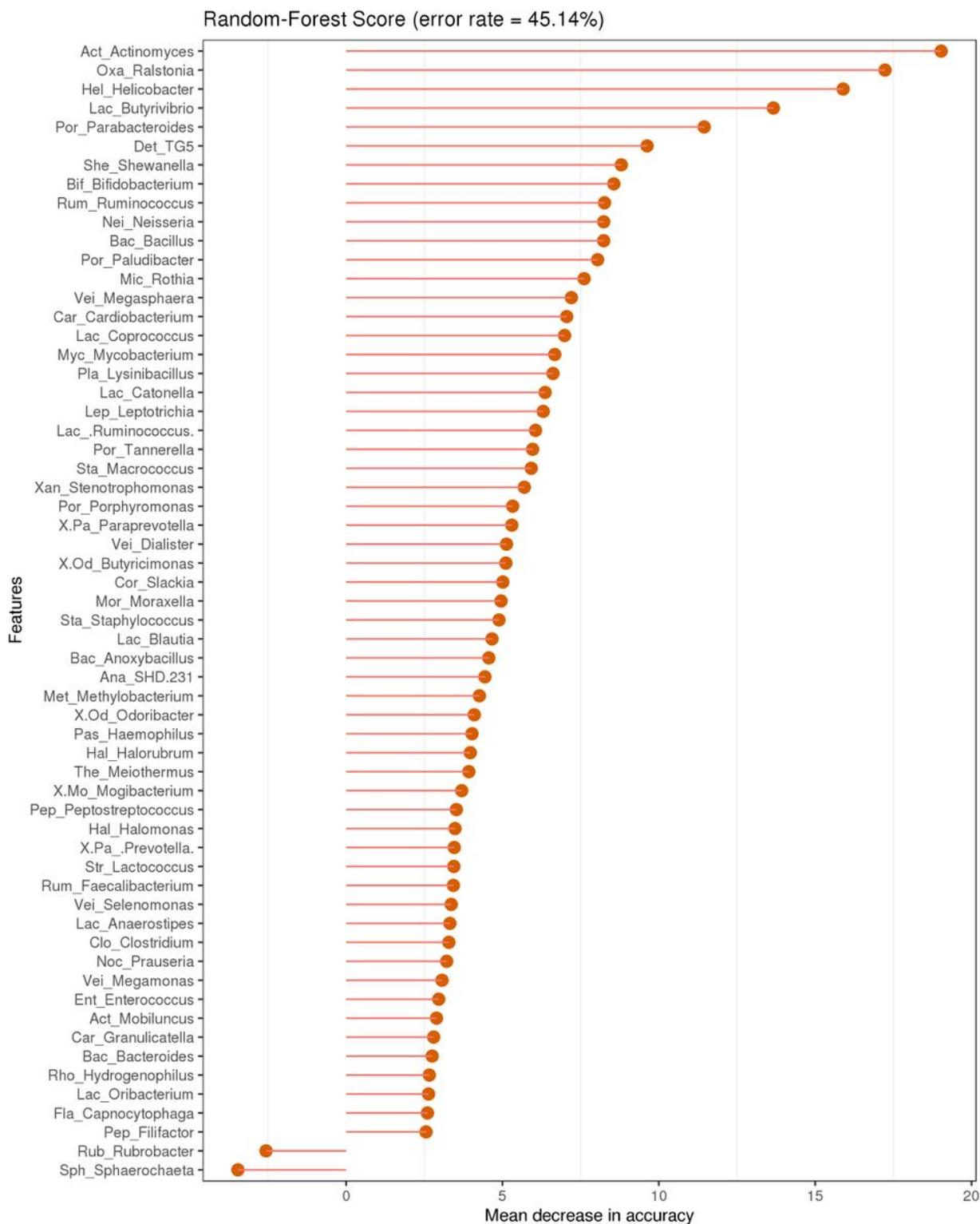


Figure 5

Effects of JZL184 treatment on the levels of AEA, PEA, OEA, 2-AG and AA in hippocampus (A-E) and in the PFC (F-J) in VPA-exposed offspring (n = 5 pups/group). The error bars represent SD. Results were analyzed by one-way analysis of variance with Tukey's post hoc test (#P<0.05, ##P<0.01, ####P<0.0001 vs. CON group; *P < 0.05, ** P<0.01, vs. VPA group). RJ, repeated JZL184 treatment.

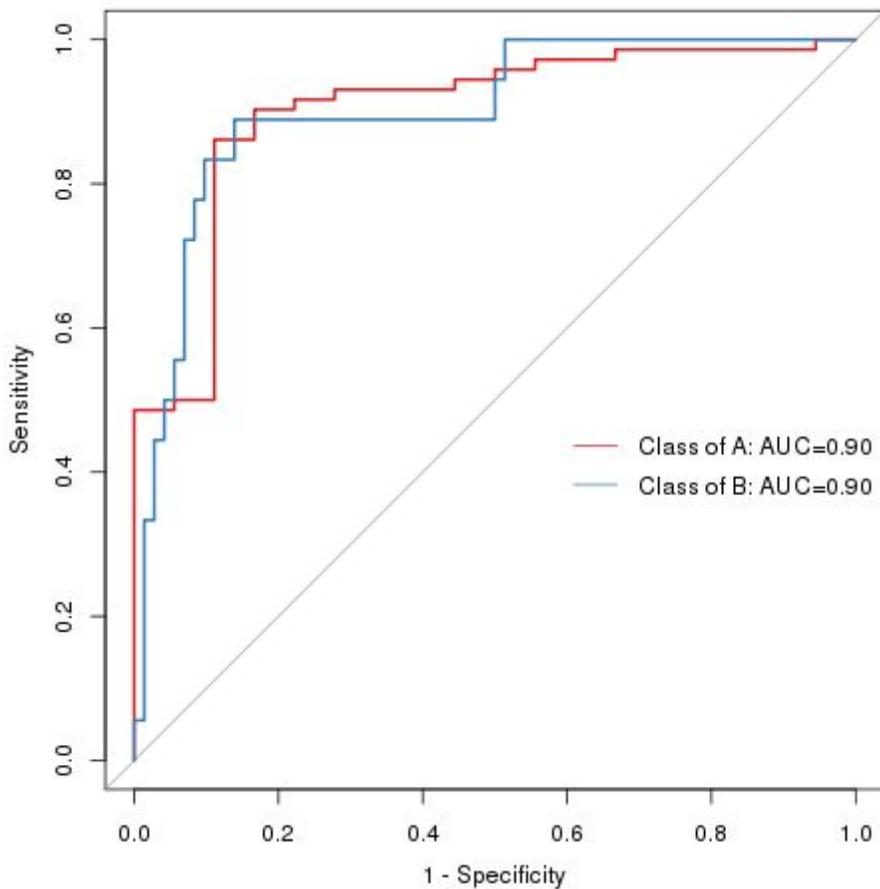


Figure 6

Effects of JZL184 treatment on the expression levels of eCB system. Data represented as means \pm SEM (n =10 pups/group). Results were analyzed by one-way ANOVA with Tukey's post hoc test (** P<0.01, ***P < 0.001, **** P<0.001, vs. VPA group). RJ, repeated JZL184 treatment.

Supplementary Files

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- [supplementarytables.docx](#)