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Peripubertal cannabidiol treatment rescues behavioral and neurochemical abnormalities in the MAM model of schizophrenia

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HIGHLIGHTS

- Prenatal MAM exposure affects CB1 receptor in the prefrontal cortex of adult rats.
- Peripubertal cannabidiol treatment prevents behavioral and molecular alterations in MAM rats at adulthood.
- Peripubertal cannabidiol treatment does not negatively affect control animals.

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ABSTRACT

In agreement with the neurodevelopmental hypothesis of schizophrenia, prenatal exposure of rats to the antimitotic agent methylazoxymethanol acetate (MAM) at gestational day 17 produced long-lasting behavioral alterations such as social withdrawal and cognitive impairment in the social interaction test and in the novel object recognition test, respectively. At the molecular level, an increased cannabinoid receptor type-1 (CB1) mRNA and protein expression, which might be due to reduction in DNA methylation at the gene promoter in the prefrontal cortex (PFC), coincided with deficits in the social interaction test and in the novel object recognition test in MAM rats. Both the schizophrenia-like phenotype and altered transcriptional regulation of CB1 receptors were reversed by peripubertal treatment (from PND 19 to PND 39) with the non-psychotropic phytocannabinoid cannabidiol (30 mg/kg/day), or, in part, by treatment with the cannabinoid CB1 receptor antagonist/inverse agonist AM251 (0.5 mg/kg/day), but not with haloperidol (0.6 mg/kg/day). These results suggest that early treatment with cannabidiol may prevent both the appearance of schizophrenia-like deficits as well as CB1 alterations in the PFC at adulthood, supporting that peripubertal cannabidiol treatment might be protective against MAM insult.

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1. Introduction

Schizophrenia (SCZ) is now well recognized as a developmental disease. The belief is that anomalies in neurodevelopment during early-life may affect brain functions and physiology resulting in the emergence of psychosis later in life, usually during early adulthood (Marenco and Weinberger, 2000; Millan et al., 2016). Early treatment during the prodromal phase of the disease has shown to reduce the risk of progression to first-episode psychosis in patients (Sommer et al., 2016); as well as to prevent behavioral and structural abnormalities in different neurodevelopmental animal models of SCZ (Gomes et al., 2016). However, the development of preventive strategies in the field of psychosis raises several clinical and ethical problems, since only 1/3 of individuals with prodrome symptoms develop SCZ (Piras et al., 2014). Therefore, preventive intervention should both reduce transition to SCZ and be safe for individuals who do not convert to psychosis (Mokhtari and Rajarethinam, 2013).

The methylazoxymethanol acetate (MAM) rat model, which involves prenatal exposure to methylazoxymethanol acetate, displays many SCZ-relevant functional and neuropathological deficits at adulthood (Lodge and Grace, 2009; Micale et al., 2013). Interestingly, these deficits can be reversed by prepubertal pharmacological (Du and Grace, 2013, 2016) or adolescent non-pharmacological (i.e., environmental enrichment strategy) interventions (Bator et al., 2018) to some degree. Therefore, the MAM model is an invaluable tool that reproduces the human condition in terms of construct, face and predictive validity to investigate potential antipsychotic compounds.

The endocannabinoid system (ECS) encompasses a large group of endogenous molecules including the two major lipid transmitters anandamide (AEA), 2-arachidonoylglycerol (2-AG) and the endocannabinoid-related molecules *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA), the class of enzymes deputed to their biosynthesis (nape-pld, gde-1, abdh4 and ptpn22 for AEA; daglα and daglβ for 2-AG) and degradation (faah for AEA; abdh6, abdh12 and magl for 2-AG) and at least two G-protein-coupled receptors named cannabinoid receptors of type 1 (CB1), type 2 (CB2) and transient receptor potential of vanilloid type-1 (TRPV1) channel (Iannotti et al., 2016).

Increasing evidence suggests that disturbances in ECS activity in the brain (in terms of CB1 dysregulation and/or altered endocannabinoids levels) are associated with the development of SCZ (Ruggiero et al., 2017; Saito et al., 2013). Pharmacological modulation of the ECS has therefore been viewed as a promising therapeutic approach (Kucerova et al., 2014). However, the majority of work linking the ECS with SCZ originates from epidemiological data, which can only suggest a non-causal association between early *cannabis* abuse and the development of psychiatric conditions later in life, including SCZ (Arseneault et al., 2002; Koethe et al., 2006). Currently, little is known about the therapeutic effect of early intervention with cannabinoids.

To this purpose, we investigated whether the development of SCZ-like symptoms, as induced by MAM (Lodge and Grace, 2009; Micale et al., 2013), could be paralleled by changes in ECS elements of specific brain regions involved in SCZ symptoms (prefrontal cortex–PFC, hippocampus–HIP and nucleus accumbens–NAc) (Lodge and Grace, 2009). Furthermore, we also attempted to reverse the SCZ-like abnormalities in the MAM model by peripubertal treatment with: 1) the non-psychotropic cannabinoid cannabidiol (CBD) (Campos et al., 2017; Osborne et al., 2017b), 2) the CB1 antagonist/inverse agonist AM251 (Kucerova et al., 2014; Roser et al., 2010; Roser and Haussleiter, 2012), and 3) first-generation antipsychotic haloperidol (HAL), used here as positive control (Valenti et al., 2011).

2. Material and methods

2.1. Animals and MAM model

Pregnant Sprague-Dawley rats were obtained from Charles River (Germany) at gestational day (GD) 13 and housed individually. They were randomly assigned to experimental groups and injected intraperitoneally (i.p.) with methylazoxymethanol acetate (MAM: 22 mg/kg) or vehicle (CNT: 0.9% NaCl) on GD 17, as previously described (D'Addario et al., 2017; Ruda-Kucerova et al., 2017a, 2017b; Večeřa et al., 2018). The mothers were regularly weighed and no differences were observed between the two experimental groups. No cross-fostering was used in this study, since in previous studies it did not impact the MAM phenotype (Moore et al., 2006). Newborn litters found up to 5 p.m. were considered to be born on that day (postnatal day 0 = PND 0). At birth, no difference was found in pregnancy length (CNT = 21.40 ± 0.24; MAM = 21.67 ± 0.21; $t = 0.8301$, $p > 0.05$), total number of pups per litter (CNT = 9.6 ± 0.5; MAM = 11.5 ± 0.9; $t = 1.598$, $p > 0.05$) or eye opening time (MAM = 16.40 ± 0.24; CNT = 16.67 ± 0.21; $t = 0.7401$, $p > 0.05$). Male pups were weaned on PND 22 and housed in groups of 2–3 with littermates until adolescent (PND 35–40) or adult (PND 100), at which time they were used for behavioral and neurochemical experiments, with food and water available *ad libitum* and under constant environmental conditions: relative humidity 50–60%, temperature 23 °C ± 1 °C, 12-h light-dark cycle (lights on at 6 a.m.).

2.2. Drugs and experimental design

All compounds were administered i.p. in a volume of 5 ml/kg of body weight. The CB1 antagonist/inverse agonist AM251 (Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO), Tween80 and saline (1:1:8). The non-psychotropic cannabinoid cannabidiol (CBD) kindly provided by Prof. Raphael Mechoulam (Hebrew University, Jerusalem, Israel) was dissolved in Tween80 (2%) and saline (98%). The typical antipsychotic haloperidol (HAL, Haloperidol-Richter®, Czech Republic) used here as positive control was dissolved in saline. Three groups of control animals were injected i.p. with AM251, CBD or HAL vehicle (VHC), respectively. As similar results were obtained from these three control groups, VHC data were pooled. As described in Fig. 1., from PND 19 to PND 39 [the period prior to puberty defined as PND 43.6 + 1 in Sprague-Dawley rats based on previous observations of balano-preputial separation and increases in circulating androgens (Clark, 1999; Korenbrot et al., 1977)] different groups of rats (n = 12–15) were treated i.p. with CBD (10 or 30 mg/kg/day), AM251 (0.5 mg/kg/day), HAL (0.6 mg/kg/day) or VHC, based on previous results (Gomes et al., 2015; Valenti et al., 2011; Zamberletti et al., 2012a, 2012b). The drug treatment period (PND 19 - PND 39) in the rats was carried out at the equivalent time of the childhood/periadolescent phase in humans (Andersen, 2003). To avoid litter effects each experimental group consisted of animals chosen randomly from different litters (at least four MAM-exposed litters and four vehicle-exposed litters). The experimental design resulted in 10 final groups: (1) offspring of control dams administered vehicle (CNT/VHC), (2) offspring of control dams administered CBD 10 mg/kg (CNT/CBD10), (3) offspring of control dams administered CBD 30 mg/kg (CNT/CBD30), (4) offspring of control dams administered AM251 (CNT/AM251), (5) offspring of control dams administered HAL (CNT/HAL), (6) offspring of MAM dams administered vehicle (MAM/VHC), (7) offspring of MAM dams administered CBD 10 mg/kg (MAM/CBD10), (8) offspring of MAM dams administered CBD 30 mg/kg (MAM/CBD30), (9) offspring of MAM dams administered AM251 (MAM/AM251) and (10) offspring of MAM dams administered HAL (MAM/HAL). As adults (from PND 100) the animals were submitted to a battery of behavioral tests with 5 days in between two consecutive tests in the following order, as previously described (Terzian et al., 2011): a) exploration-based approach

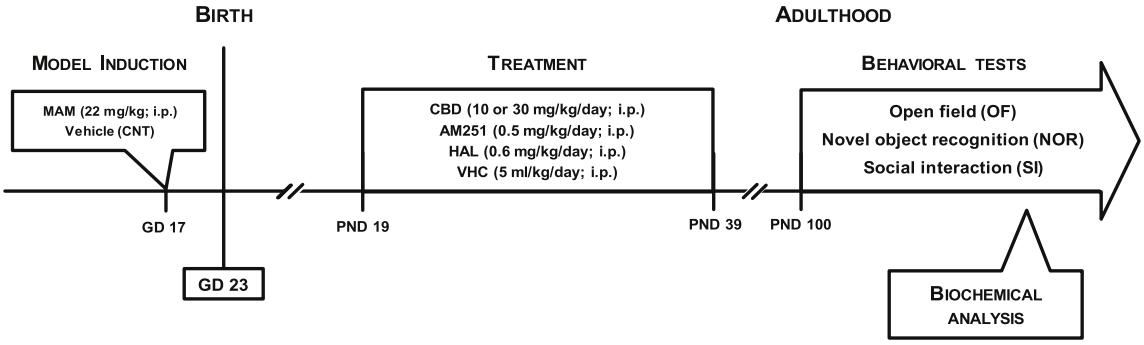

Fig. 1. Experimental design used to investigate the effects of peripubertal cannabidiol (CBD) treatment on offspring in MAM rat model of schizophrenia. Pregnant rats were exposed to methylazoxymethanol acetate (MAM; 22 mg/kg; i.p.) or saline (CNT; 1 ml/kg; i.p.) on gestational day (GD) 17. From PND 19 to PND 39 the resulting male offspring were subjected to repeated treatment with vehicles (VHC), cannabidiol (CBD: 10 or 30 mg/kg/day; i.p.), AM251 (0.5 mg/kg/day; i.p.) or haloperidol (HAL: 0.6 mg/kg/day; i.p.). Behavioral tests of the offspring were conducted at adulthood from PND 100. After completion, the neurochemical analyses were performed.

tests: open field (OF) test and novel object recognition (NOR) test; b) social approach: social interaction (SI) test. Immediately after the SI, the rats were decapitated in short aether anaesthesia and their brains were removed. The PFC [(corresponding to an area that included the rostral pole of the brain, and delimited medially by the interhemispheric fissure, laterally by the corpus callosum and caudally extended to AP +2.7 according to Paxinos and Watson (1998)], the HIP (dorsal and ventral) and the NAc (core and shell) were obtained by regional dissection on ice, immediately frozen on liquid nitrogen and stored at −80 °C until analysis. For binding assay coronal sections (20 mm-thick) were cut on a cryostat, mounted on gelatin-coated slides and stored at −80 °C until processing.

2.3. Behavioral testing

2.3.1. Spontaneous locomotor activity in the open field (OF) test

Exploratory activity was evaluated in moderately illuminated (80 lx) cubic metal arena (60 × 60 × 60 cm), as previously described (Drago et al., 2001; Tamburella et al., 2009). Animals were placed gently in the center of the arena and allowed to explore. The horizontal (number of squares crossed with all paws) and the vertical (number of rearing episodes) exploratory activity was recorded for 30 min and scored offline by two observers blinded to the treatment groups. The arena was cleaned with 0.1% acetic acid and dried after each trial.

2.3.2. Social interaction (SI) test

The test was carried out in a moderately illuminated room (120 lx), as previously described (Zamberletti et al., 2012b). Each animal was allowed to freely explore an unfamiliar congener in a metal arena (60 × 60 × 60 cm) for 10 min. The arena was cleaned with 0.1% acetic acid and dried after each trial. Social behaviors were defined as sniffing, following, grooming, mounting, and nosing. The whole testing phase was recorded and analyzed by two observers blinded to the treatment groups. We recorded the time spent in social behaviors and the number of interactions.

2.3.3. Novel object recognition (NOR) test

The experimental apparatus used for the NOR test was an arena (43 × 43 × 32 cm), made of plexiglass, placed in a moderately illuminated room (120 lx). As previously described (Zamberletti et al., 2012b), each animal was placed in the arena and allowed to explore two identical, previously unseen objects for 5 min (familiarization phase). After an inter-trial interval of 3 min, one of the two familiar objects was replaced by a novel, previously unseen object and rats were returned to the arena for the 5 min test phase. This phase was recorded and analyzed separately by two observers blinded to the treatment groups. The time spent exploring the familiar object (T_f) and the novel object (T_n) were scored. The discrimination index (*DI*) was calculated as $DI = (T_n\text{-}T_f)/(T_n + T_f)$. The arena and all objects were cleaned with 0.1% acetic acid and dried after each trial.

2.4. Biochemical methods

2.4.1. Extraction, purification and quantification of endocannabinoids and endocannabinoid-related compounds

The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and endocannabinoid-related molecules N-palmitoylethanolamide (PEA) and N-oleoylethanolamide (OEA) were extracted from tissues and then purified and quantified as described elsewhere (Matias et al., 2008). First, tissues were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM, pH 7.5 (2:1:1, v/v) containing internal deuterated standards for AEA, 2-AG, PEA and OEA quantification by isotope dilution (5 pmol of $[^2H]_8$AEA, 50 pmol of $[^2H]_5$2-AG, $[^2H]_4$ PEA, $[^2H]_2$OEA (Cayman Chemicals, MI, USA). The lipid-containing organic phase was dried down, weighed and pre-purified by open bed chromatography on silica gel. Fractions were obtained by eluting the column with 99:1, 90:10 and 50:50 (v/v) chloroform/methanol. The 90:10 fraction was used for AEA, 2-AG, PEA and OEA quantification by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), as previously described and using selected ion monitoring at M + 1 values for the four compounds and their deuterated homologues, as previously described (Bisogno et al., 2008; Di Marzo et al., 2001; Micale et al., 2009).

2.4.2. Real-time qPCR (RT-qPCR)

Total RNA was isolated from native tissues by use of the PureLink® RNA Mini Kit (Cat. N.: 12183018A; Thermo Fisher Scientific, Milan, Italy) following the manufacturer's instruction, and then quantified by spectrophotometric analysis. The purified mRNA was reverse-transcribed by use of iScript reverse transcriptase enzyme (Cat. N.: 1708840; Biorad, Milan, Italy). Quantitative real-time PCR was carried out in CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy) with specific primers by the use of Advance Universal SYBR Green Supermix (Cat. N.: 1725270 Bio-Rad, Milan, Italy). Samples were amplified simultaneously in quadruplicate in one-assay run with a non-template control blank for each primer pair to control for contamination or primer-dimers formation, and the ct (cycle threshold) value for each experimental group was determined. The housekeeping genes (the ribosomal protein S16 and/or HPRT) have been used as an internal control to normalize the ct values, using the $2^{-\Delta\Delta ct}$ formula (D'Addario et al., 2017; Iannotti et al., 2013). The primers used for PCR amplification are reported in supplementary table S1.

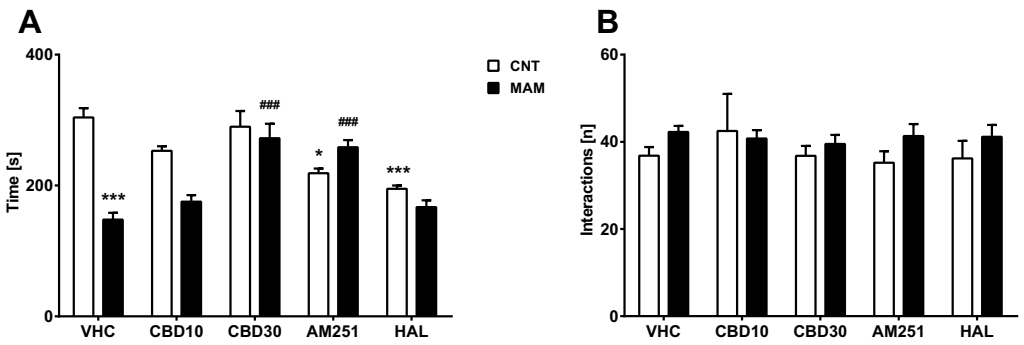

Fig. 2. Effects of peripubertal cannabidiol (CBD) treatment on the social activity of MAM rats in the social interaction (SI) test at adulthood. Data are presented as means ± S.E.M. (n = 5–12) of (A) time spent in interaction and (B) number of contacts for pairs of rats from the same treatment group. *p < 0.05 and ***p < 0.001 vs CNT/VHC; ###p < 0.001 vs MAM/VHC.

2.4.3. Western blotting analysis

The cerebral areas of interest (PFC, HIP and NAc) were dissected from whole rat brains and washed twice in cold PBS (without Ca^{2+} and Mg^{2+}, pH 7.4) and homogenized as previously described (Navarria et al., 2014). Lysates were then centrifuged for 15 min at 13000 × *g* at 4 °C, and the supernatants transferred into clear tubes and quantified by DC Protein Assay (Bio-Rad, Segrate MI, Italy). Subsequently the samples (60–80 μg of total protein) were boiled for 5 min in Laemmli SDS loading buffer and loaded on 8–10% SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. Membranes were incubated overnight at 4 °C with the following antibody: rabbit polyclonal anti-CB1 Receptor Antibody (Y080037) Applied Biological Materials Inc. (CANADA). The mouse monoclonal anti-tubulin clone B-5-1-2 (dilution 1:5000; Sigma–Aldrich, MI Italy) antibody was used to check for equal protein loading. Reactive bands were detected by chemiluminescence (ECL or ECL-plus; Perkin-Elmer). Images were acquired and analyzed on a Chemi-Doc station with Quantity-one software (Bio-Rad, Segrate MI, Italy) (Navarria et al., 2014; Panza et al., 2016). See supplementary Fig. S5 for uncropped images of key immunoblot data presented in this study.

2.4.4. Analysis of DNA methylation

Methylation status of *CNR1* promoter was determined using pyrosequencing of bisulfite converted DNA. After DNA extraction, 0.5 μg of DNA from each sample was treated with bisulfite, using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA). *CNR1* was analyzed in clinical samples with PM00122031 (Qiagen, Hilden, Germany) and in rat brain tissues using the following primers: forward: 5′-GGAAGAGAGTAGGAAGATGATAG-3′; reverse: 5′-biotin-TTCTAC CAA AACTAATATACCTAACACC-3′; and sequencing: 5′-AGAGAGTAG GAAG ATGATAGT-3′. Bisulfite treated DNA was amplified by PyroMark PCR Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. PCR conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and, finally, 72 °C for 10 min. PCR products were verified by agarose electrophoresis. Pyrosequencing methylation analysis was conducted using the PyroMark Q24 (Qiagen). The level of methylation was analyzed using PyroMark Q24 Software (Qiagen, Hilden, Germany), which calculates the methylation percentage (mC/(mC + C)) for each CpG site, allowing quantitative comparisons (mC is methylated cytosine, C is unmethylated cytosine) (D'Addario et al., 2017).

2.4.5. Cannabinoid receptor binding assays

Rat brain cortex was resuspended in 2 mM Tris–EDTA, 320 mM sucrose, 5 mM $MgCl_2$ (pH 7.4), then homogenized in a Potter homogenizer and centrifuged twice at 1000 × *g* (10 min), and the pellet was discharged. The supernatant was centrifuged at 15000 × *g* (20 min), and the pellet was resuspended in assay buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, pH 7.4), and the Bradford protein assay to measure the concentration of the sample protein was performed. These membrane fractions were used in rapid filtration assays with radiolabel agonist ([3H]CP-55,940; Perkin Elmer Life Sciences, Boston, Ma, U.S.A.), at 37 °C with incubation time of 60 min. At the beginning we performed binding assays with [3H]-CP-55,940 at different concentrations (0.1–4 nM) to create the nonlinear graph of specific binding, and to calculate the K_d, using the Prism 4 program (GraphPAD Software for Science, San Diego, CA), value necessary for subsequent evaluation of K_i. Then in all binding experiments, nonspecific binding was determined in the presence of 10 μM "cold" agonist (CP-55,940; Cayman Chemicals, Ann Arbor, Mi, U.S.A) that was tested by adding directly to the incubation medium during a preincubation time of 15 min at room temperature (Bari et al., 2013).

2.5. Statistical analysis

The results are presented as the group mean ± SEM. Behavioral data were first tested for normality distribution using the Shapiro-Wilk test. As normality tests have little power to detect non-Gaussian distributions with small data sets, we did not explicitly test for the normality of our biochemical data sets. Data were analyzed using two-way ANOVA (factor 1: MAM; factor 2: peripubertal treatment) followed by *post-hoc* Fisher's LSD if appropriate. Unpaired *t*-test was used to analyze independent data (CNT vs MAM). Statistical evaluations were performed using specialized software (Graph-Pad Prism 6.0). Statistical significance was accepted at $p < 0.05$. Detailed statistical methodology and results are provided in supplementary tables S2 and S3.

3. Results

3.1. Behavioral phenotype

The influence of peripubertal treatment, alone or combined with prenatal MAM exposure, on behavioral performance in the SItest is depicted in Fig. 2A-B. Two-way ANOVA revealed for the time of interaction a main effect of MAM ($F_{1,79} = 17.54$, $p < 0.001$), treatment ($F_{4,79} = 9.049$, $p < 0.001$) and a significant MAM × treatment interaction ($F_{4,79} = 12.37$, $p < 0.001$). *Post-hoc* analysis revealed that MAM/VHC group spent less time in social interaction compared to CNT/VHC rats ($p < 0.001$), indicating impaired social behavior. Intraperitoneal treatment with CBD 30 mg/kg (CBD30) and AM251, but neither CBD 10 mg/kg (CBD10) nor HAL, improved social performance in the MAM group as compared to the MAM/VHC group ($p < 0.001$). However, in CNT groups both AM251 and HAL reduced the social activity ($p < 0.05$, $p < 0.001$), while CBD did not (Fig. 2A). Neither prenatal MAM exposure ($F_{1,79} = 3.514$, $p > 0.05$) nor treatment ($F_{4,79} = 0.3396$, $p > 0.05$) affected the number of interaction (MAM × treatment interaction $F_{4,79} = 0.5328$, $p > 0.05$), as index of locomotor activity (Fig. 2B).

In the rats tested in the NOR test (Fig. 3A-B), two-way ANOVA showed a main effect of MAM ($F_{1,116} = 24.28$, $p < 0.001$), a significant MAM × treatment interaction ($F_{4,116} = 2.757$, $p < 0.05$) but not a main effect of treatment ($F_{4,116} = 1.221$, $p > 0.05$) for the discrimination index. *Post-hoc* analysis revealed that prenatal MAM exposure affected the recognition memory as demonstrated by a

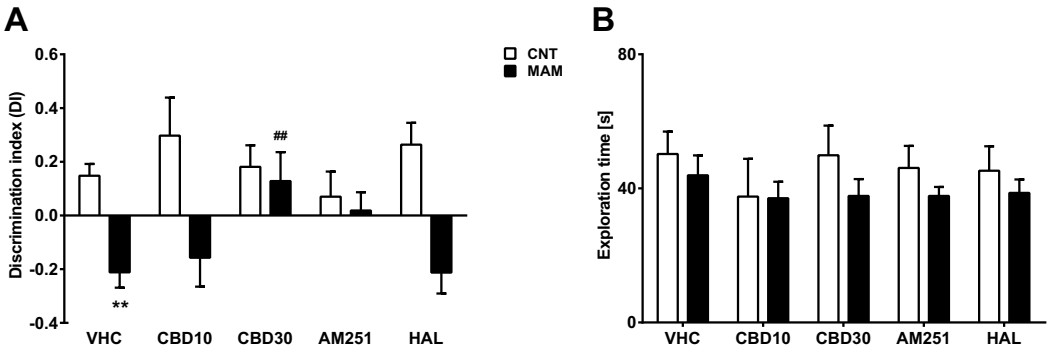

Fig. 3. Effects of peripubertal cannabidiol (CBD) treatment on the behavioral phenotype of MAM in the testing phase of novel object recognition (NOR) test at adulthood. Data are presented as means ± S.E.M. (n = 12–15 rats/group) of (A) the discrimination index and (B) total exploration time. **p < 0.01 vs CNT/VHC; ##p < 0.01 vs MAM/VHC.

significant reduction (p < 0.01) in the discrimination index during the test phase, which was reversed by CBD30 (p < 0.01; Fig. 3A). However, no difference was found in the total exploration time among the groups (two-way ANOVA, factor MAM: $F_{1,116} = 3.002$, p > 0.05; factor treatment: $F_{4,116} = 0.2028$, p > 0.05; MAM × treatment interaction: $F_{4,116} = 0.4640$, p > 0.05; Fig. 3B), as well as in the time spent to explore the two identical object in the familiarization phase (see supplementary data and supplementary Fig. S1).

In addition, neither prenatal MAM exposure nor peripubertal treatments affected the spontaneous horizontal (number of crossings, factor MAM: $F_{1,82} = 3.348$, p > 0.05; factor treatment: $F_{4,82} = 1.597$, p > 0.05; MAM × treatment interaction: $F_{4,82} = 0.5224$, p > 0.05; Fig. 4A) or vertical (number of rearings, factor MAM: $F_{1,82} = 0.1926$, p > 0.05; factor treatment: $F_{4,82} = 1.890$, p > 0.05; MAM × treatment interaction: $F_{4,82} = 0.8153$, p > 0.05; Fig. 4B) locomotor activity in a novel environment at adulthood.

3.2. Biochemical analyses

3.2.1. Cannabinoid CB1 receptors in the PFC of adult MAM rats

Among the brain regions (PFC, HIP and NAc) in our analysis CB1 receptor in the PFC was the most significant canonical target affected (Fig. 5A–C). Consistent with the increase of CB1 mRNA expression (p < 0.01 vs CNT/VHC) (two-way ANOVA, factor MAM: $F_{1,30} = 0.01859$, p > 0.05; factor treatment: $F_{3,30} = 2.610$, p > 0.05; factor MAM × treatment interaction: $F_{3,30} = 4.658$, p < 0.01, Fig. 5B), we observed a significant reduction in DNA methylation of the CB1 gene (*CNR1*) promoter in the 5 CpGs average of MAM/VHC group (p < 0.01 vs CNT/VHC) (two-way ANOVA, factor MAM: $F_{1,31} = 21.96$, p < 0.001; factor treatment: $F_{3,31} = 8.649$, p < 0.001; factor MAM × treatment interaction: $F_{3,31} = 3.585$, p < 0.05, Fig. 5A). An increase in CB1 protein levels (p < 0.001 vs CNT/VHC) (two-way ANOVA, factor MAM: $F_{1,23} = 16.01$, p < 0.01; factor treatment: $F_{3,23} = 11.87$, p < 0.001; MAM × treatment interaction: $F_{3,23} = 7.423$; p < 0.01, Fig. 5C) was observed, but not in CB1 receptor binding activity (CNT: 23.00 ± 4.398 fmol/mg; MAM: 25.68 ± 1.348 fmol/mg; unpaired *t*-test = 0.5834; p > 0.05). Peripubertal treatment with CBD30 reversed MAM-induced changes in DNA methylation (p < 0.05), mRNA (p < 0.001) and protein (p < 0.001) expression at adulthood (Fig. 5A–C).

AM251 treatment reversed CB1 mRNA (p < 0.001) but not protein (p > 0.05) expression in MAM rats; as well it increased DNA methylation in the CNT rats (p < 0.01) (Fig. 5A–C). This apparent discrepancy is likely due to the different turnover between mRNA and protein. HAL did not induce notable modifications in CB1 receptors of adult MAM rats, except for an increased DNA methylation (p < 0.001). The expression of further genes closely associated with the ECS at level of PFC, HIP or NAC are described in supplementary data and depicted in supplementary Fig. S2-S3.

3.2.2. Endocannabinoid (EC) levels in the PFC of adult MAM rats

We found a non-significant increase in 2-AG level (p > 0.05) in the PFC of MAM/VHC animals (two-way ANOVA, factor MAM: $F_{1,27} = 27.98$, p < 0.001; factor treatment: $F_{3,27} = 6.237$, p < 0.01; MAM × treatment interaction, $F_{3,27} = 8.242$; p < 0.001), which was significantly decreased by CBD30 and AM251 (p < 0.05), but not by HAL (p > 0.05). However, the latter two compounds increased the 2-AG content in CNT rats (p < 0.001, Fig. 6B). Two-way ANOVA revealed for the AEA PFC content a main effect of MAM ($F_{1,28} = 38.00$, p < 0.001) a significant MAM × treatment interaction, $F_{3,28} = 6.761$, p < 0.01; but not of treatment ($F_{3,28} = 2.311$, p > 0.05). *Post-hoc* analyses revealed that AEA content was enhanced by CBD30 in the CNT group (p < 0.001) (Fig. 6A). No difference was found between the two experimental groups in the PFC levels of PEA (two-way ANOVA, factor

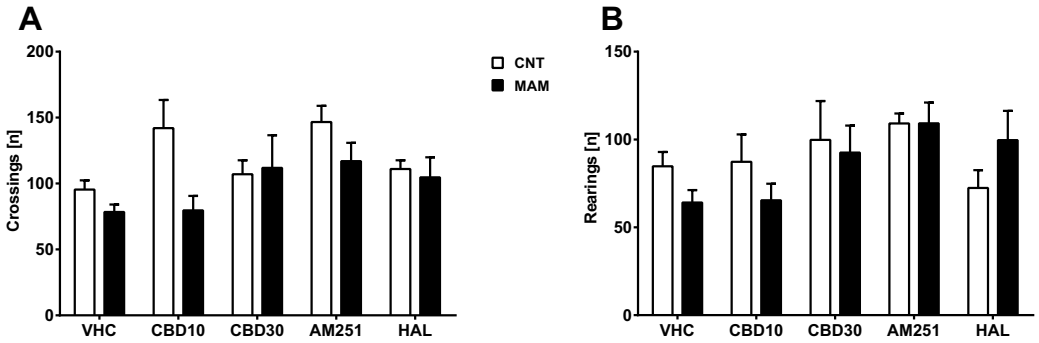

Fig. 4. Effects of peripubertal cannabidiol (CBD) treatment on the behavioral phenotype of MAM in the open field (OF) test at adulthood. Data are presented as means ± S.E.M. (n = 7–12 rats/group) of (A) number of crossings and (B) number of rearings.

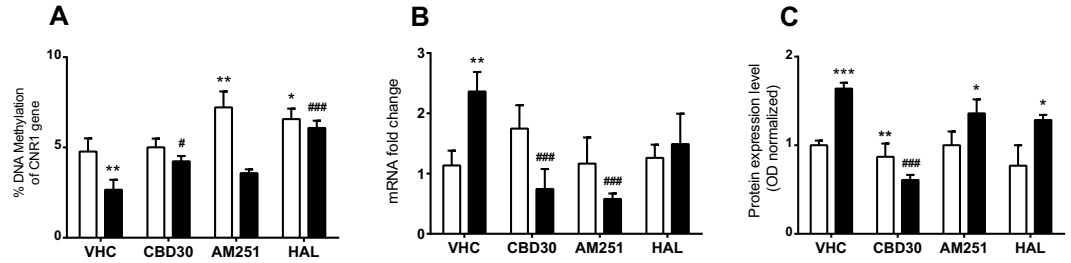

Fig. 5. Effects of peripubertal cannabidiol (CBD) treatment on CB1 receptor in the prefrontal cortex of MAM rats at adulthood. Data are presented as means ± S.E.M (n = 3–7 rats/group) of (A) DNA methylation of *CNR1* gene, (B) CB1 mRNA expression, (C) CB1 protein expression. *p < 0.05, **p < 0.01 and ***p < 0.001 vs CNT/VHC; #p < 0.05 and ###p < 0.001 vs MAM/VHC.

MAM: $F_{1,30} = 0.0088$, $p > 0.05$; factor treatment: $F_{1,30} = 1.866$, $p > 0.05$; MAM × treatment interaction, $F_{3,30} = 1.187$, $p > 0.05$; Fig. 6C) and OEA (two-way ANOVA, factor MAM: $F_{1,30} = 0.7649$, $p > 0.05$; factor treatment: $F_{1,30} = 0.8796$, $p > 0.05$; MAM × treatment interaction, $F_{3,30} = 0.3431$, $p > 0.05$; Fig. 6D). The EC levels in HIP and NAc are described in supplementary data and depicted in supplementary Fig. S3.

4. Discussion

MAM administration at gestational day 17 induced cognitive and social deficits in rats (Lodge and Grace, 2009; Micale et al., 2013), as seen by the reduced time of interaction in the SI (as index of social withdrawal) and lower discrimination ratio in the NOR (as index of impaired recognition), which are often considered the two signs of SCZ-like symptoms (Young et al., 2009). The object recognition model is a spontaneous recognition test based on the natural bias of rats toward exploring novel objects (Ennaceur and Delacour, 1988) and has been listed by the MATRICS initiative as a relevant test to study visual learning and memory deficits in SCZ (Young et al., 2009). In our study, MAM offspring, which spent the same time exploring the two identical objects during familiarization phase, showed no preference for the novel object during the testing phase, indicating an inability to recognize the familiar object. Considering that the total object exploration time did not differ among treatment groups, the MAM rats are thought to have a deficit in short-term object recognition memory in contrast to a deficit in object exploration (i.e., impaired locomotor activity). This is consistent with observations in preclinical (Flagstad et al., 2005; Micale et al., 2013) and human studies (Heckers et al., 2000; Zanto et al., 2011), further supporting the face validity of the MAM model.

Similarly, the social deficit observed in SI is also not related to changes in motor activity, since no difference was found in the number of interactions. The locomotor activity paradigms served as an internal control for possible unspecific stimulant effects. Given that no spontaneous hyperlocomotor activity was found in MAM animals, partially in line with previous results (Flagstad et al., 2004; Moore et al., 2006; Perez and Lodge, 2012; Ruda-Kucerova et al., 2017a, 2017b), overall our study reinforces the original findings that social withdrawal in the SI and cognitive impairment in the NOR are a robust phenotype in the MAM model. Nevertheless, the behavioral assays (i.e. NOR, SI and spontaneous locomotor activity) used in the present study are not strictly specific for SCZ and could be applicable to assess symptoms domains shared with other neuropsychiatric disorders (i.e. autism,

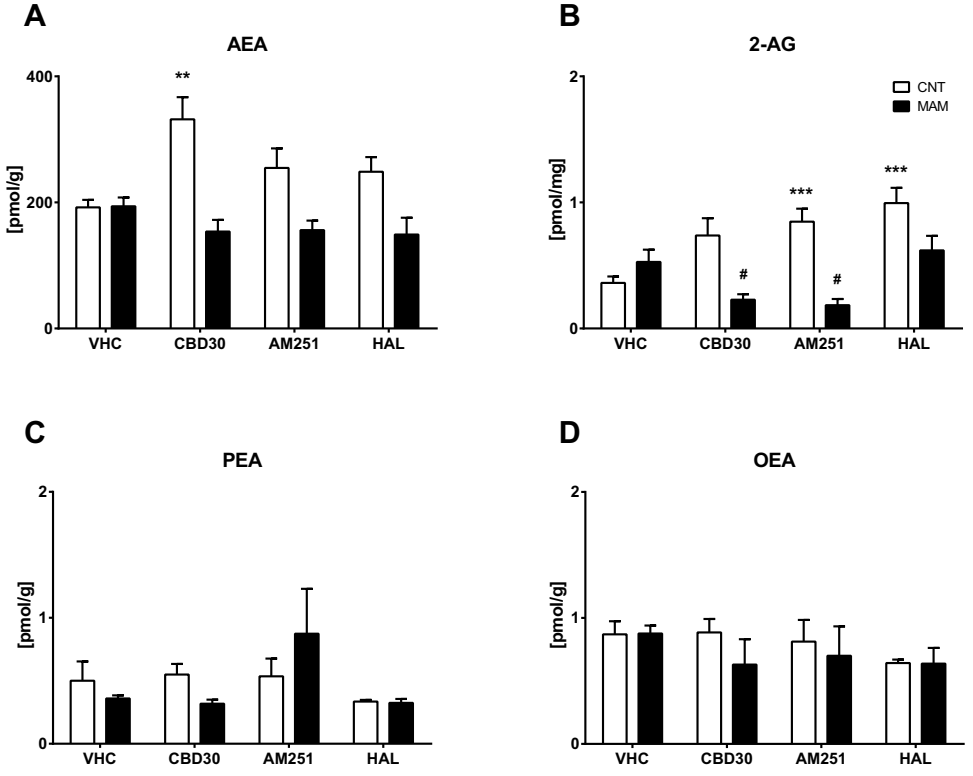

Fig. 6. Effects of peripubertal cannabidiol (CBD) treatment on (A) anandamide (AEA), (B) 2-arachidonoylglycerol (2-AG), (C) *N*-palmitoylethanolamide (PEA) and (D) *N*-oleoylethanolamide (OEA) in the prefrontal cortex (PFC) of rats at adulthood. Data are presented as means ± SEM (n = 5–6 rats/group). #p < 0.05 vs MAM/VHC; **p < 0.01 and ***p < 0.001 vs CNT/VHC.

depression, anxiety). Thus, further behavioral tasks to evaluate both the aberrant response to amphetamine or NMDA antagonist as index of positive-like deficits and the different cognitive or negative-like symptoms would be useful.

At the molecular level, a consistent reduction in DNA methylation at the *CNR1* promoter was observed, which was associated with a significant elevation in CB1 gene and protein expression restricted to the PFC. Excitingly, this modification at the cannabinoid CB1 receptor level coincided with the negative- and cognitive-like symptoms in the MAM rats. This was expected considering that the PFC is a key region for the integration of cognitive and negative signs of SCZ (Guidali et al., 2011; Pratt et al., 2009; Young et al., 2009). This observation expands on our previous findings and thereby further supports the translational value of the MAM model (D'Addario et al., 2017). Our results are consistent with previous findings showing increased CB1 mRNA expression at the level of PFC in a diverse variety of neurodevelopmental models (Marco et al., 2014; Robinson et al., 2010). However, in *post mortem* studies contradictory results have been found since decreased (Eggan et al., 2008; Kucerova et al., 2014), increased (Saito et al., 2013) or unchanged (Ruggiero et al., 2017) CB1 expression or activity at the PFC level have been detected. Conflicting data in the literature may be due to differences in patient symptom severity, pharmacological treatments or diagnostic methods in studies. Considering that alterations in the PFC seem to affect recognition memory in individuals suffering from SCZ (Heckers et al., 2000; Zanto et al., 2011) and in animal models (Ragozzino et al., 2002; McLean et al., 2017) alike, our data suggests that specific CB1 alterations could mediate the involvement of the PFC in the short-term recognition memory deficits observed in MAM rats. This would be of particular impact in the development of new therapeutic strategies for SCZ symptoms given the reversible nature of epigenetics (Ovenden et al., 2018). Further investigations are warranted to understand the epigenetic mechanisms regulating CB1 expression in PFC and, most importantly, their impact on negative and/or cognitive-like symptoms in MAM rats. Consistently with some preclinical and human studies (Kucerova et al., 2014; Saito et al., 2013), the ECs content and partially the metabolic enzymes both in the PFC (see Fig. S2) and in HIP (see Fig. S3C) were not altered by prenatal MAM exposure; thus presenting the possibility that the abnormal behavior of MAM offspring could be in part due to a maladaptation of ECS tone (in terms of CB1 receptor expression) in the PFC. However, cannabinoid CB1 receptors are present at very high levels in inhibitory (GABAergic interneurons) and at a lesser extent in excitatory (glutamatergic) terminals (Marsicano and Lutz, 1999), playing a specific role in the repertoire of different emotional behaviors (Terzian et al., 2011, 2014; Micale et al., 2017); thus, we cannot exclude that the SCZ-like phenotype in MAM animals could be specifically due to alterations of CB1 receptors on different neuronal subpopulations at the level of the PFC.

Interestingly, at prepuberty ECS signaling was only mildly altered (increased nape mRNA expression in PFC, decreased ptpn22 mRNA expression in HIP and lower OEA content in the NAc; Fig. S4). These subtle alterations might be also partially relatable to transition into a SCZ-like phenotype which is observed in prepuberty/adolescent MAM rats (Le Pen et al., 2006; Gomes et al., 2016; Kallai et al., 2017). However, CB1 receptor does not seem to be altered in the early neurodevelopmental phase until a later stage of the MAM model. Further studies are necessary to assess the potential early alterations of different neurotransmitter systems (i.e. DAergic, GLUergic, GABAergic) whose dysfunction in adulthood is well recognized in human studies and in experimental models (Gomes et al., 2016).

According to the neurodevelopmental hypothesis of SCZ, environmental risk factors (i.e. trauma, infection, malnutrition) during the perinatal period could affect the neuronal circuit development and contribute to the transition into psychosis at adulthood (Marenco and Weinberger, 2000). In this context, human studies and preclinical data have suggested that childhood or early adolescence could represent the promising window of opportunity for a course-altering strategy (Gomes et al., 2016; Millan et al., 2016; Sommer et al., 2016). In the MAM model, early pharmacological intervention or enriched living conditions prevented the development of SCZ-related deficits at adulthood (Bator et al., 2018; Du and Grace, 2013, 2016). Here, we showed that peripubertal treatment with a high dose of CBD or, in part, with AM251 rescued the decreased sociability and recognition memory deficit in MAM offspring. The peripubertal age corresponds to mid-to-late adolescence in humans, which is a pivotal period for PFC development. Dysregulation in the PFC during this period is assumed to be involved in the pathophysiology of SCZ (Volk and Lewis, 2002). Thus, our results further support the idea that early adolescent intervention could modify the appearance of SCZ-like alterations induced by prenatal MAM exposure. The present data also align with the therapeutic benefit of CBD to improve the negative symptoms and cognitive deficits in clinical studies (Fasinu et al., 2016; McGuire et al., 2018; Osborne et al., 2017b) and in most of preclinical models (Kucerova et al., 2014; Osborne et al., 2017a; Peres et al., 2016). By contrast, HAL failed to reproduce the CBD effect in MAM rats and, much alike AM251, impaired the social behavior in control rats. Notably, HAL lack of efficacy in MAM rats is in line with human studies (Young et al., 2009) and preclinical evidence in the MAM model (Brown et al., 2013), where antipsychotics showed poor effectiveness towards negative and cognitive symptoms; thus stimulating the development of innovative pharmacological approaches (Kapur and Mamo, 2003). However, further investigations are needed to assess the effects of CBD on additional cognitive and social domains, which are impaired in SCZ.

The mechanisms underlying the beneficial effects of CBD on SCZ-like symptoms are still elusive. Aberrant DAergic transmission in the brain is a common target of all current antipsychotics and a well-established neuropathological feature both in SCZ and in MAM model (Grace, 2016). Recently, CBD has been found to attenuate DAergic hyperfunction in the mesolimbic pathway (Renard et al., 2017) and, in agreement with its pharmacological profile as atypical antipsychotic (Zuardi et al., 1991; Guimarães et al., 2004), also showed partial agonistic activity at dopamine D2 receptors, similarly to aripiprazole (Seeman, 2016), which may at least in part account for its antipsychotic effects. However, in our study we used the typical antipsychotic HAL as positive control, since it reduced the hyperdopaminergic activity in the MAM model (Valenti et al., 2011), while the second generation antipsychotic risperidone failed to improve the MAM-induced cognitive deficits (Brown et al., 2013) and only modestly improved performance in the context of pharmacologically induced NOR deficits (Young et al., 2009). Another explanation is that CBD attenuated SCZ-like abnormalities at adulthood by targeting aberrant stress responsivity during the peripubertal period. In the MAM model, rats exhibited altered stress responsivity during early development (Zimmerman et al., 2013), which was circumvented by peripubertal diazepam treatment through a reduction of the hyperdopaminergic state (Du and Grace, 2013, 2016). As such, it is possible that peripubertal CBD treatment attenuated the stress responses induced by prenatal MAM treatment in agreement with its well shown stress relief/anxiolytic activity through a 5-HT_{1A} mechanism (Fogaça et al., 2014, 2018; Campos et al., 2012, 2013, 2016; Marinho et al., 2015). Further investigations into the impact of CBD on altered DAergic system may shed light on the mechanisms underlying the improvement of social behavior and recognition memory in MAM offspring. However, we also cannot exclude other targets of CBD since the typical antipsychotic HAL did not prevent the development of behavioral abnormalities in our study (see Fig. 2A and Fig. 3A), while it previously reduced DAergic neuron population activity in MAM rats (Valenti et al., 2011).

At the molecular level, repeated peripubertal CBD treatment completely normalized MAM-induced cannabinoid CB1 receptor alterations in the PFC, without having *per se* any effect in control rats. Specifically, CBD attenuated the decreases in DNA methylation and the paralleled mRNA and protein expression. These effects may in part contribute to

its antipsychotic-like effects seen by the improved cognitive and social performance of MAM/CBD30 rats in the NOR and SI, respectively. Interestingly, the CB1 antagonist/inverse agonist AM251 partially normalized the altered CB1 receptor (just the mRNA expression), which at least could be involved in its partial efficacy against MAM insult. In contrast, HAL, which increased just DNA methylation of CB1 receptor, failed to reverse the SCZ-like deficits in MAM rats. However, higher DNA methylation induced by HAL and AM251 treatment in CNT rats was not accompanied by decreased gene expression, even if there might be a delay in the molecular outcomes. These discrepancies further support the concept that although several antipsychotics act as epigenetic modifiers, the precise mechanisms are still unclear. In our study, CBD treatment alone restored both *CNR1* expression levels and DNA methylation at gene promoter presumably via an indirect mechanism, which could involve several neurotransmitter systems (Ovenden et al., 2018). Given the heterogeneity of the disease, the higher rate of non-responders to antipsychotics, the severe side effects of the latter and the limits of experimental models *per se*, this complex scenario suggests that the effects of antipsychotics on the DNA methylation status of genes involved in the pathophysiology of SCZ are still not fully elucidated.

Our results do not support the previous findings that associated the antipsychotic effect of CBD with increased AEA levels in serum (Osborne et al., 2017b). A higher AEA concentration was found in the PFC of CNT/CBD30 but not of MAM/CBD30 rats (see Fig. 6A). Given that CBD can interact with several molecular targets that elevate AEA content, such as the hydrolyzing enzyme FAAH (De Petrocellis et al., 2011) and the AEA transporter FABPs (Elmes et al., 2015), only speculations can be made on the possibility that CBD reduced AEA inactivation in the PFC of control but not MAM rats. The underlying mechanisms that could be involved in its antipsychotic activity are likely multifaceted, given that CBD modifies the function of several receptors in the central nervous system (CNS) including CB1, CB2, TRPV1, GPR55, 5-HT_{1A}, PPARγ, μ and δ opioid receptors. Moreover, we cannot exclude that its beneficial effect to prevent the development of SCZ-like alterations as well as its safety profile could be also due to its activity as negative allosteric modulator of the cannabinoid CB1 receptors (Laprairie et al., 2015). By contrast, the CNS adverse effects and the lack of significant effect on psychopathology, could be associated with orthosteric CB1 antagonist/inverse agonists, such as rimonabant or AM251 (Ross, 2007; Jones, 2008; Laprairie et al., 2015). Besides its effect on CB1 receptors, we cannot rule out that the antipsychotic-like activity of CBD on MAM rats could also be based on its capacity to reduce 2-AG content in PFC. However, a possibility that CBD-induced reduction of 2-AG levels was a mere consequence of the amelioration of the SCZ-like signs, or the reduction of CB1 expression by the cannabinoid cannot be neglected.

Another intriguing finding of the present study is that AM251 and HAL increased 2-AG levels in CNT rats (see Fig. 6B), an effect that could be in part responsible for the social withdrawal similar to that observed in MAM rats. The mechanisms underlying the 2-AG increase in the PFC of CNT rats remain undetermined and may be linked to changes in the dopaminergic and/or glutamatergic neurotransmission, as already suggested (Guidali et al., 2011). However, these results provide evidence that AM251 and HAL trigger different behavioral responses in the context of negative-like symptoms based on experimental groups (CNT vs. MAM), consistent with previous reports in healthy subjects (Mas et al., 2013) and in laboratory-based studies (Guidali et al., 2011; Seillier et al., 2013).

5. Conclusion

In agreement with the hypothesis of preventive antipsychotic treatment in individuals that are at risk of developing SCZ (Gomes et al., 2016; Millan et al., 2016; Sommer et al., 2016), repeated peripubertal CBD treatment prevented MAM-induced negative- and cognitive-like symptoms at adulthood, which are insensitive to currently used antipsychotics. Conveniently, CBD did not negatively affect control offspring, supporting its safety profile (Iffland and Grotenhermen, 2017), which is a pivotal ethical issue when we consider both the preventive treatment as intervention strategy and the rates of individuals (~30%) developing the disease (Mokhtari and Rajarethinam, 2013; Piras et al., 2014). We also confirmed that a dysregulation of the ECS may play a role in the pathophysiology of the disease, which seems to be a promising target for innovative treatment. As highlighted in a recent review (Osborne et al., 2017b), several possible mechanisms of action have been suggested. However, to the best of our knowledge, none of them have been conclusively identified as the prime mechanism for the antipsychotic effect of CBD. Based on our results, peripubertal age may be a promising window for CBD treatment to prevent the emergence of SCZ-like deficits at adulthood, which may in part relate to the reversal of CB1 dysregulation in the PFC. Further studies are necessary to assess ECS effects on different neurotransmitter systems (i.e., dopaminergic, glutamatergic, GABAergic).

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2018.11.035.

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