Pharmacological Research 203 (2024) 107176

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Cannabidiol and positive effects on object recognition memory in an in vivo model of Fragile X Syndrome: Obligatory role of hippocampal GPR55 receptors

Antonia Manduca [a,b,c,*,1,2], Valeria Buzzelli [b,1], Alessandro Rava [b], Alessandro Feo [b], Emilia Carbone [b], Sara Schiavi [b], Barbara Peruzzi [d], Valentina D'Oria [e], Marco Pezzullo [f], Andrea Pasquadibisceglie [b], Fabio Polticelli [b], Vincenzo Micale [g], Martin Kuchar [h,i], Viviana Trezza [b,c,**,3]

[a] Dept. Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy
[b] Dept. Science, Roma Tre University, Rome, Italy
[c] Neuroendocrinology, Metabolism and Neuropharmacology Unit, IRCCS Fondazione Santa Lucia, Rome, Italy
[d] Bone Physiopathology Research Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy
[e] Confocal Microscopy Core Facility, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy
[f] Histology Core Facility, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy
[g] Dept. Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy
[h] Forensic Laboratory of Biologically Active Substances, Dept. Chemistry of Natural Compounds, University of Chemistry and Technologies, Prague, Czech Republic
[i] Psychedelic Research Center, National Institute of Mental Health, Klecany, Czech Republic

ARTICLE INFO

Keywords:
Cannabidiol
Fragile X syndrome
GPR55 receptors
Fatty acid amide hydrolase
Cognitive performance

ABSTRACT

Cannabidiol (CBD), a non-psychotomimetic constituent of *Cannabis sativa*, has been recently approved for epileptic syndromes often associated with Autism spectrum disorder (ASD). However, the putative efficacy and mechanism of action of CBD in patients suffering from ASD and related comorbidities remain debated, especially because of the complex pharmacology of CBD. We used pharmacological, immunohistochemical and biochemical approaches to investigate the effects and mechanisms of action of CBD in the recently validated *Fmr1-$^{\Delta}$exon 8* rat model of ASD, that is also a model of Fragile X Syndrome (FXS), the leading monogenic cause of autism. CBD rescued the cognitive deficits displayed by juvenile *Fmr1-$^{\Delta}$exon 8* animals, without inducing tolerance after repeated administration. Blockade of CA1 hippocampal GPR55 receptors prevented the beneficial effect of both CBD and the fatty acid amide hydrolase (FAAH) inhibitor URB597 in the short-term recognition memory deficits displayed by *Fmr1-$^{\Delta}$exon 8* rats. Thus, CBD may exert its beneficial effects through CA1 hippocampal GPR55 receptors. Docking analysis further confirmed that the mechanism of action of CBD might involve competition for brain fatty acid binding proteins (FABPs) that deliver anandamide and related bioactive lipids to their catabolic enzyme FAAH. These findings demonstrate that CBD reduced cognitive deficits in a rat model of FXS and provide initial mechanistic insights into its therapeutic potential in neurodevelopmental disorders.

Abbreviations: AEA, anandamide; ASD, Autism spectrum disorder; CB1, CB1 cannabinoid receptor; CB2, CB2 cannabinoid receptor; CBD, cannabidiol; DMSO, dimethyl sulfoxide; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; FABPs, fatty acid binding proteins; FMR1, fragile X messenger ribonucleoprotein 1; FXS, Fragile X Syndrome; GPR55, G protein-coupled receptor 55; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; SERT, serotonin transporter; VEH, vehicle; WT, wild-type.

* Correspondence to: Department of Physiology and Pharmacology, Sapienza University of Rome, Piazzale Aldo Moro 5, Rome 00185, Italy.
** Correspondence to: Department of Science, Section of Biomedical Sciences and Technologies, Roma Tre University, Viale G. Marconi 446, Rome 00146, Italy.
E-mail addresses: antonia.manduca@uniroma1.it (A. Manduca), viviana.trezza@uniroma3.it (V. Trezza).
[1] These authors are joint first authors.
[2] ORCID: 0000-0001-9730-2467
[3] ORCID: 0000-0002-3922-6045

https://doi.org/10.1016/j.phrs.2024.107176
Received 13 November 2023; Received in revised form 3 April 2024; Accepted 4 April 2024
Available online 5 April 2024

1. Introduction

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental disorders for which no treatment currently exists. Fragile X syndrome (FXS) is the leading monogenic cause of ASD. FXS and ASD share several behavioral characteristics, with FXS patients often experiencing ASD-like symptoms [25]. FXS is caused by the silencing of the Fragile X Messenger Ribonucleoprotein 1 (*FMR1*) gene. FMRP, the protein product of *FMR1*, has a key role in the development of synapses and in underlying learning and memory processes, as it serves as a key regulator in the translation of numerous RNAs crucial for synaptic plasticity [35].

Recently, there has been growing interest in the potential benefits of medical Cannabis to manage behavioral disturbances in children with ASD and co-occurring conditions, including FXS [68]. This interest arises from the evidence that dysregulations in the endocannabinoid system (ECS) have been found both in ASD patients [32,4,63] and in animal models of ASD [10,48], thus reinforcing the idea that fine changes in ECS contribute to ASD-related traits and that pharmacological manipulation of ECS is a relevant target for ASD. Additional interest arises from the therapeutic potential of cannabidiol (CBD), an abundant non-psychotropic constituent of Cannabis, approved for two refractory epileptic syndromes (i.e., Dravet and Lennox-Gastaut syndromes) and tuberous sclerosis complex in pediatric patients at increased risk of developing ASD [16,69]. To support its therapeutic potential in neurodevelopmental disorders, early clinical data reported that CBD may be beneficial in relieving some core and comorbid symptoms with favorable benefit-risk ratio in ASD patients [24,45,5] and in FXS patients with $\geq$ 90% methylation of the *FMR1* gene [43,68,7]. However, intrinsic limitations of these studies (e.g., lack of standardized clinical assessments and placebo-controlled studies, open label design, small cohort size, inconsistencies in CBD dosing) make the interpretation of these findings challenging and the use of CBD in neurodevelopmental disorders remains controversial [50]. Furthermore, the underlying mechanisms are largely unexplored, and this is relevant as CBD has multiple molecular targets [13,45,66,73].

Here, we used pharmacological, immunohistochemical and biochemical approaches to investigate the effects and mechanism of action of CBD in the *Fmr1-$^{\Delta}$exon 8* rat model of FXS, that is a valuable tool to study common neurobiological aspects of both FXS and ASD [14, 22,56]. We first tested the effects of CBD in the short-term novel object recognition deficits displayed by *Fmr1-$^{\Delta}$exon 8* rats. As we reported that reduced endocannabinoid signaling in the hippocampus due to increased fatty acid amide hydrolase (FAAH) activity may underlie the short-term cognitive deficits displayed by *Fmr1-$^{\Delta}$exon 8* rats [58], we performed biochemical experiments in the hippocampus to detect potential changes in the ECS following CBD treatment. We then performed intracranial pharmacology experiments to explore the possibility that the beneficial effects of CBD in *Fmr1-$^{\Delta}$exon 8* rats may be mediated by increased endocannabinoid signaling on GPR55 receptors in the CA1 region of the hippocampus as a consequence of inhibition of endocannabinoid hydrolysis by FAAH. Finally, we performed *in silico* docking analysis to investigate the molecular complementarity of CBD for both FAAH and fatty acid binding proteins (FABPs), that deliver anandamide and related bioactive lipids to their catabolic enzymes [28].

2. Material and methods

2.1. Animals

Juvenile (40-day-old; weight 150 $\pm$ 15 g) male wild-type (WT) (Charles River Laboratories, Italy) and *Fmr1-$^{\Delta}$exon 8* rats (Horizon Discovery, USA) on a Sprague-Dawley background were used. We and others have shown that *Fmr1-$^{\Delta}$exon 8* male rats display FXS-like behavioral traits [22,26,57,56], suggesting the validity of this animal model in mimicking the key behavioral deficits that characterize FXS and some of the core and comorbid features of non-syndromic ASD. Rats were housed in groups of three (same sex and same genotype) under controlled conditions (temperature 20–21°C, 55–65% relative humidity and 12/12 h light cycle with lights on at 07:00 h). For each experiment, animals were randomized for treatment.

Sample size (n), based on our previous experiments and power analysis (assuming 80% power at a significance level of 0.05, G*Power software), is indicated in the figure legends for each experimental group. Potential outliers within each data set were calculated using the ROUT method. Scoring of the behavioral experiments was done in blind conditions (i.e., the researchers who performed treatments did not test or score the animals and vice versa) using the Observer 3.0 software (Noldus, The Netherlands). The experiments were approved by the Italian Ministry of Health and performed in agreement with the ARRIVE 2.0 guidelines [46], the Italian Ministry of Health Directive (D.L. 26/14; Project authorization n. 306/2020-PR), and the European Community Directive 2010/63/EU.

2.2. Experimental design

WT and *Fmr1-$^{\Delta}$exon 8* male rats were tested following the experimental design shown in Fig. 1.

In experiment 1, CBD (or its vehicle, VEH) was administered intraperitoneally (i.p.) 2 hours before testing in juvenile (postnatal day (PND) 40) *Fmr1-$^{\Delta}$exon 8* rats and WT controls tested in the novel object recognition task.

In experiment 2, to evaluate whether tolerance occurred to the effects of CBD after repeated treatment, different groups of animals were treated with CBD or VEH once daily for three weeks (PND 19–39) [17, 66,65] and tested at PND 40 in the novel object recognition task. This treatment schedule for CBD administration has been chosen based on literature data showing its efficacy in a neurodevelopmental model of schizophrenia in juvenile rats [66,65] at a time point that should be considered as equivalent to childhood/periadolescence in humans [1].

In experiment 3, we collected hippocampal tissues for qPCR, western blotting and immunohistochemistry analysis of the main ECS components in both naïve rats and animals treated acutely or repeatedly with CBD or its VEH, searching for possible mechanistic targets underlying the behavioral effects of CBD.

Based on the results of experiment 3 and literature data [31], in experiment 4 we evaluated whether the effects of CBD in juvenile *Fmr1-$^{\Delta}$exon 8* rats were mediated by hippocampal GPR55 receptors. CBD was administered 2 hours before testing while the GPR55 receptor antagonist CID16020046 was bilaterally infused in the dorsal hippocampus (CA1 subregion) immediately after the training phase of the novel object recognition task.

Since GPR55 receptors are also activated by anandamide (AEA) and other bioactive N-acylethanolamines metabolized by FAAH, in experiment 5 we systemically administered the FAAH inhibitor URB597 2 h before testing and we bilaterally infused the GPR55 antagonist CID16020046 in the hippocampus (CA1) immediately after the training phase of the novel object recognition task. This experiment aimed at testing whether URB597 had the same behavioral effects of CBD at GPR55 receptors, thus exploring the possibility that the beneficial effects of CBD in *Fmr1-$^{\Delta}$exon 8* rats may be mediated by increased endocannabinoid signaling on GPR55 receptors in the CA1 region of the hippocampus as a consequence of inhibition of endocannabinoid hydrolysis by FAAH.

In experiment 6, docking analyses were performed to computationally predict binding geometries of CBD for FABPs (FABP3, FABP5 and FABP7) and FAAH.

2.3. Surgery

Rats were anesthetized with Zoletil® (50 mg kg^{-1}, i.p.) and Rompun® (7 mg kg^{-1}, i.p.) and placed in a stereotaxic frame (2biological

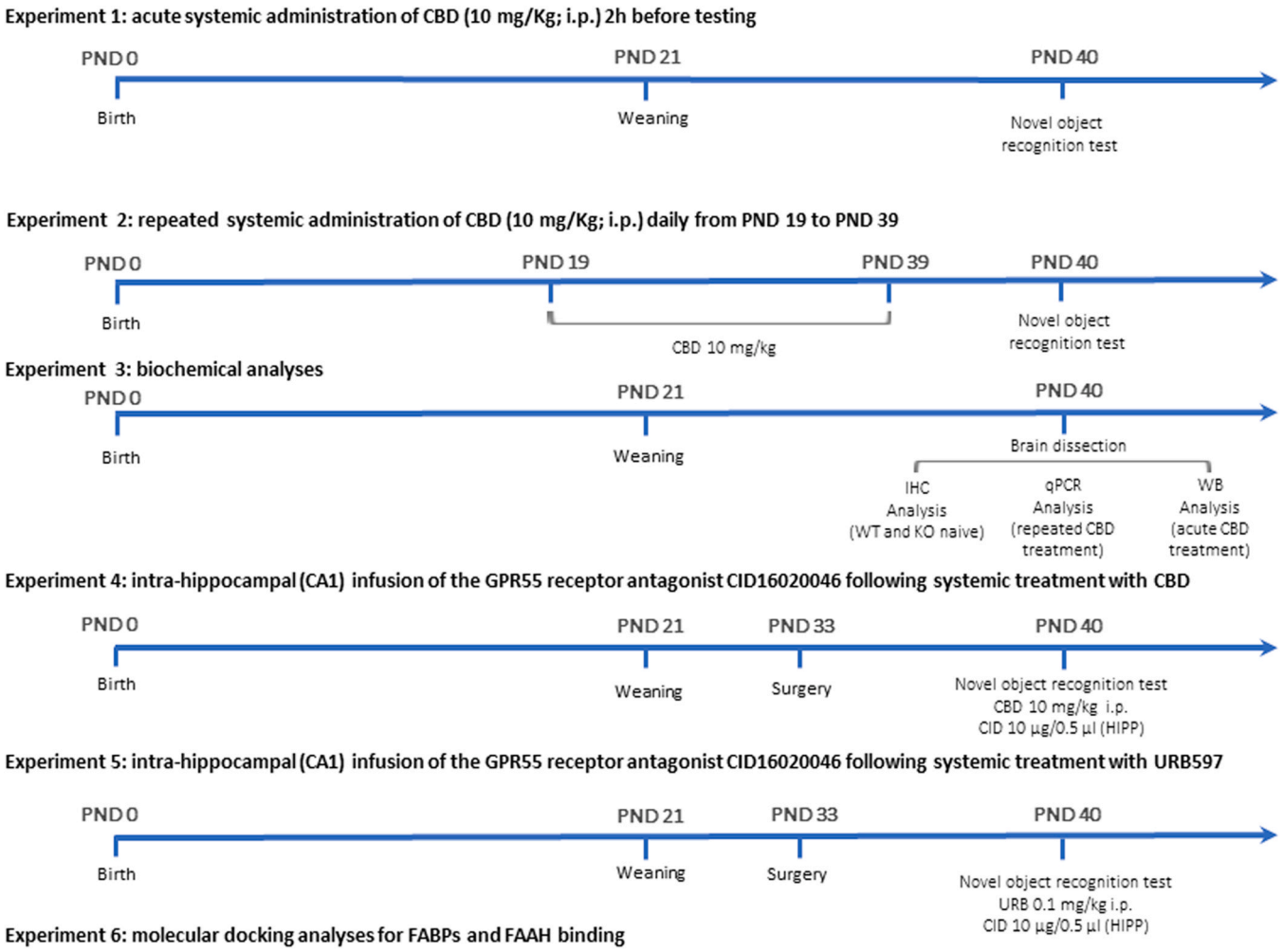

Fig. 1. Timeline of the experiments.

Instruments, Italy). Two stainless-steel 24-gauge cannulae (Cooper's Needleworks, UK) were implanted bilaterally above the CA1 region of the dorsal hippocampus [11 mm-long cannulae; coordinates: AP, −3.3 mm; ML, ±1.7 mm; DV, −2.7 mm]. Coordinates for the brain region were based on previous studies [58] and according to the atlas of Paxinos and Watson (2013). Stylets (Cooper's Needleworks, UK) were inserted into each cannula to maintain patency. After surgery, animals received saline (2 mL once, s.c.) and carprofen for pain relief (5 mg kg^{-1}, 2 × daily, s.c.). The rats were individually housed and allowed to recover for 4 days. On the fifth day, they were re-housed in groups with their original cage mates. Behavioral testing began 1 week after surgery.

2.4. Drugs

Cannabidiol (2-[(1 R, 6 R)-6-Isopropenyl-3-methylcyclohex-2-en-1-yl]-5pentylbenzene-1,3-diol) (CBD), extracted by the Forensic Laboratory of Biologically Active Substances, University of Chemistry and Technology, Prague, Czech Republic (purity (NMR) > 99%) [27] was dissolved in 5% Tween 80, 5% polyethylene glycol, 90% saline and administered i.p. at the dose of 10 mg kg^{-1}. Dosing was based on previous findings [17,66] and pilot experiments. The GPR55 receptor antagonist CID16020046 (Selleck Chemicals, Italy) was dissolved in 5% DMSO and infused in the hippocampus (CA1, 10 ng/0.5 µL per side) [34]. URB597 (Sigma-Aldrich, Italy) was dissolved in 5% Tween 80, 5% polyethylene glycol, 90% saline and administered i.p. (0.1 mg kg^{-1}) [58]. Solutions were administered in a volume of 2 mL kg^{-1}.

2.5. Histological confirmation of injection sites

Injection sites were verified as previously described [58]. After testing, animals were sacrificed by carbon dioxide inhalation and microinjected with 0.5 µL of black ink over 60 s through the guide cannulae. Animals were immediately decapitated, and their brains removed. Slices (30 µm thick) were collected throughout the forebrain and analyzed under a dissecting microscope for the location of the infusion sites according to the atlas of Paxinos and Watson (2013). Only pairs in which both animals had bilateral needle tracks terminating in the CA1 subregion of the hippocampus and no damage to the target tissues were included in the final analysis (Fig. 5E).

2.6. Behavioral tests

Scoring of the behavioral experiments was done in blind conditions using the Observer 3.0 software (Noldus, The Netherlands). The behavioral experiments were conducted between 10:00 a.m. and 2:30 p.m.

2.6.1. Novel object recognition test

The task procedure consisted of three phases. In the habituation phase, each rat was allowed to freely explore the open-field arena (45 × 45 cm) in the absence of objects for 5 min. Immediately after, the animal was individually placed in the arena containing two identical objects (A1 and A2), equidistant from each other, and allowed to explore the objects for 5 min (training). Thirty min later, one copy of the familiar

object (A3) and a new object (B) were placed in the same location as during training. Each rat was placed in the apparatus for 5 minutes, and the time spent exploring each object was recorded [9]. The discrimination index was calculated as the difference in time exploring the novel and the familiar objects, expressed as percentage ratio of the total time spent exploring both objects.

2.6.2. Locomotor activity

Locomotor activity was assessed during the 5-min habituation phase (i.e., in the absence of objects) of the novel object recognition test and scored as follows: a grid, dividing the arena into equally sized squares, was projected over the recordings, and the number of line crossings made by the animal (i.e., the frequency of the animal's passage from one section of the grid to another) was manually scored using the Observer 3.0 software (Noldus Information Technology, NL). The crossing was counted at the time when the animal passed from one section to another with all four paws.

2.7. Biochemical analysis

2.7.1. Brain samples collection

Rats were decapitated and their brains were rapidly removed from the skull on a cold plate. Based on our recent evidence indicating brain region-specific changes in the main ECS components in *Fmr1-$^{\Delta}$exon 8* rats [58], experiments were performed in the hippocampus.

2.7.2. Quantitative PCR (qPCR) analysis

The experiments were performed as previously described [58]. Total RNA isolation was performed using Total RNA purification Kit (Norgen-Biotek Corp., Canada). RNA was retrotranscribed into complementary DNA (cDNA) using Oligo(dt) primer and SuperScript II Reverse Transcriptase system (Invitrogen, Thermo Fisher Scientific, USA). cDNA was amplified using SYBR Green Supermix (Bio-Rad, USA) in AriaMx RTPCR system (Agilent, USA), for 2 min at 95 °C, followed by 40 cycles (15 s at 95°C and 30 s at 60°C). Primer sequences used for gene expression analysis are provided in Table S1. β-actin was used as reference gene. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method [59] and results are expressed as fold changes relative to the correspondent WT group.

2.7.3. Western blotting

We performed western blot experiments to evaluate FAAH protein levels in the hippocampus of *Fmr1-$^{\Delta}$exon 8* rats compared to WT control animals following acute systemic administration of CBD. Specifically, 2 h after treatment, tissue samples were collected and stored at −80°C. Dorsal hippocampus was sonicated in ice-cold RIPA buffer supplemented with protease (Roche, cat. 05,892,791,001) and phosphatase (Roche, cat. 4906,845,001) inhibitors, centrifuged at 13.000 rpm for 15 min and supernatants were collected. Protein content was quantified by using the colorimetric Breadford assay and 30 μg of proteins for each sample were loaded and resolved through SDS-PAGE electrophoresis at 120 V. Subsequently, proteins were transferred on a nitrocellulose membrane by Turbo-blot system (2.5 V, 25 mA, 3 min), and a Ponceau staining was performed to verify the quality of transfer. Membrane was then blocked in 5% milk blocking buffer for 1 h and incubated with the primary antibody anti-FAAH (1:2000, Santa Cruz, cat. sc-100739) in TBS with 2% milk + 0.1% Tween-20 overnight at + 4 °C. After washings, membranes were incubated with secondary antibody anti-mouse IgG HRP-linked antibody (1:10.000, Cell Signaling Technology, cat. 7076) for 1 hour at RT. Bands were visualized by Clarity ECL (Bio-Rad, cat. 1705,060) and the densitometry analysis performed by ImageLab Software (Version 6.1, Bio-Rad). Densitometric values were normalized to Ponceau staining [54] and the relative expression of FAAH in *Fmr1-$^{\Delta}$exon 8* rats was reported as fold change on WT controls. We analyzed the total amount of proteins by Ponceau stain as it represents a viable methodological alternative for normalization in immunoblotting, offering several advantages compared to housekeeping proteins [38]. Blots are shown in Fig. S2. In this experiment only, to compare the FAAH protein expression between the two genotypes (WT vs *Fmr1-$^{\Delta}$exon 8*) following the two treatments (VEH vs CBD) on the same nitrocellulose membrane (gel/blot), we used less than 5 animals per group.

2.7.4. Immunohistochemistry

Brains were fixed in 4% PFA for 72 hours, washed in phosphate-buffered saline (PBS), embedded in paraffin and coronally cut into 2.5 μm thick sections [21]. Sections were mounted on slides, dewaxed, rehydrated and stained with hematoxylin/eosin to confirm the presence of the hippocampus. Sections were incubated with 3% hydrogen peroxide for 10 min at RT to inactivate endogenous peroxidases, blocked with 5% bovine serum albumin for 1 h at RT and incubated with anti-CB1 (1:200, Abcam, Cat# ab3558), anti-CB2 (1:100, Abcam, Cat# ab3561), anti-GPR55 (1:100, Biorbyt, Cat# orb101191), and anti-FAAH (1:50, Abcam, Cat# ab54615) antibodies at 4°C over-night. Secondary biotinylated antibody (K8024, Dako, Carpinteria, USA) and the peroxidase DAB kit (Dako, Carpinteria, USA) were used to reveal the primary antibodies. Slices were counterstained with hematoxylin, dehydrated with ascending alcohol concentrations, followed by three changes of Diasolv and mounted with Diamount (Diapath). Sections were acquired by Nanozoomer-S60 (Hamamatsu) and original 40x magnifications of the CA1 hippocampus were collected for each staining by using NDP. view 2 software (Hamamatsu). The DAB intensity was measured by Fiji-Image-J2 software.

2.8. Molecular docking

AlphaFold 2 is a deep learning-based algorithm that predicts the three-dimensional structure of proteins from their amino acid sequence. AlphaFold-predicted structural models of the rat proteins FABP3 (Uniprot ID: P07483), FABP5 (Uniprot ID: P55053) and FABP7 (Uniprot ID: P55051) were downloaded from Uniprot database (https://www.uniprot.org/); the FAAH crystal structure (PDB ID: 3QJ8) [37] was downloaded from Protein Data Bank (https://www.rcsb.org/); ligands were downloaded from ZINC database (https://zinc.docking.org/) and individually docked with the selected target (*i.e.*, FABPs and FAAH), as separate docking runs. Docking analysis was performed using the AutoDock Vina (version 1.1.2) and AutoDock Tools software [39,72]. The grid box dimension was set as 40 × 40 × 40 Å having the following center at: −0.815 × 1.765 × 0.053 Å for FABP3; 0.175 × 2.987 × 2.045 Å for FABP5; −4.507 × 2.03 × 2.883 Å for FABP7; −10.27 × −4.5 × 1.807 Å for FAAH. Grid spacing was set to 1 Å per grid unit with the "exhaustiveness" set to 8. The docking simulations performed are rigid, i.e., only the flexibility of the ligand is considered. Rotatable torsions were released during docking. The predicted CBD and AEA complexes with FABPs (i.e., FABP3, FABP5 and FABP7) and FAAH are shown in Figs. 6 and S3, respectively. The predicted binding affinity values are shown in Table S2.

2.9. Data and statistical analysis

The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology [11]. All studies were designed to generate groups of equal size using randomization and blinded analysis. The minimum number of animals and sample sizes required to achieve statistical significance were determined by power analysis and prior experience, assuming 80% power at a significance level of 0.05. Data are expressed as mean ± SEM. To assess the effects of CBD in WT and *Fmr1-$^{\Delta}$exon 8* rats, data were analyzed by two-way ANOVA, with genotype and treatments as factors. For the immunohistochemical experiments, differences in the level of DAB intensity distributions for each staining between WT and *Fmr1-$^{\Delta}$exon 8* rats were analyzed with non-parametric Mann–Whitney rank-sum test. To assess the ability of CID16020046 to counteract or mitigate the effects of CBD

and URB597 in WT and *Fmr1-Δexon 8* rats, data were analyzed by three-way ANOVA, with genotype, intracranial and systemic treatments as factors. The accepted value for significance was set at $p<0.05$. The Tukey's *post hoc* test was used for individual group comparisons. *Post hoc* tests were only conducted when F in ANOVA achieved $p<0.05$ and there was no significant inhomogeneity of variance. Data were analyzed using GraphPad Prism Software (Version 8.0.2). The individual data points presented in this study are available from the corresponding author upon request.

3. Results

3.1. CBD restored cognitive performance in Fmr1-Δexon 8 rats

Acute administration of CBD rescued the altered discrimination index of juvenile *Fmr1-Δexon 8* rats in the novel object recognition task ($F_{(genotype)\ 1,29} = 4.22$, $p < 0.05$; $F_{(treatment)\ 1,29} = 3.92$, p =n.s.; $F_{(genotype\ x\ treatment)\ 1,29} = 7.57$, $p < 0.05$, Fig. 2A), without altering the total time spent in object exploration (Fig. S1A).

The beneficial effects of CBD in *Fmr1-Δexon 8* rats were not subject to tolerance, as repeated CBD treatment rescued the altered object recognition abilities of *Fmr1-Δexon 8* rats ($F_{(genotype)\ 1,36} = 0.22$, p = n.s; $F_{(treatment)\ 1,36} = 1.63$, p = n.s.; $F_{(genotype\ x\ treatment)\ 1,36} = 13.61$, $p < 0.05$, Fig. 2C), without altering the total time spent in object exploration (Fig. S1B). CBD did not affect locomotor activity either after acute administration (number of crossings: [$F_{(genotype)\ 1,36} = 8.63$, $p < 0.05$; $F_{(treatment)\ 1,36} = 1.51$, p = n.s.; $F_{(genotype\ x\ treatment)\ 1,36} = 0.21$, p = n.s.], Fig. 2B) or after repeated administration (number of crossings: $F_{(genotype)\ 1,36} = 5.80$, $p < 0.05$; $F_{(treatment)1,36} = 0.97$, p = n.s.; $F_{(genotype\ x\ treatment)\ 1,36} = 1.09$, p = n.s., Fig. 2D). Although we did find a significant effect of genotype, the effect of CBD treatment and the interaction between genotype and treatment on the number of crossings were not significant.

3.2. CBD-induced changes in the ECS in the hippocampus of Fmr1-Δexon 8 rats

To gain insights into the molecular effects of CBD treatment, we first performed qPCR experiments to evaluate the transcriptional expression of the main ECS components in hippocampal lysates of WT and *Fmr1-Δexon 8* rats repeatedly treated with either CBD or its VEH. The expression levels of CB1 ($F_{(genotype)\ 1,16} = 0.0009$, p =n.s.; $F_{(treatment)\ 1,16} = 0.013$, p = n.s.; $F_{(genotype\ x\ treatment)\ 1,16} = 1.775$, p = n.s., Fig. 3A), CB2 ($F_{(genotype)\ 1,16} = 0.0027$, p = n.s; $F_{(treatment)\ 1,16} = 0.319$, p = n.s.; $F_{(genotype\ x\ treatment)\ 1,16} = 0.427$, p = n.s., Fig. 3B) and GPR55 receptors ($F_{(genotype)\ 1,16} = 0.725$, p = n.s; $F_{(treatment)\ 1,16} = 0.497$, p = n.s.; $F_{(genotype\ x\ treatment)\ 1,16} = 0.071$, p = n.s., Fig. 3C) were spared across genotypes and treatment. FAAH mRNA expression ($F_{(genotype)\ 1,15} = 0.0183$, p = n.s; $F_{(treatment)\ 1,15} = 7.126$, $p < 0.05$; $F_{(genotype\ x\ treatment)\ 1,15} = 6.400$, $p < 0.05$, Fig. 3D) was reduced in CBD-treated WT rats, in line with the evidence that CBD inhibits FAAH expression in rodents [33,8]. However, FAAH transcriptional expression was unaffected in CBD-treated *Fmr1-Δexon 8* rats. In line with qPCR results, Western blot analysis showed that CBD changed FAAH protein expression in the hippocampus of WT but not *Fmr1-Δexon 8* rats ($F_{(genotype)\ 1,10} = 10.18$, $p < 0.01$; $F_{(treatment)\ 1,10} = 4.087$, p = n.s.; $F_{(genotype\ x\ treatment)\ 1,10} = 6.927$, $p < 0.05$, Fig. 3E). This may be due to a floor effect as FAAH expression was reduced in the hippocampus of *Fmr1-Δexon 8* rats compared to WT controls ($p<0.05$ vs WT-VEH).

Given the critical role of the CA1 hippocampus in object recognition [6] and in the cognitive deficits observed in FXS [55], we focused on this subregion to assess region-specific alterations in the main ECS components. While the qPCR analysis performed on the whole hippocampus did not reveal significant differences between *Fmr1-Δexon 8* rats and WT controls treated with VEH, immunohistochemistry showed that CB1 receptor expression in CA1 was increased in *Fmr1-Δexon 8* rats compared to WT controls ($p < 0.05$; Fig. 4B). CA1 immunohistochemistry also revealed higher CB2 receptor expression in naïve *Fmr1-Δexon 8* rats compared to WT controls ($p < 0.05$; Fig. 4C) and reduced CA1 expression of GPR55 receptors in *Fmr1-Δexon 8* rats ($p < 0.05$; Fig. 4D). In line with western blot analyses, FAAH expression in CA1 was reduced in naïve *Fmr1-Δexon 8* rats compared to WT animals ($p < 0.05$; Fig. 4E).

3.3. The effects of CBD and of the FAAH inhibitor URB597 on novel object discrimination in Fmr1-Δexon 8 rats depend on CA1 hippocampal GPR55 receptors

The GPR55 selective antagonist CID16020046 (CID), infused in CA1, counteracted the beneficial effects of systemic CBD on novel object

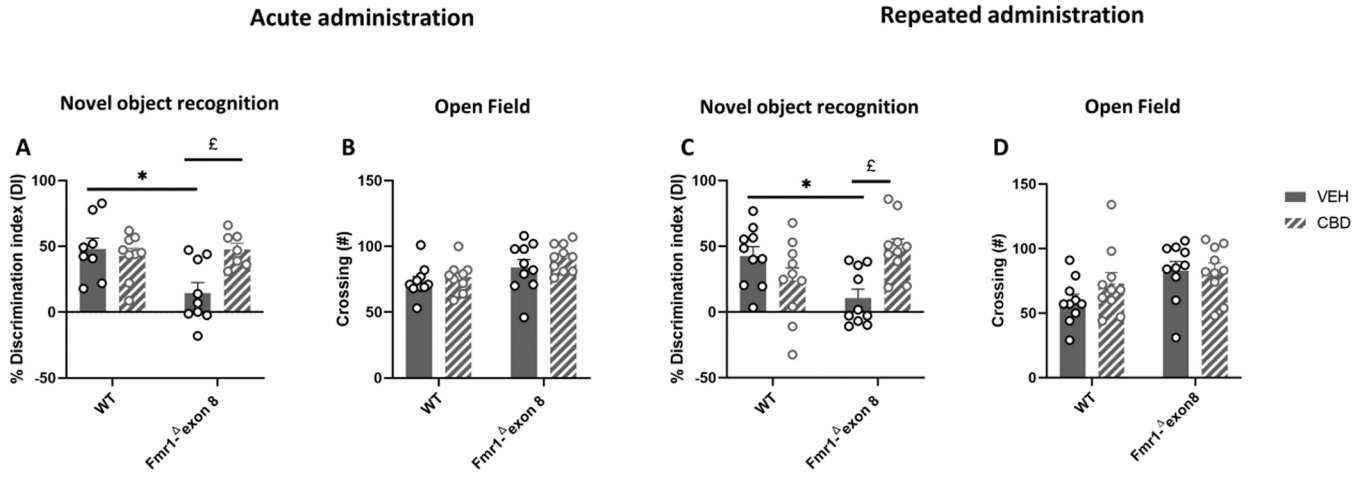

Fig. 2. Systemic administration of CBD rescued the short-term cognitive deficits displayed by *Fmr1-Δexon 8* rats. Acute administration of CBD (10 mg kg^{-1}, 2 h prior testing) rescued the altered discrimination index displayed by *Fmr1-Δexon 8* rats in the novel object recognition test (A) (WT-VEH = 8, WT-CBD = 9, *Fmr1-Δexon 8*-VEH = 9, *Fmr1-Δexon 8*-CBD = 7 animals per group). Repeated administration of CBD (10 mg kg^{-1}, i.p.) from PND 19 to PND 39 rescued the altered discrimination index of *Fmr1-Δexon 8* rats in the novel object recognition test (C) (WT-VEH = 10, WT-CBD = 10, *Fmr1-Δexon 8*-VEH = 10, *Fmr1-Δexon 8*-CBD = 10 animals per group). No differences in locomotor activity (i.e., the number of crossings) were found following acute (B) (WT-VEH = 10, WT-CBD = 10, *Fmr1-Δexon 8*-VEH = 10, *Fmr1-Δexon 8*-CBD = 10 animals per group) and repeated (D) (WT-VEH = 10, WT-CBD = 10, *Fmr1-Δexon 8*-VEH = 10, *Fmr1-Δexon 8*-CBD = 10 animals per group) administration of CBD. Data represent mean ± SEM, *$p < 0.05$ vs WT-VEH group, $^{£}p < 0.05$ vs *Fmr1-Δexon 8*-VEH group (two-way ANOVA with Tukey's *post hoc* test).

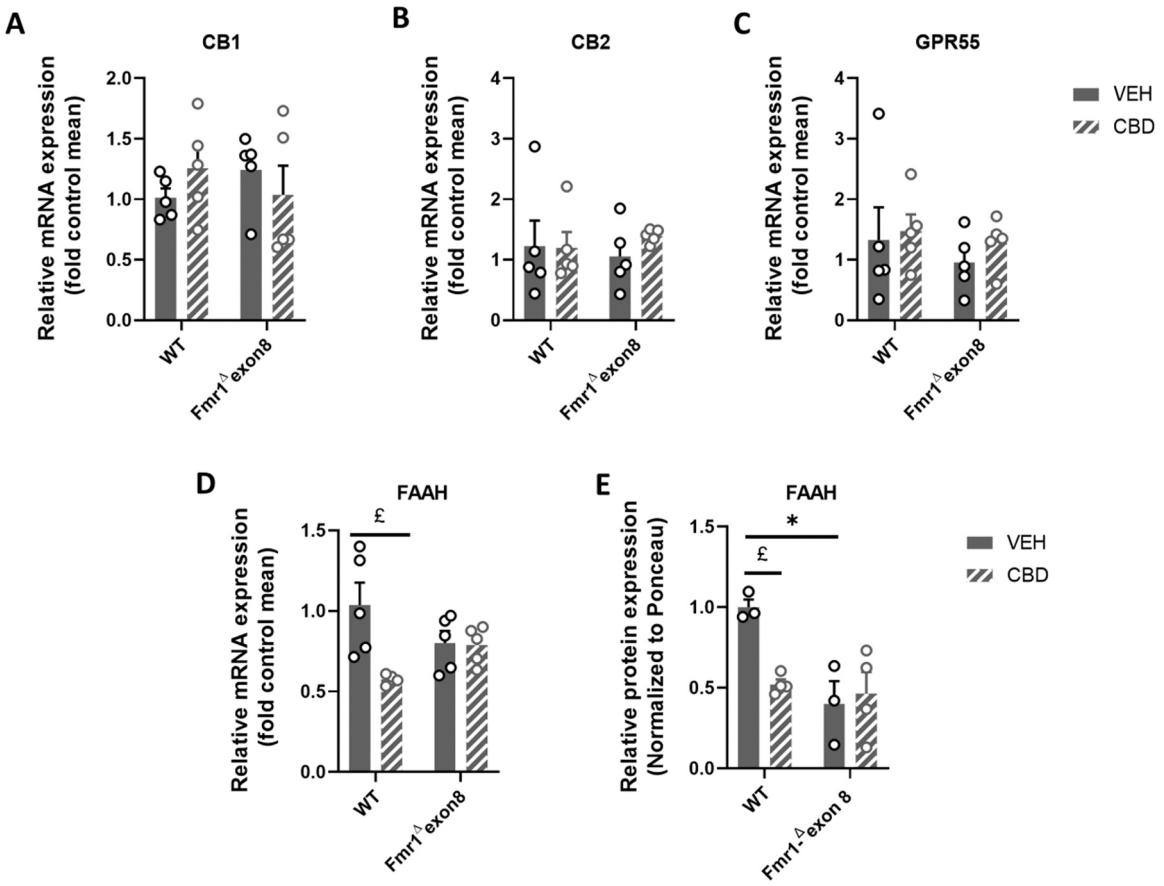

Fig. 3. qPCR and Western Blot analysis of the main ECS components in the hippocampus of juvenile *Fmr1-*$^{\Delta}$*exon 8* rats. Relative mRNA expression levels of CB1 (A), CB2 (B), GPR55 (C) receptors and FAAH (D) obtained by qPCR (WT-VEH = 5, WT-CBD = 5, *Fmr1-*$^{\Delta}$*exon 8*-VEH = 5, *Fmr1-*$^{\Delta}$*exon 8*-CBD = 5 animals per group for CB1, CB2 and GPR55 receptors; WT-VEH = 5, WT-CBD = 4, *Fmr1-*$^{\Delta}$*exon 8*-VEH = 5, *Fmr1-*$^{\Delta}$*exon 8*-CBD = 5 animals per group for FAAH), together with FAAH protein expression obtained by Western Blot analysis (E) (WT-VEH = 3, WT-CBD = 4, *Fmr1-*$^{\Delta}$*exon 8*-VEH = 3, *Fmr1-*$^{\Delta}$*exon 8*-CBD = 4 animals per group) in the hippocampus of *Fmr1-*$^{\Delta}$*exon* 8 rats and WT controls. Data represent mean ± SEM, *$p < 0.05$ *Fmr1-*$^{\Delta}$*exon 8*-VEH vs WT-VEH group, $^{£}p < 0.05$ WT-CBD vs WT-VEH group (two-way ANOVA with Tukey's *post hoc* test). Abbreviations: CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; GPR55, G protein-coupled receptor 55; FAAH, fatty acid amide hydrolase.

discrimination in *Fmr1-*$^{\Delta}$*exon 8* rats ($F_{(genotype)\ 1,61} = 51.10$, $p < 0.05$; $F_{(intracranic)\ 1,61} = 13.59$, $p < 0.05$; $F_{(systemic)\ 1,61} = 11.21$, $p < 0.05$; $F_{(gen\ x\ intra)\ 1,61} = 1.673$, $p =$ n.s; $F_{(gen\ x\ syst)\ 1,61} = 0.78$, $p =$ n.s.; $F_{(intra\ x\ syst)\ 1,61} = 8.11$, $p < 0.05$; $F_{(intra\ x\ syst\ x\ gen)\ 1,61} = 13.52$, $p < 0.05$; Fig. 5A), without altering the total time spent in object exploration ($F_{(genotype)\ 1,61} = 2.48$, $p =$ n.s; $F_{(intracranic)\ 1,61} = 0.07$, $p =$ n.s; $F_{(systemic)\ 1,61} = 3.14$, $p =$ n.s;: $F_{(gen\ x\ intrac)\ 1,61} = 1.89$, $p =$ n.s; $F_{(gen\ x\ system)\ 1,61} = 0.14$, $p =$ n.s; $F_{(intrac\ x\ system)\ 1,61} = 0.49$, $p =$ n.s; $F_{(gen\ x\ intrac\ x\ system)\ 1,61} = 0.50$, $p =$ n.s; Fig. 5B). Locomotor activity was unaffected (number of crossings: $F_{(genotype)\ 1,61} = 17.92$, $p < 0.001$; $F_{(intracranic)\ 1,61} = 0.76$, $p =$ n.s.; $F_{(systemic)\ 1,61} = 0.15$, $p = 0.15$; $F_{(gen\ x\ intra)\ 1,61} = 0.014$, $p =$ n.s; $F_{(gen\ x\ syst)\ 1,61} = 0.79$, $p =$ n.s.; $F_{(intra\ x\ syst)\ 1,61} = 0.10$, $p =$ n.s.; $F_{(intra\ x\ syst\ x\ gen)\ 1,61} = 1.02$, $p =$ n.s., **data not shown**).

Intra-CA1 administration of CID also counteracted the beneficial effects of the FAAH inhibitor URB597 on novel object discrimination in *Fmr1-*$^{\Delta}$*exon 8* rats ($F_{(genotype)\ 1,47} = 34.40$, $p < 0.05$; $F_{(intracranic)\ 1,47} = 3.92$, $p = 0.054$; $F_{(systemic)\ 1,47} = 1.92$, $p =$ n.s.; $F_{(gen\ x\ intra)\ 1,47} = 4.38$, $p < 0.05$; $F_{(gen\ x\ syst)\ 1,47} = 1.37$, $p =$ n.s.; $F_{(intra\ x\ syst)\ 1,47} = 13.25$, $p < 0.05$; $F_{(intra\ x\ syst\ x\ gen)\ 1,47} = 14.30$, $p < 0.05$ Fig. 5C), without affecting the total object exploration time ($F_{(genotype)\ 1,47} = 3.60$, $p =$ n.s.; $F_{(intracranic)\ 1,47} = 0.89$, $p =$ n.s.; $F_{(systemic)\ 1,47} = 0.10$, $p =$ n.s.; $F_{(gen\ x\ intra)\ 1,47} = 0.25$, $p =$ n.s.; $F_{(gen\ x\ syst)\ 1,47} = 0.31$, $p =$ n.s.; $F_{(intra\ x\ syst)\ 1,47} = 2.31$, $p =$ n.s.; $F_{(intra\ x\ syst\ x\ gen)\ 1,47} = 0.61$, $p =$ n.s. Fig. 5D). Thus, increased endocannabinoid signaling at CA1 hippocampal GPR55 receptors, possibly as a consequence of FAAH inhibition, mediates the short-term object recognition deficits observed in this genetic rat model of ASD.

3.4. Molecular docking

CBD is a FAAH inhibitor in rodents [8] but it also inhibits the cellular uptake and catabolism of endocannabinoids by targeting FABPs [19], that mediate transport of bioactive N-acylethanolamines to their catabolic enzyme FAAH. To explore the molecular complementarity of CBD with rat FAAH and FABPs, we performed *in silico* docking analysis of CBD binding to rat FABPs (FABP3, FABP5 and FABP7, mainly expressed in the brain) and FAAH.

The analysis of the best docking pose allows the following considerations (Fig. 6):

1. CBD-FABP3 binding is stabilized by hydrophobic interactions with residues Tyr20, Leu24, Pro39, Phe58, Lys59, Thr61, Ala76 and Leu105 (Fig. 6A; best binding affinity: −7.8 kcal mol^{-1}).
2. CBD-FABP5 binding is stabilized by hydrophobic interactions with Tyr22, Leu26, Ala39, Pro41, Lys61, Thr63 and Ile107 (Fig. 6B; best binding affinity: −7.7 kcal mol^{-1}).
3. CBD-FABP7 binding is stabilized by a hydrogen bond with Arg127 and by hydrophobic interactions with Pro39, Val41, Thr54, Ile76, Val116 and Leu118 (Fig. 6C; best binding affinity: −7.2 kcal mol^{-1}).
4. CBD-FAAH binding is stabilized through hydrophobic interactions with Ile407, Leu429, Phe432, Leu433, Ile530, Trp531 (Fig. 6D; best binding affinity: −6.5 kcal mol^{-1}).

Overall, CBD showed overlapped conformations for FABPs and

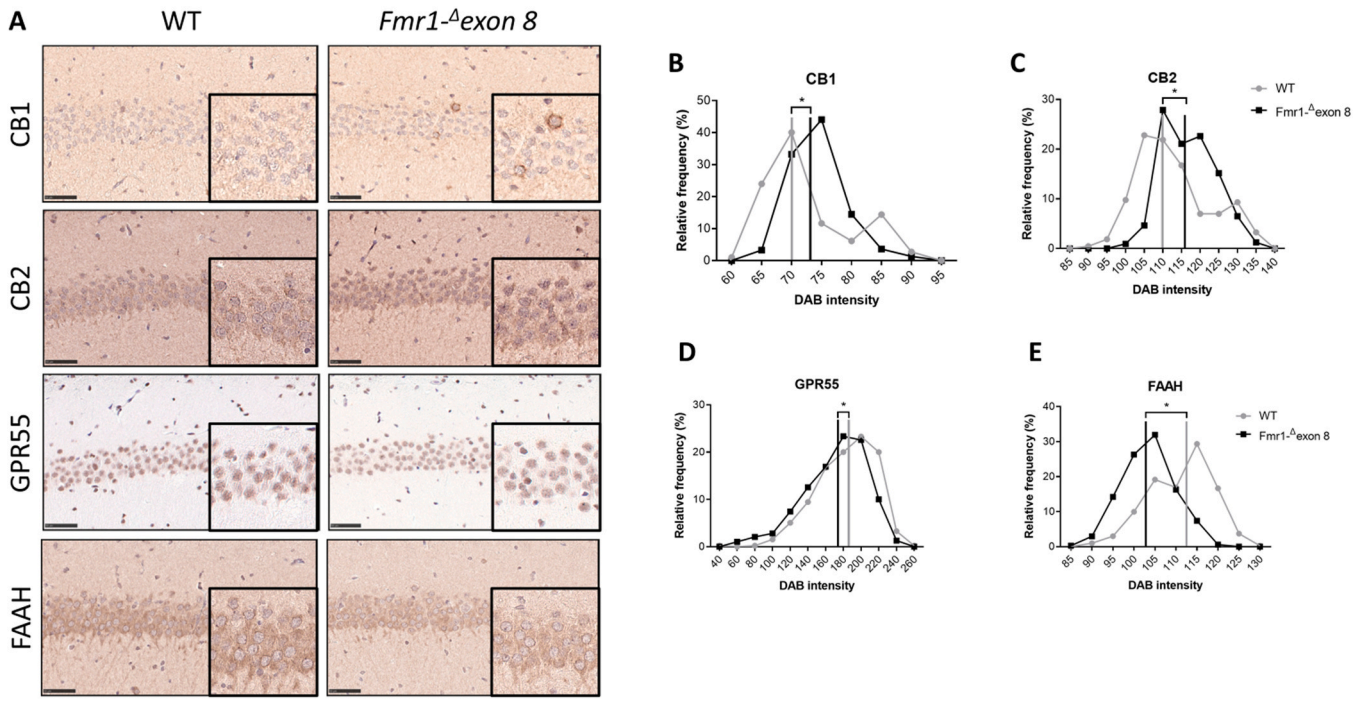

Fig. 4. Immunohistochemical analysis of the main components of the ECS in the CA1 hippocampal sections of juvenile *Fmr1-Δexon 8* rats. (A) Immunohistochemical staining of WT and *Fmr1-Δexon* 8 naïve rats for different ECS components. The inserts show higher magnification of the same picture. Nuclei were counterstained with hematoxylin. Original magnification: 40x. Scale bar: 50 μm. (B-E) Relative frequencies of DAB staining distribution of CB1 (B), CB2 (C), GPR55 (D) receptors and FAAH (E). Grey vertical lines are the median values of WT controls, while black vertical lines are the median values of *Fmr1-Δexon* 8 rats. Cells assessed: n > 200 (WT = 6 and *Fmr1-Δexon* 8 = 6 animals per group). *p < 0.05 vs WT group (Mann–Whitney rank-sum test).

FAAH, as reported in literature [15,19].

The binding affinity of AEA to both FABPs and FAAH is shown in Fig. S3.

The relative similarity in binding affinity between CBD and AEA for FABPs and FAAH suggests that competition for these binding sites may exist (Table S2).

4. Discussion

Our results show that CBD rescued the aberrant performance of *Fmr1-Δexon 8* rats in the novel object recognition task, without inducing tolerance after repeated administration. Immunohistochemical analysis revealed CA1 hippocampal changes in different components of the ECS (i.e., CB1, CB2 and GPR55 receptors, and FAAH) in juvenile *Fmr1-Δexon 8* rats compared to their WT controls. Moreover, qPCR and western blot analysis in the hippocampus revealed a decrease in FAAH expression following CBD treatment in WT but not *Fmr1-Δexon 8* rats. Interestingly, intra-hippocampal (CA1) blockade of GPR55 receptors counteracted the beneficial effect of systemic administration of either CBD or the FAAH inhibitor URB597 in the short-term recognition memory deficits displayed by *Fmr1-Δexon 8* rats. Thus, CBD may exert its beneficial effects via endocannabinoid signaling through CA1 hippocampal GPR55 receptors.

The extensively characterized *Fmr1*-KO mouse model of FXS has been shown to display motor alterations and hyperactivity [18,64,74], although normal locomotor activity has also been reported in rat models of FXS [26,70]. These differences could depend on the animal strain, the behavioral task, and/or the experimental protocols used. In line with our previous study [14] and with studies in the mouse models of FXS [18,64,74], we found a significant effect of the genotype on locomotion; however, the effect of CBD treatment and the interaction between genotype and treatment on locomotion were not significant, indicating that the effects of CBD on object recognition were not secondary to changes in locomotor activity.

Since 2018, when it was approved by FDA for refractory pediatric epilepsies, CBD raised growing interest as potential treatment for neurodevelopmental disorders. Clinical studies and anecdotes from families turning toward CBD-related products to treat their children with ASD and comorbidities, including FXS, reported that prolonged CBD treatment is associated with reduced ASD-related behavioral symptoms, with no major adverse events [3,24,62,68]. Furthermore, CBD was well tolerated in FXS patients and demonstrated efficacy in patients with-$\geq$ 90% methylation of the *FMR1* gene, in whom the disease is most severe [7]. Despite this evidence, public health professionals have expressed concerns about the increasing acceptance of CBD as a treatment for neurodevelopmental disorders, especially for the paucity of data on CBD interactions with other drugs, the selection of patients who could benefit from its use and the underlying mechanisms.

Studies in well-validated animal models of neuropsychiatric disorders are crucial to probe CBD efficacy and mechanism of action: thus, CBD improved social and cognitive dysfunctions in neurodevelopmental rat models of schizophrenia [17,42,66,65] and corrected repetitive and anxiety-like behaviors observed in a Shank3 mouse model of ASD [49]. Furthermore, CBD increased seizure resistance, mitigated social dysfunctions and hyperactivity in a mouse model of epileptic encephalopathy [60], and it improved social deficits in BTBR and SERT knock-out mice [20]. Interestingly, CBD mitigated seizures and social deficits in a mouse model of Dravet Syndrome [2,44] acting via GPR55 receptors [31]. In line with this finding, we found that pharmacological blockade of GPR55 receptors in the CA1 hippocampus mediates the ability of CBD to counteract the short-term memory deficit displayed by *Fmr1-Δexon 8* rats. We recently suggested that impairment in hippocampal AEA signaling, possibly as a consequence of an increased FAAH activity, mediates the short-term novel object and social discrimination deficits displayed by juvenile *Fmr1-Δexon 8* rats [58]. Given that CBD acts as a FAAH inhibitor in rodents [8], it is plausible that it could exert, at least in part, its action through FAAH inhibition and subsequent increase in endocannabinoid tone. In support of this hypothesis, the FAAH inhibitor

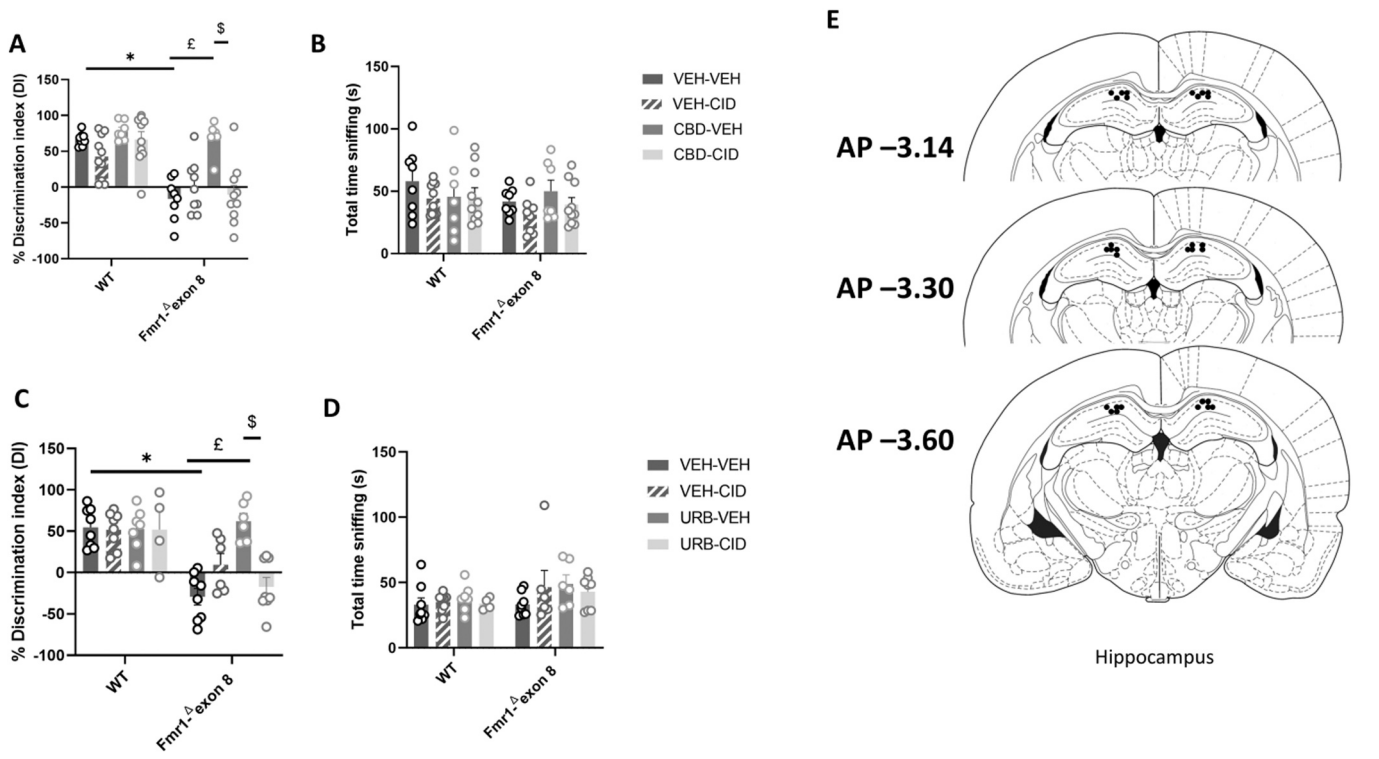

Fig. 5. Blockade of GPR55 receptors in the CA1 region of the hippocampus counteracted the beneficial effects of systemic treatment with either CBD or the FAAH inhibitor URB597. Bilateral infusion of the GPR55 selective antagonist CID16020046 in the CA1 region of the hippocampus of *Fmr1-$^{\Delta}$exon 8* rats (10 ng/0.5 μl) counteracted the beneficial effects of systemic treatment with either CBD (10 mg kg^{-1}; i.p.) (A) or URB597 (0.1 mg kg^{-1}; i.p.) (C) in the cognitive deficit displayed by *Fmr1-$^{\Delta}$exon 8* in the novel object recognition test, without altering the total time spent in object exploration (B, D) (CBD experiment: WT-VEH/VEH = 8, WT-VEH/CID = 10, WT-CBD/VEH = 7, WT-CBD/CID = 10, *Fmr1-$^{\Delta}$exon 8*-VEH/VEH = 8, *Fmr1-$^{\Delta}$exon 8*-VEH/CID = 9, *Fmr1-$^{\Delta}$exon 8*-CBD/VEH = 7, *Fmr1-$^{\Delta}$exon 8*-CBD/CID = 10 animals per group; URB597 experiment: WT-VEH/VEH = 8, WT-VEH/CID = 8, WT-URB/VEH = 7, WT-URB/CID = 4, *Fmr1-$^{\Delta}$exon 8*-VEH/VEH = 8, *Fmr1-$^{\Delta}$exon 8*-VEH/CID = 6, *Fmr1-$^{\Delta}$exon 8*-URB/VEH = 6, *Fmr1-$^{\Delta}$exon 8*-URB/CID = 8 animals per group). Data represent mean ± SEM, *p < 0.05 vs WT-VEH/VEH group, $^{£}$p < 0.05 vs *Fmr1-$^{\Delta}$exon 8*-VEH/VEH group; $^{\$}$p < 0.05 vs *Fmr1-$^{\Delta}$exon 8*-CBD/VEH and *Fmr1-$^{\Delta}$exon 8*-URB597/VEH group (three-way ANOVA with Tukey's post hoc test). (E) Diagrams of rat brain sections showing representative microinjection sites (filled circles) in the CA1 hippocampus.

URB597 also improved the short-term cognitive deficits displayed by *Fmr1-$^{\Delta}$exon 8* animals, and intra-hippocampal (CA1) blockade of GPR55 receptors counteracted its beneficial effects, highlighting that CBD and URB597 had the same behavioral effects at GPR55 receptors. Moreover, CBD reduced FAAH hippocampal expression in WT but not in *Fmr1-$^{\Delta}$exon 8* rats, probably due to a floor effect, as FAAH expression was reduced in the dorsal hippocampus of *Fmr1-$^{\Delta}$exon 8* rats compared to WT controls, as we consistently showed by western blot and immunohistochemistry. As CBD does not inhibit human FAAH [19], the competition of CBD for FABPs, intracellular carriers that transport AEA and related N-acylethanolamines to FAAH for hydrolysis [15], has been considered as a potential mechanism underlying the increased circulating levels of AEA reported after consumption of CBD. We therefore used *in silico* molecular docking to investigate the molecular complementarity of CBD for both FAAH and FABPs, that are expressed in three subtypes in the mammalian brain (FABP3, FABP5, FABP7) [29,30], in order to clarify whether CBD could exert its action in a combined manner through binding to both FABPs and FAAH, or its target is either the hydrolysis or transport process. In line with previous results [19], we found that CBD binds to both rat FABPs and FAAH. Therefore, a partial mechanism of action underlying the effects of CBD may involve competition for brain FABPs and FAAH, with subsequent increase of N-acylethanolamines signaling: the relative similarity in binding affinity between CBD and AEA for FABPs and FAAH suggests that competition for these binding sites may indeed exist. Although the docking experiments helped us to confirm a drug target rather than a mechanism, they allow us to provide further evidence for the dual action of CBD both in AEA transport and metabolism, thus strengthening the hypothesis of a key role of AEA signaling in the autism-like traits observed in this genetic model of autism based on deletion of *FMR1* in rats.

Notably, as only males were used in the present study, our findings might not be replicated in female *Fmr1-$^{\Delta}$exon 8* rats. Since sex-dependent differences in preclinical models of ASD have been documented [40] and the loss of FMRP can produce different effects in male and female rodent models [41], we will consider the inclusion of both male and female animals in a follow-up study by characterizing the behavioral phenotype of *Fmr1-$^{\Delta}$exon 8* female rats across development and evaluating the (possible) sex-dependent effects of CBD.

Moreover, this study offers many opportunities for further investigation: (1) to test the effects of CBD in other behavioral tasks to assess, for instance, its impact on the socioemotional domain in *Fmr1-$^{\Delta}$exon 8* rats; (2) to measure the levels of bioactive N-acylethanolamines following CBD treatment in *Fmr1-$^{\Delta}$exon 8* rats and to examine the behavioral effects of FABPs inhibitors, together with the contribution of other enzymes involved in AEA catabolism including NAPE-PLD; (3) as CBD appears to function as an antagonist at GPR55 receptors [53], it would be interesting to test whether higher doses of CBD could exert different effects at this receptor; (4) another hypothesis to be explored is whether the GPR55 antagonist CID counteracted the beneficial effects of CBD by competing for the same binding site at the GPR55 receptor. For instance, biased antagonism at GPR55 receptors has been reported for CBD [61].

5. Conclusions

We have taken three important steps in this study: first, we found that CBD reduced the short-term object recognition deficits of *Fmr1-$^{\Delta}$exon 8* rats and that intra-hippocampal (CA1) blockade of GPR55

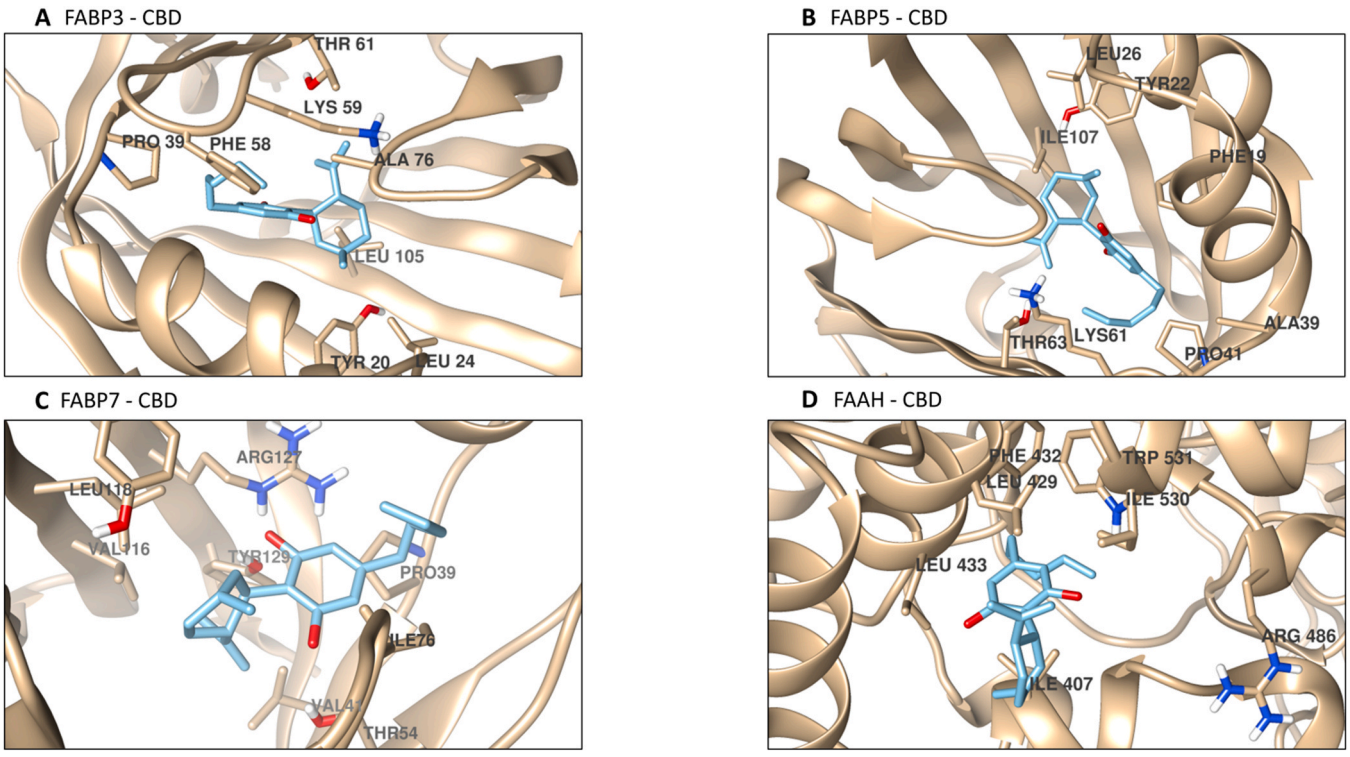

Fig. 6. Schematic representation of predicted CBD complexes with FABPs and FAAH. The CBD-FABP3 predicted binding affinity value is −7.8 kcal mol^{-1}(A). Docking simulations between CBD and FABP5 predicted a binding affinity value of −7.7 kcal mol^{-1} (B). The CBD-FABP7 predicted binding affinity value is −7.2 kcal mol^{-1} (C). Finally, the CBD-FAAH best pose is characterized by a predicted binding affinity value of −6.5 kcal mol^{-1}(D). The pictures were drawn with the UCSF-Chimera 1.16 software [47].

receptors counteracted its positive effects; second, we confirmed the role of endocannabinoid signaling via GPR55 receptors in the short-term memory deficits observed in this model of ASD; third, we strengthen our hypothesis of brain region-(and behaviorally) specific effects of cannabinoid compounds in *Fmr1-*$^{\Delta}$*exon 8* rats. These findings clarify the molecular mechanisms of CBD in ASD-related cognitive impairment, but also indicate that its (poly)pharmacology should be considered.

This study also presents the following limitations: 1. only male animals were used; 2. as GPR55 and FAAH mRNAs do not appear to be prominent targets of FMRP in brain regions such as the hippocampus [12,36,55,71], investigating endocannabinoid downstream pathways should be considered to understand the effects of cannabinoid drugs (including CBD) in animal models of FXS, especially in this new developed rat model of FXS; 3. because FMRP is thought to be expressed in all cells in the brain [52,51,75], a key question is whether it binds mRNAs with cell-type specificity. In this study, we were unable to precisely identify the specific cell types involved in the observed behavioral effects due to the absence of cytological analyses. Based on pioneering studies on mRNA targets of FMRP in rodent brain regions including the hippocampus [36,55] and on the pivotal role of hippocampal pyramidal neurons in memory and learning processes [23,67], we could hypothesize the possible involvement of this cell type in mediating the behavioral findings reported in this study; 4. more data should be included to firmly confirm the ability of the GPR55 antagonist CID16020046 to counteract the effects of CBD.

Funding

This work was supported by Autism Speaks grant #11690 (AM and VT), by Regione Lazio 'Gruppi di ricerca 2020' grant # PROT. A0375–2020–36550 (VT), by Jerome Lejeune Foundation Research grant #2209 - GRT-2023A (VT), by PNRR– M4 C2 I1.1 -PRIN 2022CZTZ87- CUP F53D23006660006 -Dissecting the pharmacological mechanisms underlying the therapeutic potential of phytocannabinoids in autism- finanziato dall'Unione europea – NextGenerationEU (VT), by PRIN 2022K7YKTY (VM) and by the Italian Ministry of Health with Current Research funds (BP). The project was partly supported also by Rome Technopole, PNRR grant M4-C2-Inv. 1.5 -CUP F83B22000040006 to VT. In particular, the intracranial pharmacology and docking experiments were funded by Rome Technopole.

CRediT authorship contribution statement

Alessandro Rava: Writing – original draft, Methodology, Investigation, Data curation. **Alessandro Feo:** Writing – original draft, Methodology, Investigation, Data curation. **Emilia Carbone:** Methodology, Formal analysis, Data curation. **Sara Schiavi:** Methodology, Formal analysis, Data curation. **Barbara Peruzzi:** Methodology, Funding acquisition, Formal analysis, Data curation. **Valentina D'Oria:** Methodology, Data curation. **Marco Pezzullo:** Methodology, Data curation. **Andrea Pasquadibisceglie:** Software, Methodology, Data curation. **Fabio Polticelli:** Software, Methodology, Conceptualization. **Vincenzo Micale:** Methodology, Investigation. **Martin Kuchar:** Resources, Methodology, Investigation. **Viviana Trezza:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Antonia Manduca:** Writing – original draft, Investigation, Conceptualization. **Valeria Buzzelli:** Writing – original draft, Methodology, Formal analysis, Data curation.

Declaration of Competing Interest

I declare on behalf of all authors that we do not have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We thank Prof. Alessandra Di Masi and Dott. Giovanna De Simone, Department of Science, Roma Tre University, for critical reading of the manuscript and for helpful discussion.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2024.107176.

References

[1] S.L. Andersen, Trajectories of brain development: point of vulnerability or window of opportunity? Neurosci. Biobehav. Rev. 27 (1-2) (2003) 3–18.
[2] L.L. Anderson, I.K. Low, I.S. McGregor, J.C. Arnold, Interactions between cannabidiol and Delta(9) -tetrahydrocannabinol in modulating seizure susceptibility and survival in a mouse model of Dravet syndrome, Br. J. Pharmacol. 177 (18) (2020) 4261–4274.
[3] A. Aran, H. Cassuto, A. Lubotzky, N. Wattad, E. Hazan, Brief Report: Cannabidiol-Rich Cannabis in Children with Autism Spectrum Disorder and Severe Behavioral Problems-A Retrospective Feasibility Study, J. Autism Dev. Disord. 49 (3) (2019) 1284–1288.
[4] A. Aran, M. Eylon, M. Harel, L. Polianski, A. Nemirovski, S. Tepper, et al., Lower circulating endocannabinoid levels in children with autism spectrum disorder, Mol. Autism 10 (2019) 2.
[5] A. Aran, M. Harel, H. Cassuto, L. Polyansky, A. Schnapp, N. Wattad, et al., Cannabinoid treatment for autism: a proof-of-concept randomized trial, Mol. Autism 12 (1) (2021) 6.
[6] H.N. Asgeirsdottir, S.J. Cohen, R.W., Jr Stackman, Object and place information processing by CA1 hippocampal neurons of C57BL/6J mice, J. Neurophysiol. 123 (3) (2020) 1247–1264.
[7] E. Berry-Kravis, R. Hagerman, D. Budimirovic, C. Erickson, H. Heussler, N. Tartaglia, et al., A randomized, controlled trial of ZYN002 cannabidiol transdermal gel in children and adolescents with fragile X syndrome (CONNECT-FX), J. Neurodev. Disord. 14 (1) (2022) 56.
[8] T. Bisogno, L. Hanus, L. De Petrocellis, S. Tchilibon, D.E. Ponde, I. Brandi, et al., Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide, Br. J. Pharmacol. 134 (4) (2001) 845–852.
[9] V. Buzzelli, E. Carbone, A. Manduca, S. Schiavi, A. Feo, J.V. Peredericy, et al., Psilocybin mitigates the cognitive deficits observed in a rat model of Fragile X syndrome, Psychopharmacology 240 (1) (2023) 137–147.
[10] E. Carbone, A. Manduca, C. Cacchione, S. Vicari, V. Trezza, Healing autism spectrum disorder with cannabinoids: a neuroinflammatory story, Neurosci. Biobehav. Rev. 121 (2021) 128–143.
[11] M.J. Curtis, S. Alexander, G. Cirino, J.R. Docherty, C.H. George, M.A. Giembycz, et al., Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers, Br. J. Pharmacol. 175 (7) (2018) 987–993.
[12] J.C. Darnell, S.J. Van Driesche, C. Zhang, K.Y. Hung, A. Mele, C.E. Fraser, et al., FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism, Cell 146 (2) (2011) 247–261.
[13] D.L. de Almeida, L.A. Devi, Diversity of molecular targets and signaling pathways for CBD, Pharmacol. Res. Perspect. 8 (6) (2020) e00682.
[14] A. D'Elia, S. Schiavi, A. Manduca, A. Rava, V. Buzzelli, F. Ascone, et al., FMR1 deletion in rats induces hyperactivity with no changes in striatal dopamine transporter availability, Sci. Rep. 12 (1) (2022) 22535.
[15] D.G. Deutsch, A Personal Retrospective: Elevating Anandamide (AEA) by Targeting Fatty Acid Amide Hydrolase (FAAH) and the Fatty Acid Binding Proteins (FABPs), Front. Pharmacol. 7 (2016) 370.
[16] O. Devinsky, R. Nabbout, I. Miller, L. Laux, M. Zolnowska, S. Wright, et al., Long-term cannabidiol treatment in patients with Dravet syndrome: An open-label extension trial, Epilepsia 60 (2) (2019) 294–302.
[17] M. Di Bartolomeo, T. Stark, O.M. Maurel, F.A. Iannotti, M. Kuchar, J. Ruda-Kucerova, et al., Crosstalk between the transcriptional regulation of dopamine D2 and cannabinoid CB1 receptors in schizophrenia: Analyses in patients and in perinatal Delta9-tetrahydrocannabinol-exposed rats, Pharmacol. Res. 164 (2021) 105357.
[18] Q. Ding, F. Sethna, H. Wang, Behavioral analysis of male and female Fmr1 knockout mice on C57BL/6 background, Behav Brain Res 271 (2014) 72–78.
[19] M.W. Elmes, M. Kaczocha, W.T. Berger, K. Leung, B.P. Ralph, L. Wang, et al., Fatty acid-binding proteins (FABPs) are intracellular carriers for Delta9-tetrahydrocannabinol (THC) and cannabidiol (CBD), J. Biol. Chem. 290 (14) (2015) 8711–8721.
[20] L.F. Ferreira, N. Pathapati, S.T. Schultz, M.C. Nunn, B.L. Pierce, Y.R. Sanchez, et al., Acute cannabidiol treatment enhances social interaction in adult male mice, Adv. Drug Alcohol Res. 3 (2023).
[21] R.H. Garman, A.A. Li, W. Kaufmann, R.N. Auer, B. Bolon, Recommended Methods for Brain Processing and Quantitative Analysis in Rodent Developmental Neurotoxicity Studies, Toxicol. Pathol. 44 (1) (2016) 14–42.
[22] Golden CEM, M.S. Breen, L. Koro, S. Sonar, K. Niblo, A. Browne, et al., Deletion of the KH1 Domain of Fmr1 Leads to Transcriptional Alterations and Attentional Deficits in Rats, Cereb. Cortex 29 (5) (2019) 2228–2244.
[23] A.R. Graves, S.J. Moore, E.B. Bloss, B.D. Mensh, W.L. Kath, N. Spruston, Hippocampal pyramidal neurons comprise two distinct cell types that are countermodulated by metabotropic receptors, Neuron 76 (4) (2012) 776–789.
[24] M. Hacohen, O.E. Stolar, M. Berkovitch, O. Elkana, E. Kohn, A. Hazan, et al., Children and adolescents with ASD treated with CBD-rich cannabis exhibit significant improvements particularly in social symptoms: an open label study, Transl. Psychiatry 12 (1) (2022) 375.
[25] R.J. Hagerman, E. Berry-Kravis, H.C. Hazlett, D.B. Bailey Jr., H. Moine, R.F. Kooy, et al., Fragile X syndrome, Nat. Rev. Dis. Prim. 3 (2017) 17065.
[26] S.M. Hamilton, J.R. Green, S. Veeraragavan, L. Yuva, A. McCoy, Y. Wu, et al., Fmr1 and Nlgn3 knockout rats: novel tools for investigating autism spectrum disorders, Behav. Neurosci. 128 (2) (2014) 103–109.
[27] T. Hlozek, L. Uttl, L. Kaderabek, M. Balikova, E. Lhotkova, R.R. Horsley, et al., Pharmacokinetic and behavioural profile of THC, CBD, and THC+CBD combination after pulmonary, oral, and subcutaneous administration in rats and confirmation of conversion in vivo of CBD to THC, Eur. Neuropsychopharmacol.: J. Eur. Coll. Neuropsychopharmacol. 27 (12) (2017) 1223–1237.
[28] M. Kaczocha, S. Haj-Dahmane, Mechanisms of endocannabinoid transport in the brain, Br. J. Pharmacol. 179 (17) (2022) 4300–4310.
[29] M. Kaczocha, S.T. Glaser, D.G. Deutsch, Identification of intracellular carriers for the endocannabinoid anandamide, Proc. Natl. Acad. Sci. USA 106 (15) (2009) 6375–6380.
[30] M. Kaczocha, M.J. Rebecchi, B.P. Ralph, Y.H. Teng, W.T. Berger, W. Galbavy, et al., Inhibition of fatty acid binding proteins elevates brain anandamide levels and produces analgesia, PloS One 9 (4) (2014) e94200.
[31] J.S. Kaplan, N. Stella, W.A. Catterall, R.E. Westenbroek, Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome, Proc. Natl. Acad. Sci. USA 114 (42) (2017) 11229–11234.
[32] D.S. Karhson, K.M. Krasinska, J.A. Dallaire, R.A. Libove, J.M. Phillips, A.S. Chien, et al., Plasma anandamide concentrations are lower in children with autism spectrum disorder, Mol. Autism 9 (2018) 18.
[33] F.M. Leweke, D. Piomelli, F. Pahlisch, D. Muhl, C.W. Gerth, C. Hoyer, et al., Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia, Transl. Psychiatry 2 (3) (2012) e94.
[34] B.A. Marichal-Cancino, A. Fajardo-Valdez, A.E. Ruiz-Contreras, M. Mendez-Diaz, O. Prospero-Garcia, Possible role of hippocampal GPR55 in spatial learning and memory in rats, Acta Neurobiol. Exp. 78 (1) (2018) 41–50.
[35] T. Maurin, S. Zongaro, B. Bardoni, Fragile X Syndrome: from molecular pathology to therapy, Neurosci. Biobehav. Rev. 46 (Pt 2) (2014) 242–255.
[36] T. Maurin, K. Lebrigand, S. Castagnola, A. Paquet, M. Jarjat, A. Popa, et al., HITS-CLIP in various brain areas reveals new targets and new modalities of RNA binding by fragile X mental retardation protein, Nucleic Acids Res. 46 (12) (2018) 6344–6355.
[37] X. Min, S.T. Thibault, A.C. Porter, D.J. Gustin, T.J. Carlson, H. Xu, et al., Discovery and molecular basis of potent noncovalent inhibitors of fatty acid amide hydrolase (FAAH), Proc. Natl. Acad. Sci. USA 108 (18) (2011) 7379–7384.
[38] C.P. Moritz, Tubulin or Not Tubulin: Heading Toward Total Protein Staining as Loading Control in Western Blots, Proteomics 17 (20) (2017).
[39] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell, et al., AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility, J. Comput. Chem. 30 (16) (2009) 2785–2791.
[40] A. Napolitano, S. Schiavi, P. La Rosa, M.C. Rossi-Espagnet, S. Petrillo, F. Bottino, et al., Sex Differences in Autism Spectrum Disorder: Diagnostic, Neurobiological, and Behavioral Features, Front. Psychiatry 13 (2022) 889636.
[41] S.O. Nolan, C.D. Reynolds, G.D. Smith, A.J. Holley, B. Escobar, M.A. Chandler, et al., Deletion of Fmr1 results in sex-specific changes in behavior, Brain Behav. 7 (10) (2017) e00800.
[42] A.L. Osborne, N. Solowij, I. Babic, X.F. Huang, K. Weston-Green, Improved Social Interaction, Recognition and Working Memory with Cannabidiol Treatment in a Prenatal Infection (poly I:C) Rat Model, Neuropsychopharmacol.: Off. Publ. Am. Coll. Neuropsychopharmacol. 42 (7) (2017) 1447–1457.
[43] J.M. Palumbo, B.F. Thomas, D. Budimirovic, S. Siegel, F. Tassone, R. Hagerman, et al., Role of the endocannabinoid system in fragile X syndrome: potential mechanisms for benefit from cannabidiol treatment, J. Neurodev. Disord. 15 (1) (2023) 1.
[44] P.H. Patra, E. Serafeimidou-Pouliou, M. Bazelot, B.J. Whalley, C.M. Williams, A. J. McNeish, Cannabidiol improves survival and behavioural co-morbidities of Dravet syndrome in mice, Br. J. Pharmacol. 177 (12) (2020) 2779–2792.
[45] J.F.C. Pedrazzi, F.R. Ferreira, D. Silva-Amaral, D.A. Lima, J.E.C. Hallak, A. W. Zuardi, et al., Cannabidiol for the treatment of autism spectrum disorder: hope or hype? Psychopharmacology 239 (9) (2022) 2713–2734.
[46] N. Percie du Sert, V. Hurst, A. Ahluwalia, S. Alam, M.T. Avey, M. Baker, et al., The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, PLoS Biol. 18 (7) (2020) e3000410.
[47] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, et al., UCSF Chimera–a visualization system for exploratory research and analysis, J. Comput. Chem. 25 (13) (2004) 1605–1612.

[48] S. Pietropaolo, G. Marsicano, The role of the endocannabinoid system as a therapeutic target for autism spectrum disorder: Lessons from behavioral studies on mouse models, Neurosci. Biobehav. Rev. 132 (2022) 664–678.
[49] S. Poleg, E. Kourieh, A. Ruban, G. Shapira, N. Shomron, B. Barak, et al., Behavioral aspects and neurobiological properties underlying medical cannabis treatment in Shank3 mouse model of autism spectrum disorder, Transl. Psychiatry 11 (1) (2021) 524.
[50] Rice, L.J. Cannon, L. Dadlani, N. Cheung MMY, S.L. Einfeld, D. Efron, et al., Efficacy of cannabinoids in neurodevelopmental and neuropsychiatric disorders among children and adolescents: a systematic review, Eur. Child Adolesc. Psychiatry (2023).
[51] J.D. Richter, X. Zhao, The molecular biology of FMRP: new insights into fragile X syndrome, Nat. Rev. Neurosci. 22 (4) (2021) 209–222.
[52] J.D. Richter, G.J. Bassell, E. Klann, Dysregulation and restoration of translational homeostasis in fragile X syndrome, Nat. Rev. Neurosci. 16 (10) (2015) 595–605.
[53] R.A. Ross, The enigmatic pharmacology of GPR55, Trends Pharmacol. Sci. 30 (3) (2009) 156–163.
[54] H. Sander, S. Wallace, R. Plouse, S. Tiwari, A.V. Gomes, Ponceau S waste: Ponceau S staining for total protein normalization, Anal. Biochem. 575 (2019) 44–53.
[55] K. Sawicka, C.R. Hale, C.Y. Park, J.J. Fak, J.E. Gresack, S.J. Van Driesche, et al., FMRP has a cell-type-specific role in CA1 pyramidal neurons to regulate autism-related transcripts and circadian memory, eLife (2019) **8**.
[56] S. Schiavi, E. Carbone, F. Melancia, A. di Masi, M. Jarjat, F. Brau, et al., Phosphodiesterase 2A inhibition corrects the aberrant behavioral traits observed in genetic and environmental preclinical models of Autism Spectrum Disorder, Transl. Psychiatry 12 (1) (2022) 119.
[57] S. Schiavi, E. Carbone, F. Melancia, V. Buzzelli, A. Manduca, P. Campolongo, et al., Perinatal supplementation with omega-3 fatty acids corrects the aberrant social and cognitive traits observed in a genetic model of autism based on FMR1 deletion in rats, Nutr. Neurosci. 25 (5) (2022) 898–911.
[58] S. Schiavi, A. Manduca, E. Carbone, V. Buzzelli, A. Rava, A. Feo, et al., Anandamide and 2-arachidonoylglycerol differentially modulate autistic-like traits in a genetic model of autism based on FMR1 deletion in rats, Neuropsychopharmacol.: Off. Publ. Am. Coll. Neuropsychopharmacol. 48 (6) (2023) 897–907.
[59] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (6) (2008) 1101–1108.
[60] L. Shapiro, A. Escayg, J.C. Wong, Cannabidiol Increases Seizure Resistance and Improves Behavior in an Scn8a Mouse Model, Front. Pharmacol. 13 (2022) 815950.
[61] H. Sharir, M.E. Abood, Pharmacological characterization of GPR55, a putative cannabinoid receptor, Pharmacol. Ther. 126 (3) (2010) 301–313.
[62] E.A.D.J. Silva, W.M.B. Medeiros, J. Santos, J.M.M. Sousa, F.B.D. Costa, K. M. Pontes, et al., Evaluation of the efficacy and safety of cannabidiol-rich cannabis extract in children with autism spectrum disorder: randomized, double-blind and controlled placebo clinical trial, Trends Psychiatry Psychother. 44 (2022).
[63] D. Siniscalco, A. Sapone, C. Giordano, A. Cirillo, L. de Magistris, F. Rossi, et al., Cannabinoid receptor type 2, but not type 1, is up-regulated in peripheral blood mononuclear cells of children affected by autistic disorders, J. Autism Dev. Disord. 43 (11) (2013) 2686–2695.
[64] E.M. Sorensen, F. Bertelsen, P. Weikop, M.M. Skovborg, T. Banke, K.R. Drasbek, J. Scheel-Kruger, Hyperactivity and lack of social discrimination in the adolescent Fmr1 knockout mouse, Behav Pharmacol 26 (2015) 733–740.
[65] T. Stark, J. Ruda-Kucerova, F.A. Iannotti, C. D'Addario, R. Di Marco, V. Pekarik, et al., Peripubertal cannabidiol treatment rescues behavioral and neurochemical abnormalities in the MAM model of schizophrenia, Neuropharmacology 146 (2019) 212–221.
[66] T. Stark, M. Di Bartolomeo, R. Di Marco, E. Drazanova, C.B.M. Platania, F. A. Iannotti, et al., Altered dopamine D3 receptor gene expression in MAM model of schizophrenia is reversed by peripubertal cannabidiol treatment, Biochem. Pharmacol. 177 (2020) 114004.
[67] S. Takamiya, K. Shiotani, T. Ohnuki, Y. Osako, Y. Tanisumi, S. Yuki, et al., Hippocampal CA1 Neurons Represent Positive Feedback During the Learning Process of an Associative Memory Task, Front. Syst. Neurosci. 15 (2021) 718619.
[68] N. Tartaglia, M. Bonn-Miller, R. Hagerman, Treatment of Fragile X Syndrome with Cannabidiol: A Case Series Study and Brief Review of the Literature, Cannabis cannabinoid Res. 4 (1) (2019) 3–9.
[69] E.A. Thiele, E.M. Bebin, H. Bhathal, F.E. Jansen, K. Kotulska, J.A. Lawson, et al., Add-on Cannabidiol Treatment for Drug-Resistant Seizures in Tuberous Sclerosis Complex: A Placebo-Controlled Randomized Clinical Trial, JAMA Neurol. 78 (3) (2021) 285–292.
[70] Y. Tian, C. Yang, S. Shang, Y. Cai, X. Deng, J. Zhang, et al., Loss of FMRP Impaired Hippocampal Long-Term Plasticity and Spatial Learning in Rats, Front. Mol. Neurosci. 10 (2017) 269.
[71] S.S. Tran, H.I. Jun, J.H. Bahn, A. Azghadi, G. Ramaswami, E.L. Van Nostrand, et al., Widespread RNA editing dysregulation in brains from autistic individuals, Nat. Neurosci. 22 (1) (2019) 25–36.
[72] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, J. Comput. Chem. 31 (2) (2010) 455–461.
[73] S.E. Turner, C.M. Williams, L. Iversen, B.J. Whalley, Molecular Pharmacology of Phytocannabinoids, Prog. Chem. Org. Nat. Prod. 103 (2017) 61–101.
[74] M. Uutela, J. Lindholm, T. Rantamaki, J. Umemori, K. Hunter, V. Voikar, M. L. Castren, Distinctive behavioral and cellular responses to fluoxetine in the mouse model for Fragile X syndrome, Front Cell Neurosci 8 (2014) 150.
[75] X. Zhao, A. Bhattacharyya, Human models are needed for studying human neurodevelopmental disorders, Am. J. Hum. Genet. 103 (6) (2018) 829–857.