



## ARTICLE



# Anandamide and 2-arachidonoylglycerol differentially modulate autistic-like traits in a genetic model of autism based on FMR1 deletion in rats

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Autism spectrum disorder (ASD) has a multifactorial etiology. Major efforts are underway to understand the neurobiological bases of ASD and to develop efficacious treatment strategies. Recently, the use of cannabinoid compounds in children with neurodevelopmental disorders including ASD has received increasing attention. Beyond anecdotal reports of efficacy, however, there is limited current evidence supporting such an intervention and the clinical studies currently available have intrinsic limitations that make the interpretation of the findings challenging. Furthermore, as the mechanisms underlying the beneficial effects of cannabinoid compounds in neurodevelopmental disorders are still largely unknown, the use of drugs targeting the endocannabinoid system remains controversial. Here, we studied the role of endocannabinoid neurotransmission in the autistic-like traits displayed by the recently validated *Fmr1*<sup>Δ</sup>*exon 8* rat model of autism. *Fmr1*<sup>Δ</sup>*exon 8* rats showed reduced anandamide levels in the hippocampus and increased 2-arachidonoylglycerol (2-AG) content in the amygdala. Systemic and intra-hippocampal potentiation of anandamide tone through administration of the anandamide hydrolysis inhibitor URB597 ameliorated the cognitive deficits displayed by *Fmr1*<sup>Δ</sup>*exon 8* rats along development, as assessed through the novel object and social discrimination tasks. Moreover, blockade of amygdalar 2-AG signaling through intra-amygdala administration of the CB1 receptor antagonist SR141716A prevented the altered sociability displayed by *Fmr1*<sup>Δ</sup>*exon 8* rats. These findings demonstrate that anandamide and 2-AG differentially modulate specific autistic-like traits in *Fmr1*<sup>Δ</sup>*exon 8* rats in a brain region-specific manner, suggesting that fine changes in endocannabinoid mechanisms contribute to ASD-related behavioral phenotypes.

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## INTRODUCTION

Autism spectrum disorder (ASD) defines a group of neurodevelopmental disorders characterized by disturbed patterns of social behavior and further differentiated by other features such as atypical or deficient communication skills and restricted or repetitive patterns of behavior [1]. Other comorbid features are often associated with these core symptoms, such as intellectual disability (ID) and aberrant sensitivity to sensory stimulation [2]. Multiple factors are involved in the pathogenesis of the disease, ranging from environmental to genetic and even to combinations of the two factors [3].

Fragile X syndrome (FXS) is the most common inherited cause of ASD and ID worldwide. It is caused by the silencing of the Fragile X Messenger Ribonucleoprotein 1 (FMR1) gene which has a key role in the development of synapses and in underlying learning and memory processes [4–7]. While clinically and genetically distinct, FXS and ASD share significant comorbidity, suggesting that there may be common molecular and/or cellular bases controlling the dysregulated neuronal

networking and circuitry, leading to common aberrant patterns of behavior [8–10].

To date, there are no approved curative therapies for ASD; current approaches focus on helpful educational and behavioral strategies, together with off-label drugs that mitigate specific symptoms such as hyperactivity, anxiety and seizures [11]. Yet, for many patients, these off-label medications, when used alone or in combination, have suboptimal efficacy and tolerability and do not ameliorate the cognitive and social impairments associated with ASD.

The endocannabinoid system (ECS) is a major neuromodulatory system involved in the regulation of synaptic plasticity, emotional responses, social behavior and cognitive states [12–15], all of which are affected in ASD and FXS [16, 17]. It consists of endocannabinoids (primarily anandamide (AEA) and 2-arachidonoylglycerol (2-AG)), cannabinoid receptors and the enzymes responsible for endocannabinoid synthesis (e.g., NAPE-PLD, DAGL) and degradation (e.g., FAAH, MAGL) [18–21], together with a “non-canonical” extended signaling network

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[22]. Abnormalities in the ECS have been found both in patients with ASD [23–26] and in animal models of ASD [27–29]. Furthermore, pharmacological modulation of the ECS restored synaptic and/or behavioral functions in animal models of ASD, including animal models of FXS [30–34]. This evidence has increased the scientific and industry interest in cannabinoid compounds as a treatment for the core symptoms and comorbidities of ASD: thus, some non-psychoactive phytocannabinoids (i.e., cannabidiol (CBD) and cannabidivarin (CBVD)) are currently under clinical trial in children with neurodevelopmental disorders. However, the few clinical studies currently available provided sometimes controversial results [35–37] and they have intrinsic limitations (e.g., inconsistencies in availability, quality and purity of cannabinoid compounds, open label design and small sample size) that make the interpretation of the findings challenging. In addition, the underlying mechanisms remain largely unexplored. This points out the necessity of clarifying the role of the ECS in ASD using appropriate animal models to determine whether specific changes in brain endocannabinoid signaling differentially contribute to ASD-related behavioral phenotypes across the lifespan and to test which pharmacological approach targeting endocannabinoid neurotransmission (i.e., cannabinoid receptor agonists or antagonists, inhibitors of endocannabinoid biosynthesis or metabolism) may be promising for the core symptoms and comorbidities of ASD.

In the present work, we aimed at investigating the role of endocannabinoid neurotransmission in the autistic-like traits displayed by the recently validated *Fmr1*<sup>Δ</sup>*exon 8* rat model of ASD [38, 39]. This rat model is not a null knock-out of *Fmr1*, but instead results in a gene product with a loss of exon 8, which encodes a domain within *Fmr1* that is responsible for RNA-binding [39]. Such deletion is sufficient to cause FXS-like behavioral traits and abnormal synaptic plasticity [38–42], making the *Fmr1*<sup>Δ</sup>*exon 8* rat a valuable tool to study common neurobiological aspects of both FXS and ASD [40]. Specifically, we assessed whether a brain region-specific alteration in the ECS occurs in *Fmr1*<sup>Δ</sup>*exon 8* rats by measuring the levels of the main endocannabinoids (AEA and 2-AG) in the hippocampus, prefrontal cortex and amygdala, as these brain regions have been suggested to mediate ASD-related phenotype together with associated morphological and functional changes in patients [43–45] and in preclinical models of ASD [46, 47]. Furthermore, we performed biochemical experiments to detect changes in other main components of the ECS (i.e., enzymes modulating synthesis/degradation of AEA and 2-AG, and cannabinoid receptors) in *Fmr1*<sup>Δ</sup>*exon 8* rats compared to wild-type controls. Based on these results, we tested the behavioral effects of systemic administration of the AEA hydrolysis inhibitor URB597 in the short-term recognition memory and social deficits displayed by *Fmr1*<sup>Δ</sup>*exon 8* rats. Then, we explored the effects of intracranial modulation of the endocannabinoid tone to verify the specific brain regions and mechanisms mediating the role of either AEA or 2-AG in the altered behavioral phenotype displayed by *Fmr1*<sup>Δ</sup>*exon 8* rats.

## MATERIALS AND METHODS

### Experimental design

Juvenile (35-day-old) and adult (80-day-old) wild-type (WT) and *Fmr1*<sup>Δ</sup>*exon 8* rats were used and tested following the experimental design shown in Supplementary Fig. 1.

### Animals

WT (Charles River Laboratories, Italy) and *Fmr1*<sup>Δ</sup>*exon 8* male and female rats (Horizon Discovery, formerly SAGE Labs, USA) on a Sprague-Dawley background were mated overnight. Pregnant rats were individually housed in Macrolon cages (40 (length) × 26 (width) × 20 (height) cm), under controlled conditions (temperature 20–21 °C, 55–65% relative humidity and 12/12 h light cycle with lights on at 07:00 h). Newborn litters found up

to 17:00 h were considered to be born on that day (postnatal day (PND) 0). On PND 1, the litters were culled to eight animals (six males and two females), to reduce any litter size-induced variability in the growth and development of pups during the postnatal period. On PND 21, the pups were weaned and housed in groups of three (same sex and same genotype) and tested across development. One male pup per litter from different litters per treatment group was randomly used in each experiment. Sample size (n) is indicated in the figure legends and was based on our previous experiments and power analysis performed with the software G\*Power. Potential outliers within each data set were calculated using the GraphPad Prism 8 software (Grubbs' method). Scoring of the behavioral experiments was done in blind conditions using the Observer 3.0 software (Noldus, The Netherlands). Moreover, the operators for stereotaxic surgery, training/testing and scoring were different (i.e., the researchers who performed surgery did not test or score the animals and *vice versa*). The experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines [48], the guidelines of the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU.

### Biochemical analyses

**Brain samples collection.** Rats were decapitated and their brains were rapidly removed from the skull on a cold plate. The hippocampus, prefrontal cortex and amygdala were dissected by hand under microscopic control within 2 min and stored at –80 °C for endocannabinoid levels analysis at PND 35 [49–51]. qPCR experiments were performed in hippocampus and amygdala samples collected at PNDs 35 and 80; protein levels were assessed in samples collected at PND 35.

**Endocannabinoid extraction and quantification.** Brain tissues were weighed and placed into borosilicate glass tubes with 2 ml of acetonitrile and 5 pmol of [<sup>2</sup>H<sub>8</sub>] AEA and 5 nmol of [<sup>2</sup>H<sub>8</sub>] 2-AG for extraction and homogenized with a glass rod. Tissues were sonicated for 30 min on ice water and incubated overnight at –20 °C to precipitate proteins, then centrifuged at 1500 × g to remove particulates. Supernatants were transferred to a new glass tube and evaporated to dryness under N<sub>2</sub> gas. The samples were reconstituted in 300 μl of acetonitrile and dried again under N<sub>2</sub> gas. Lipid extracts were suspended in 200 μl of acetonitrile and stored at –80 °C until analysis. Analysis of AEA and 2-AG was performed by liquid chromatography tandem mass spectrometry analysis as previously detailed [52].

**Quantitative PCR (qPCR) analysis.** Total RNA isolation was performed by 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 RT-PCR 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 Supplementary Table 1. β-actin was used as reference gene. Data were analyzed using the 2<sup>–ΔΔCt</sup> method [53], and results are expressed as fold changes relative to the correspondent WT group.

### Western blot analysis

See Supplementary material.

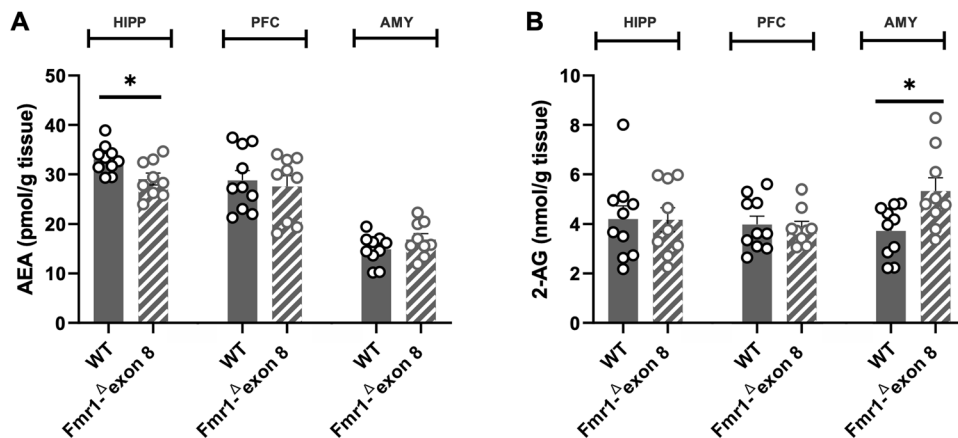
### FAAH activity assay

FAAH activity was assessed by performing a 96-well fluorimetric assay (Cat. ab252895, Abcam, UK). Hippocampal samples were rapidly sonicated in ice-cold FAAH assay buffer, followed by centrifugation at 10,000 × g, 4 °C and collection of supernatants. Protein concentration was measured by a colorimetric Bradford assay. FAAH hydrolytic activity of test sample was evaluated by measuring the production of the 7-amino-4-methylcoumarin (AMC) in kinetic mode for 60 min at 37 °C. The fluorescent output (Excitation/Emission = 360/465 nm) was recorded each 120 s for 30 cycles. Values are expressed as fold change on the average of the WT control group.

### Surgical procedures

**Surgery.** Rats were anesthetized with a mixture of Zoletil® (50 mg/kg, i.p.) and Rompun® (7 mg/kg, i.p.) and placed in a stereotaxic frame (2biological Instruments, Italy). Two stainless-steel 24-gauge cannulae (Cooper's

## Endocannabinoid levels



**Fig. 1** AEA and 2-AG levels were altered in a region-specific manner in the brain of *Fmr1-Δexon 8* rats. Compared to WT animals, *Fmr1-Δexon 8* rats showed reduced levels of AEA in the hippocampus (HIPP) (A) and increased levels of 2-AG into the amygdala (AMY) (B), while no differences in AEA and 2-AG levels were found in the prefrontal cortex (PFC) (A, B) (WT = 10; *Fmr1-Δexon 8* = 9 animals per group). Data represent means  $\pm$  SEM, \* $p < 0.05$  vs WT group (Student's *t* test).

Needleworks, UK) were implanted bilaterally above the CA1 region of the dorsal hippocampus [coordinates: AP,  $-3.3$  mm; ML,  $\pm 1.7$  mm; DV,  $-2.7$  mm] or above the basolateral amygdala [coordinates: AP,  $-1.9$  mm; ML,  $\pm 4.6$  mm; DV,  $-7.5$  mm]. Coordinates for each brain region were based on our previous studies [54, 55] and on the atlas of Paxinos and Watson (2014). Stylets (Cooper's Needleworks, UK) were inserted into each cannula to maintain patency. After surgery, rats were individually housed and allowed to recover for 4 days. On the fifth day, they were re-housed in groups of three with their original cage mates. Behavioral testing began 1 week after surgery.

**Drug and infusion procedures.** URB597 (Sigma-Aldrich, Italy) was dissolved in 5% Tween 80/5% polyethylene glycol/saline and administered intraperitoneally (i.p.) at the dose of 0.1 mg/kg 2 h before testing in the novel object recognition, three-chamber and social discrimination tests. Drug doses and pre-treatment intervals were based on our previous findings [56] and on literature data in order to have a maximum augmentation of AEA levels [57–62]. Solutions were administered in a volume of 2 ml/kg in juvenile rats and 1 ml/kg in adult rats. For intracranial experiments, URB597 and the CB1 cannabinoid receptor antagonist/inverse agonist SR141716A (Sigma-Aldrich, Italy) were dissolved in 5% Tween 80/5% polyethylene glycol/saline. URB597 was infused in the hippocampus of juvenile and adult rats at the dose of 10 ng per side [50]. SR141716A was infused in the basolateral amygdala of juvenile rats at the dose of 0.1  $\mu$ g per side. This dose did not alter the behavior per se and antagonized the effects of systemic URB597 treatment on social play [55]. Bilateral infusions of the drugs or the corresponding vehicle were made using 30-gauge injection needles (Bilaney, Germany) connected to 10  $\mu$ l Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 1.5 mm beyond the cannula tips, and a 0.5  $\mu$ l (for hippocampus) or 0.2  $\mu$ l (for basolateral amygdala) injection volume per hemisphere was infused over 60 s using a syringe pump (Harvard Apparatus, USA). The injection needles remained within the guide cannulae for 60 s following drug infusion to facilitate diffusion and to prevent backflow of drug along the cannula track. Infusion of URB597 occurred immediately after training session (30 min before testing) in the novel object and social discrimination tests. Infusion of SR141716A occurred immediately before testing in the three-chamber test.

**Histological confirmation of injection sites.** Injection sites were verified as previously described [55, 63]. After testing, animals were sacrificed and microinjected with black ink over 60 s through the guide cannulae. Animals were immediately decapitated and their brains removed. Slices (20  $\mu$ m 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 (2014). Only samples with bilateral needle tracks terminating into the target area and no damage to the target tissues were included in the final analysis (Supplementary Fig. 2).

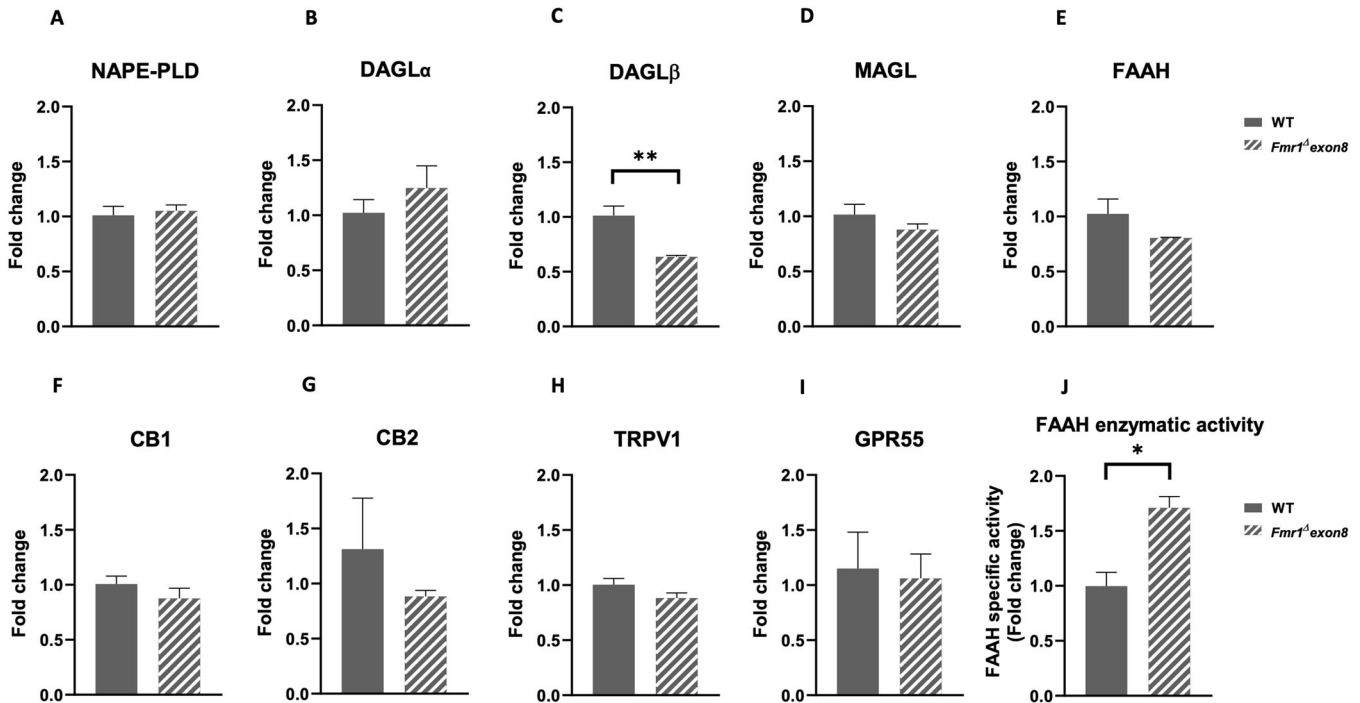
### Behavioral test

**Novel object recognition test.** The test was performed as previously described [38, 64]. On the training trial, each rat was individually placed into an open-field arena containing two identical objects (A1 and A2) for 5 min. Thirty minutes later, one copy of the familiar object (A3) and a new object (B) were placed in the same location as during the training trial. Each rat was placed in the apparatus for 5 min, and the time spent exploring each object was recorded. The discrimination index was calculated as the difference in time spent by each animal exploring the novel compared with the familiar object divided by the total time spent exploring both objects [65, 66] in percentage. Exploration was scored when the animal was sniffing or touching the object with the nose and/or forepaws.

**Three-chamber test.** The test was performed as previously described [59, 67]. Each rat was individually allowed to explore a three-chamber apparatus for 10 min and then confined in the central compartment. An unfamiliar stimulus animal was confined in a cage located in one chamber of the apparatus, while the cage in the other chamber was left empty. Both doors to the side chambers were then opened, allowing the experimental animal to explore the apparatus for 10 min. The time spent in social approach (sniffing the stimulus animal) and the time spent exploring the empty chamber were scored. The discrimination index was calculated as the difference in time spent by each animal sniffing the cage with the stimulus animal compared with the empty cage divided by the total time spent exploring both cages in percentage.

**Social discrimination test.** The test was performed as previously described [49, 59]. Adult animals were isolated for 7 days before testing. The test consisted of a learning trial and a retrieval trial, separated by a 30 min inter-trial interval. During the learning trial, a juvenile unfamiliar rat was introduced into the home cage of the experimental rat for 5 min. The time spent by the experimental rat investigating (sniffing, allogrooming and following) the juvenile was measured. Thirty minutes after, the juvenile used in the learning trial was returned to the same adult's cage together with a novel juvenile. The time spent by the adult exploring the novel and the familiar juveniles was monitored for 5 min. The discrimination index was calculated as the difference in time spent by each animal exploring the novel compared with the familiar juvenile divided by the total time spent exploring both juveniles in percentage.

**Statistical analysis.** Data are expressed as mean  $\pm$  SEM. To assess the effects of the genotype in the biochemical experiments, data were analyzed by unpaired two sample Student's *t* tests. To assess the effects of URB597 or SR141716A in the behavioral experiments, data were analyzed by two-way ANOVA, with genotype and treatments as factors. The Tukey's post hoc test was used for individual group comparisons. The accepted value for significance was set at  $p < 0.05$ . Data were analyzed using GraphPad Prism 8.



**Fig. 2** qPCR analysis of the main components of the ECS in the hippocampus of juvenile *Fmr1*<sup>Δexon 8</sup> rats. Fold induction of NAPE-PLD (A), DAGL $\alpha$  (B), DAGL $\beta$  (C), MAGL (D), FAAH (E), CB1 (F), CB2 (G), TRPV1 (H), GPR55 (I) gene expression in the hippocampus of *Fmr1*<sup>Δexon 8</sup> rats and WT animals, evaluated at PND 35, together with FAAH enzymatic activity (J) (WT = 3–4; *Fmr1*<sup>Δexon 8</sup> = 3–4 animals per group). Data represent mean  $\pm$  SEM, \* $p$  < 0.05 and \*\* $p$  < 0.01 vs WT group (Student's  $t$  test). NAPE-PLD, N-acylphosphatidylethanolamine-phospholipid D; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase; FAAH, fatty acid amide hydrolase; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; TRPV1, transient receptor potential vanilloid 1; GPR55, G protein-coupled receptor 55.

## RESULTS

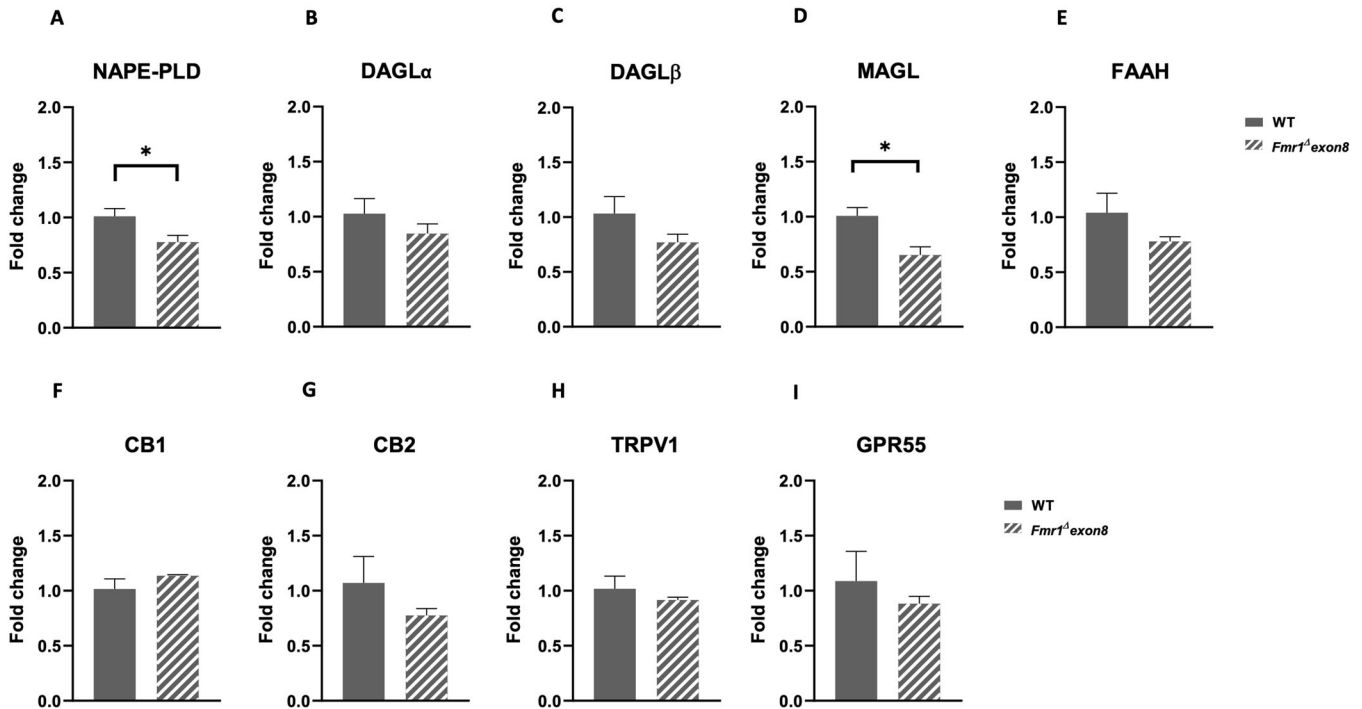
### *Fmr1*<sup>Δexon 8</sup> rats showed brain region-specific changes in AEA and 2-AG levels, together with discrete alterations in the main endocannabinoid enzymes

Juvenile *Fmr1*<sup>Δexon 8</sup> rats showed reduced AEA levels in the hippocampus ( $t = 2.684$ ,  $df = 17$ ,  $p < 0.05$ , Fig. 1A) and increased 2-AG levels in the amygdala ( $t = 2.604$ ,  $df = 17$ ,  $p < 0.05$ , Fig. 1B), while no differences in AEA and 2-AG levels were found in the prefrontal cortex of *Fmr1*<sup>Δexon 8</sup> rats compared to WT animals (AEA;  $t = 0.427$ ,  $df = 17$ ,  $p = n.s.$ ; 2-AG,  $t = 0.303$ ;  $df = 17$ ;  $p = n.s.$ , Fig. 1A, B).

Based on these results, we further characterized the ECS in the hippocampus (Fig. 2) and amygdala (Fig. 3) of WT and *Fmr1*<sup>Δexon 8</sup> rats. In the hippocampus, we found a significant reduction in the expression of the enzyme DAGL $\beta$ , involved in the synthesis of 2-AG ( $t = 4.261$ ,  $df = 6$ ,  $p < 0.01$ ; Fig. 2C) in juvenile *Fmr1*<sup>Δexon 8</sup> rats compared to WT controls; other enzymes involved in endocannabinoid synthesis were spared, such as NAPE-PLD ( $t = 0.432$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 2A) and DAGL $\alpha$  ( $t = 0.962$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 2B). The 2-AG degrading enzyme MAGL ( $t = 1.268$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 2D) and the AEA catabolizing enzyme FAAH ( $t = 1.634$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 2E) were also spared. No significant differences were found in the mRNA expression levels of CB1 ( $t = 1.107$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 2F), CB2 ( $t = 0.780$ ,  $df = 5$ ,  $p = n.s.$ ; Fig. 2G), TRPV1 ( $t = 1.683$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 2H) and GPR55 ( $t = 0.221$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 2I) receptors. Interestingly, FAAH activity was increased in the hippocampus of juvenile *Fmr1*<sup>Δexon 8</sup> rats compared to WT controls ( $t = 4.403$ ,  $df = 4$ ,  $p < 0.05$ ; Fig. 2J). This suggests that the decreased levels of AEA found in the hippocampus of juvenile *Fmr1*<sup>Δexon 8</sup> rats (Fig. 1A) could be due to an increased activity of FAAH. Western blot analyses of the protein levels of the main cannabinoid targets are in line with qPCR results (DAGL $\alpha$ :  $t = 2.216$ ,  $df = 6$ ,  $p = 0.068$ ; Supplementary Fig. 3A; MAGL:  $t = 0.891$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 3B;

FAAH:  $t = 1.826$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 3C; CB1:  $t = 0.461$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 3D; CB2:  $t = 0.503$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 3E). Consistent data were obtained by qPCR analysis of the main endocannabinoid targets at adulthood, with only a significant decrease in DAGL $\beta$  expression ( $t = 3.008$ ,  $df = 6$ ,  $p < 0.05$ ; Supplementary Fig. 4C) in *Fmr1*<sup>Δexon 8</sup> rats compared to WT controls; the other main cannabinoid enzymes NAPE-PLD ( $t = 0.481$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4A), DAGL $\alpha$  ( $t = 1.579$ ,  $df = 5$ ,  $p = n.s.$ ; Supplementary Fig. 4B), MAGL ( $t = 0.942$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4D), FAAH ( $t = 1.845$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4E) and the CB1 ( $t = 1.418$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 4F), CB2 ( $t = 0.199$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4G), TRPV1 ( $t = 0.085$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 4H) and GPR55 ( $t = 0.124$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4I) receptors were spared.

In the amygdala of juvenile *Fmr1*<sup>Δexon 8</sup> rats, we found a significant reduction in the expression of the enzymes NAPE-PLD ( $t = 2.506$ ,  $df = 6$ ,  $p < 0.05$ ; Fig. 3A) and MAGL ( $t = 3.380$ ,  $df = 6$ ,  $p < 0.05$ ; Fig. 3B), whereas DAGL $\alpha$  ( $t = 1.119$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 3B), DAGL $\beta$  ( $t = 1.536$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 3C) and FAAH ( $t = 1.423$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 3E) were spared. No changes in the expression levels of CB1 ( $t = 1.307$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 3F), CB2 ( $t = 1.185$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 3G), TRPV1 ( $t = 0.865$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 3H), GPR55 ( $t = 0.735$ ,  $df = 6$ ,  $p = n.s.$ ; Fig. 3I) receptors were found. These data correlate with the results in the protein levels of the main components of the ECS (DAGL $\alpha$ :  $t = 0.019$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 3F; MAGL:  $t = 1.283$ ,  $df = 5$ ,  $p = n.s.$ ; Supplementary Fig. 3G; FAAH:  $t = 2.033$ ,  $df = 6$ ,  $p = 0.09$ ; Supplementary Fig. 3H; CB1:  $t = 1.202$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 3I; CB2:  $t = 0.241$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 3J). At adulthood, we found a significant reduction in DAGL $\beta$  ( $t = 6.054$ ,  $df = 5$ ,  $p < 0.01$ ; Supplementary Fig. 4L) and TRPV1 ( $t = 5.707$ ,  $df = 6$ ,  $p < 0.01$ ; Supplementary Fig. 4Q) expression in *Fmr1*<sup>Δexon 8</sup> rats compared to WT controls; the other main



**Fig. 3** qPCR analysis of the main components of the ECS in the amygdala of juvenile *Fmr1*<sup>Δexon 8</sup> rats. Fold induction of NAPE-PLD (A), DAGL $\alpha$  (B), DAGL $\beta$  (C), MAGL (D), FAAH (E), CB1 (F), CB2 (G), TRPV1 (H), GPR55 (I) gene expression in the amygdala of *Fmr1*<sup>Δexon 8</sup> rats and WT animals, evaluated at PND 35 (WT = 3–4; *Fmr1*<sup>Δexon 8</sup> = 3–4 animals per group). Data represent mean  $\pm$  SEM, \* $p$  < 0.05 vs WT group (Student's  $t$  test).

cannabinoid enzymes NAPE-PLD ( $t = 1.812$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4J), DAGL $\alpha$  ( $t = 1.403$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4K), MAGL ( $t = 0.084$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4M), FAAH ( $t = 0.959$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4N) and CB1 ( $t = 0.608$ ,  $df = 6$ ,  $p = n.s.$ ; Supplementary Fig. 4O), CB2 ( $t = 1.031$ ,  $df = 5$ ,  $p = n.s.$ ; Supplementary Fig. 4P), and GPR55 ( $t = 1.331$ ,  $df = 5$ ,  $p = n.s.$ ; Supplementary Fig. 4R) receptors were spared.

These results further confirm a discrete dysregulation of the brain ECS in *Fmr1*<sup>Δexon 8</sup> rats across development.

#### Potentiation of AEA tone through systemic administration of URB597 restored the impaired novel object and social discrimination abilities of *Fmr1*<sup>Δexon 8</sup> rats

Systemic administration of URB597 (0.1 mg/kg, i.p., 2 h before testing) rescued the altered discrimination index of juvenile and adult *Fmr1*<sup>Δexon 8</sup> rats in the novel object recognition task (PND 35:  $F_{(\text{genotype})} 1,49 = 12.39$ ,  $p < 0.001$ ;  $F_{(\text{treatment})} 1,49 = 4.289$ ,  $p < 0.05$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,49 = 6.141$ ,  $p < 0.05$ , Fig. 4A; PND 80:  $F_{(\text{genotype})} 1,26 = 24.36$ ,  $p < 0.001$ ;  $F_{(\text{treatment})} 1,26 = 4.269$ ,  $p < 0.05$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,26 = 15.49$ ,  $p < 0.001$ , Fig. 4C) without altering the total time spent in object exploration (PND 35:  $F_{(\text{genotype})} 1,49 = 0.537$ ,  $p = n.s.$ ;  $F_{(\text{treatment})} 1,49 = 0.047$ ,  $p = n.s.$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,49 = 0.451$ ,  $p = n.s.$ , Fig. 4B; PND 80:  $F_{(\text{genotype})} 1,26 = 1.881$ ,  $p = n.s.$ ;  $F_{(\text{treatment})} 1,26 = 4.411$ ,  $p < 0.05$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,26 = 1.136$ ,  $p = n.s.$ , Fig. 4D).

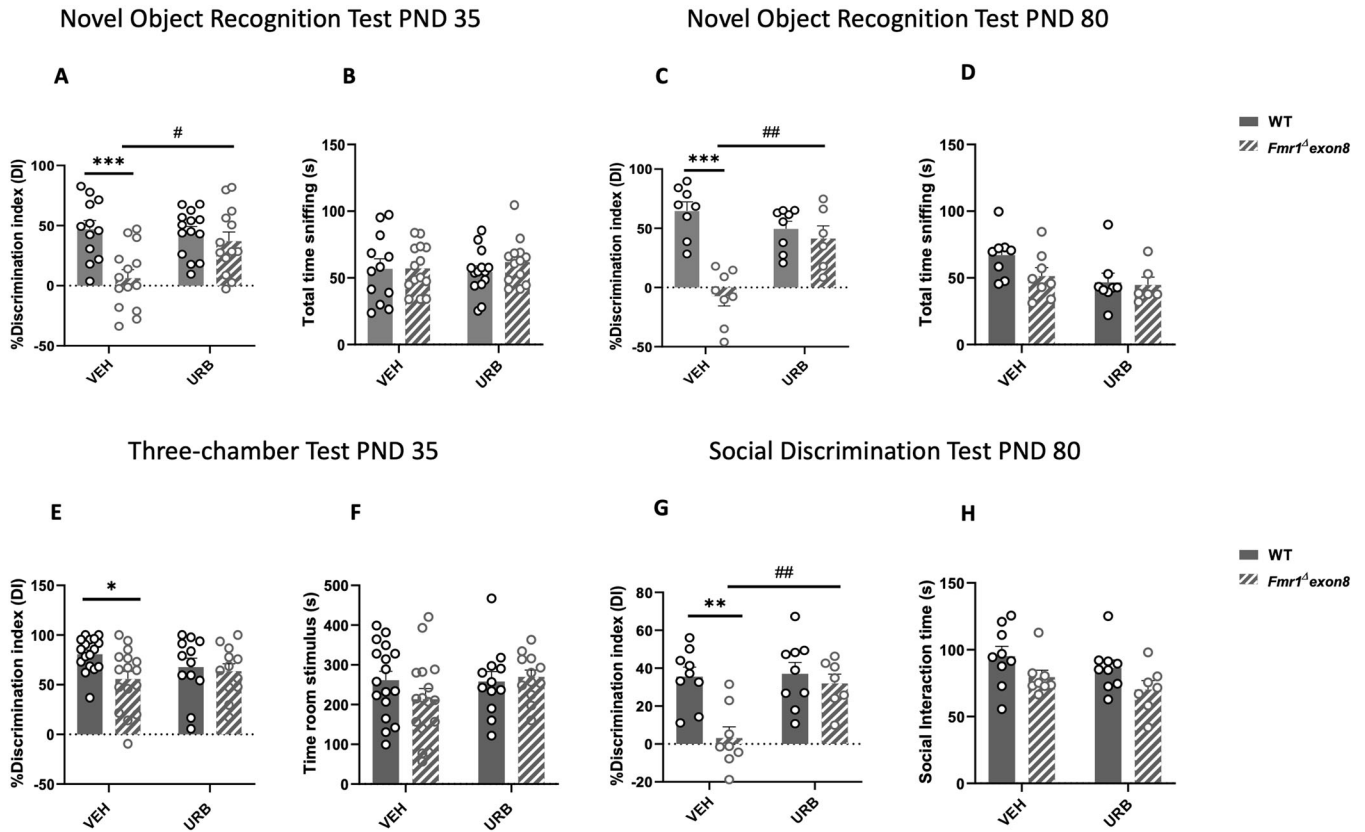
To explore whether potentiation of AEA tone also restored the deficits in sociability displayed by *Fmr1*<sup>Δexon 8</sup> rats [38], we performed a three-chamber test at PND 35 following systemic administration of URB597 (0.1 mg/kg, i.p., 2 h before testing). *Fmr1*<sup>Δexon 8</sup> displayed altered sociability, as they showed a lower discrimination index during testing; this deficit was mitigated but not fully antagonized when *Fmr1*<sup>Δexon 8</sup> rats were treated with URB597 ( $F_{(\text{genotype})} 1,53 = 4.252$ ,  $p < 0.05$ ;  $F_{(\text{treatment})} 1,53 = 0.128$ ,  $p = n.s.$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,53 = 2.212$ ,  $p = n.s.$ , Fig. 4E), as no

differences were found between *Fmr1*<sup>Δexon 8</sup> rats treated with either URB597 or vehicle ( $p = n.s.$ ). URB597 did not affect the time spent in the stimulus room ( $F_{(\text{genotype})} 1,53 = 0.542$ ,  $p = n.s.$ ;  $F_{(\text{treatment})} 1,53 = 1.209$ ,  $p = n.s.$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,53 = 1.544$ ,  $p = n.s.$ , Fig. 4F).

Conversely, at adulthood, URB597 (0.1 mg/kg, i.p., 2 h before testing) rescued the impaired discrimination index of *Fmr1*<sup>Δexon 8</sup> rats while performing the social discrimination test ( $F_{(\text{genotype})} 1,29 = 11.36$ ,  $p < 0.01$ ;  $F_{(\text{treatment})} 1,29 = 7.489$ ,  $p < 0.05$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,29 = 6.073$ ,  $p < 0.05$ , Fig. 4G), without altering the total time spent in general social exploration of WT and *Fmr1*<sup>Δexon 8</sup> rats ( $F_{(\text{genotype})} 1,29 = 6.113$ ,  $p < 0.05$ ;  $F_{(\text{treatment})} 1,29 = 1.968$ ,  $p = n.s.$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,29 = 0.001$ ,  $p = n.s.$ , Fig. 4H). These results indicate that potentiation of AEA tone through systemic administration of URB597 restored the impaired novel object and social discrimination abilities of *Fmr1*<sup>Δexon 8</sup> rats, but only attenuated their impaired sociability in the three-chamber test.

#### Intra-hippocampal potentiation of AEA tone rescued the impaired novel object and social discrimination abilities of *Fmr1*<sup>Δexon 8</sup> rats

To evaluate whether the reduced levels of AEA found in the hippocampus of *Fmr1*<sup>Δexon 8</sup> rats contributed to their impaired short-term object recognition memory, we bilaterally infused the AEA hydrolysis inhibitor URB597 in the hippocampus of juvenile and adult *Fmr1*<sup>Δexon 8</sup> and WT animals immediately after the training phase of the novel object recognition test. URB597 rescued the altered discrimination index of *Fmr1*<sup>Δexon 8</sup> rats (PND 35:  $F_{(\text{genotype})} 1,43 = 8.772$ ,  $p < 0.01$ ;  $F_{(\text{treatment})} 1,43 = 7.517$ ,  $p < 0.01$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,43 = 6.831$ ,  $p < 0.05$ , Fig. 5A; PND 80:  $F_{(\text{genotype})} 1,25 = 6.154$ ,  $p < 0.05$ ;  $F_{(\text{treatment})} 1,25 = 14.48$ ,  $p < 0.001$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,25 = 32.93$ ,  $p < 0.001$ , Fig. 5C), without altering the total time spent exploring the objects during the test phase (PND 35:  $F_{(\text{genotype})} 1,43 = 0.563$ ,  $p = n.s.$ ;  $F_{(\text{treatment})} 1,43 = 0.218$ ,  $p = n.s.$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,43 = 1.051$ ,  $p = n.s.$ , Fig. 5B; PND 80:  $F_{(\text{genotype})} 1,25 = 0.209$ ,  $p = n.s.$ ;  $F_{(\text{treatment})} 1,25 = 6.313$ ,  $p < 0.05$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,25 = 0.153$ ,  $p = n.s.$ , Fig. 5D).



**Fig. 4 Potentiation of AEA tone through systemic administration of URB597 rescued the impaired object and social discrimination abilities of *Fmr1-Δexon 8* rats.** Systemic administration of URB597 (0.1 mg/kg, 2 h prior testing) rescued the altered discrimination index of both juvenile and adult *Fmr1-Δexon 8* rats (**A, C**) without altering their total time spent in object exploration (**B, D**) (PND 35: WT-VEH = 12, WT-URB = 14, *Fmr1-Δexon 8*-VEH = 14, *Fmr1-Δexon 8*-URB = 13; PND 80: WT-VEH = 8, WT-URB = 8, *Fmr1-Δexon 8*-VEH = 8, *Fmr1-Δexon 8*-URB = 6 animals per group). In the three-chamber test, systemic administration of URB597 (0.1 mg/kg, 2 h prior testing) did not rescue but only attenuated the impaired sociability of juvenile *Fmr1-Δexon 8* rats (**E**), as no significant differences were found between *Fmr1-Δexon 8* rats treated with URB597 and WT rats treated with vehicle (WT-VEH = 17, WT-URB = 12, *Fmr1-Δexon 8*-VEH = 16, *Fmr1-Δexon 8*-URB = 12 animals per group). No differences among experimental groups were found in the time spent in the stimulus room (**F**). In the social discrimination test, URB597 normalized the impaired discrimination index of *Fmr1-Δexon 8* adult rats (**G**) without altering the time spent in general social exploration by both WT and *Fmr1-Δexon 8* rats (**H**) (WT-VEH = 9, WT-URB = 9, *Fmr1-Δexon 8*-VEH = 8, *Fmr1-Δexon 8*-URB = 7 animals per group). Data represent means  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs WT-VEH group, # $p < 0.05$ , ## $p < 0.01$  vs *Fmr1-Δexon 8* VEH group (two-way ANOVA with Tukey's *post hoc* test).

Intra-hippocampal infusion of URB597 also normalized the impaired discrimination index of *Fmr1-Δexon 8* adult rats in the social discrimination task ( $F_{(\text{genotype})} 1,41 = 6.448$ ,  $p < 0.05$ ;  $F_{(\text{treatment})} 1,41 = 9.220$ ,  $p < 0.01$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,41 = 4.902$ ,  $p < 0.05$ , Fig. 5E), without affecting the total time spent by the animals in general social exploration ( $F_{(\text{genotype})} 1,41 = 4.074$ ,  $p = \text{n.s.}$ ;  $F_{(\text{treatment})} 1,41 = 0.001$ ,  $p = \text{n.s.}$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,41 = 0.012$ ,  $p = \text{n.s.}$ , Fig. 5F). These results indicate that AEA in the hippocampus is specifically involved in the short-term cognitive deficits displayed by *Fmr1-Δexon 8* rats in the novel object and social discrimination tasks.

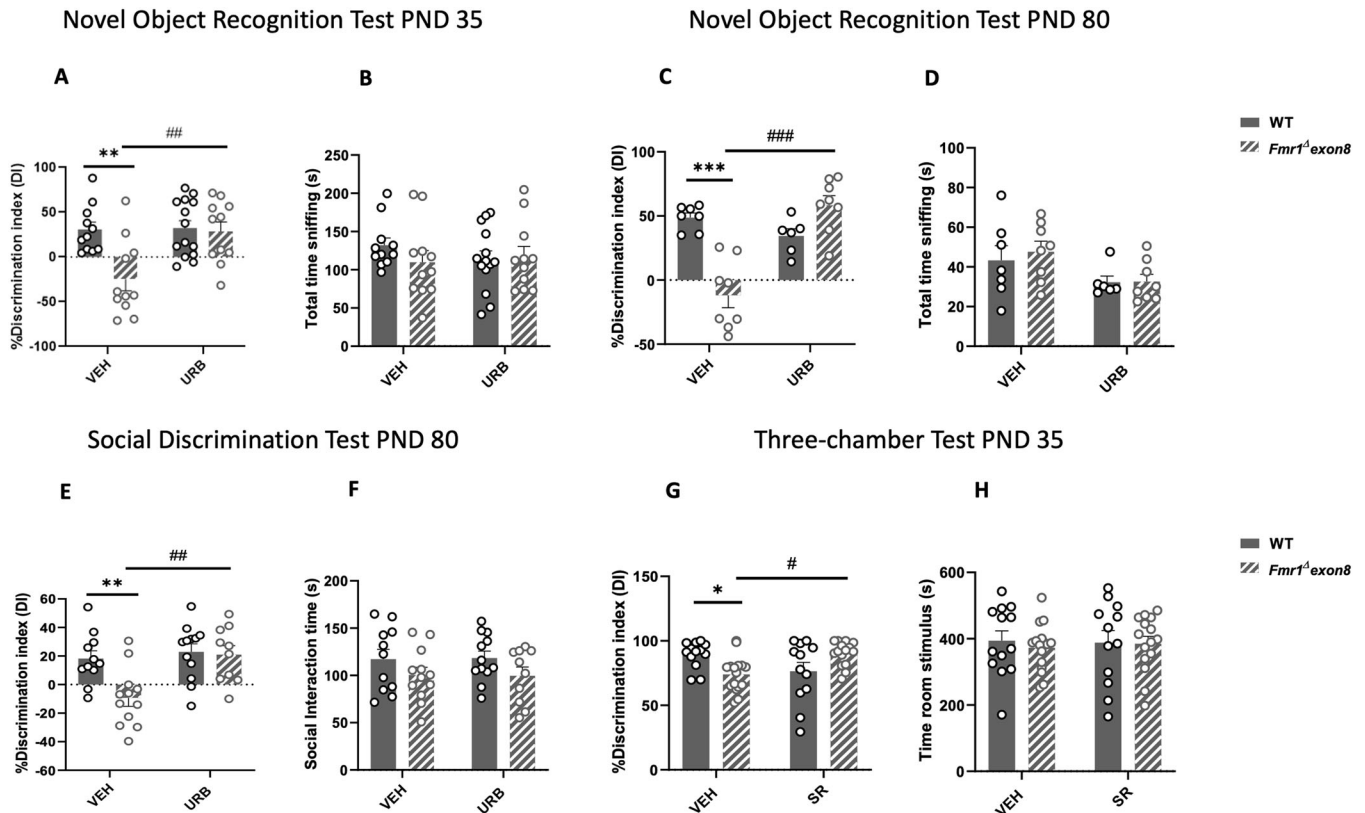
#### CB1 cannabinoid receptors in the amygdala modulate sociability in *Fmr1-Δexon 8* rats

Based on the evidence that *Fmr1-Δexon 8* rats showed abnormal 2-AG levels in the amygdala and that pharmacological/genetic blockade of CB1 receptors normalized some FXS-like traits in the mouse model of FXS [31, 68], we hypothesized that blocking CB1 receptors into the amygdala could restore the impaired sociability displayed by *Fmr1-Δexon 8* rats in the three-chamber test possibly interfering with 2-AG signaling. In line with this possibility, intra-amygdala infusion of SR141716A immediately before the test phase rescued the atypical sociability of *Fmr1-Δexon 8* rats by normalizing their discrimination index ( $F_{(\text{genotype})} 1,52 = 0.036$ ,  $p = \text{n.s.}$ ;

$F_{(\text{treatment})} 1,52 = 0.159$ ,  $p = \text{n.s.}$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,52 = 13.26$ ,  $p < 0.001$ , Fig. 5G), without affecting the time spent in the stimulus room ( $F_{(\text{genotype})} 1,52 = 0.211$ ,  $p = \text{n.s.}$ ;  $F_{(\text{treatment})} 1,52 = 0.014$ ,  $p = \text{n.s.}$ ;  $F_{(\text{genotype} \times \text{treatment})} 1,52 = 0.123$ ,  $p = \text{n.s.}$ , Fig. 5H).

#### DISCUSSION

Our results revealed region-specific changes in endocannabinoid levels in the brain of *Fmr1-Δexon 8* rats, i.e., reduced levels of AEA in the hippocampus and increased levels of 2-AG into the amygdala, together with discrete alterations in the expression of the enzymes modulating synthesis/degradation of AEA and 2-AG, reinforcing the idea that brain region-specific changes in endocannabinoid signaling contribute to the altered molecular, functional and behavioral profile that characterize this genetic rat model of ASD. Systemic injection and intra-hippocampal infusion of the AEA hydrolysis inhibitor URB597 rescued the aberrant cognitive performance of *Fmr1-Δexon 8* rats in the novel object and social discrimination tasks, suggesting that the hippocampus is an important brain area where reduced AEA tone contributes to the short-term cognitive deficits displayed by *Fmr1-Δexon 8* rats. Conversely, blockade of CB1 cannabinoid receptors in the amygdala by the antagonist/inverse agonist SR141716A rescued the altered sociability of *Fmr1-Δexon 8* rats in the three-chamber



**Fig. 5** Intra-hippocampal potentiation of AEA tone rescued the impaired object and social discrimination abilities of *Fmr1*<sup>Δexon 8</sup> rats, while blockade of CB1 cannabinoid receptors in the amygdala restored their altered sociability. The bilateral infusion of URB597 (10 ng/0.5 μl) in the hippocampus rescued the altered discrimination index of *Fmr1*<sup>Δexon 8</sup> juvenile and adult rats in the novel object recognition test (A, C), without altering the total time spent in object exploration by both juvenile and adult WT and *Fmr1*<sup>Δexon 8</sup> rats (B, D) (PND 35: WT-VEH = 11, WT-URB = 14, *Fmr1*<sup>Δexon 8</sup>-VEH = 11, *Fmr1*<sup>Δexon 8</sup>-URB = 11; PND 80: WT-VEH = 7, WT-URB = 6, *Fmr1*<sup>Δexon 8</sup>-VEH = 8, *Fmr1*<sup>Δexon 8</sup>-URB = 8 animals per group). In the social discrimination task, hippocampal infusion of URB597 (10 ng/0.5 μl) normalized the impaired discrimination index of *Fmr1*<sup>Δexon 8</sup> adult rats (E) without altering the time spent in social exploration by both WT and *Fmr1*<sup>Δexon 8</sup> rats (F) (WT-VEH = 11, WT-URB = 12, *Fmr1*<sup>Δexon 8</sup>-VEH = 10, *Fmr1*<sup>Δexon 8</sup>-URB = 12 animals per group). Bilateral infusion of SR141716A (0.1 μg/0.2 μl) in the amygdala rescued the atypical sociability of *Fmr1*<sup>Δexon 8</sup> rats normalizing their discrimination index (G). Conversely, no differences among groups were found in the time spent in the stimulus room (H) (WT-VEH = 13, WT-URB = 12, *Fmr1*<sup>Δexon 8</sup>-VEH = 16, *Fmr1*<sup>Δexon 8</sup>-URB = 15 animals per group). Data represent means ± SEM, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs WT-VEH group, #*p* < 0.01, ##*p* < 0.01, ###*p* < 0.001 vs *Fmr1*<sup>Δexon 8</sup>-VEH group (two-way ANOVA with Tukey's post hoc test).

test, indicating that the amygdala is likely a prominent site of action for 2-AG to underlie the altered sociability of *Fmr1*<sup>Δexon 8</sup> rats.

We have shown that *Fmr1*<sup>Δexon 8</sup> rats display cognitive, communicative and social impairments [38], 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. Converging evidence suggested a region-specific alteration of the ECS in the brain of *Fmr1* KO mice [31, 32, 34, 69]. For instance, alterations in mGluR5 and DAGLα coupling led to disruption of mGluR5-mediated production of 2-AG in the ventral striatum and prefrontal cortex of *Fmr1* KO mice [32, 70, 71], together with decreased 2-AG levels in the hippocampus [34]. On the other hand, pioneering studies in *Fmr1* KO mice reported no differences in brain levels of 2-AG and AEA or in the hippocampal expression of several components of the ECS [69, 72]. For the first time, we here found that *Fmr1*<sup>Δexon 8</sup> rats displayed reduced basal AEA levels in the hippocampus and increased 2-AG levels into the amygdala, suggesting that an imbalance of endocannabinoid signaling in selected brain regions such as the hippocampus and amygdala could drive ASD-like behavioral features in these animals. Interestingly, *Fmr1*<sup>Δexon 8</sup> rats showed an increase in the hydrolytic activity of FAAH compared to WT controls at juvenile age. This led us to

hypothesize that the decreased AEA levels in the hippocampus of juvenile *Fmr1*<sup>Δexon 8</sup> rats could be due to an increased FAAH activity. Concerning the amygdala, we found a decrease in the expression of the enzyme MAGL, that could correlate to the increased levels of 2-AG we found in the amygdala of juvenile *Fmr1*<sup>Δexon 8</sup> rats. Interestingly, these discrete alterations in the main endocannabinoid synthesis/degradation enzymes appeared consistent across development.

The hippocampus and amygdala have a key role in the modulation of cognitive performance and emotional states, which are profoundly impaired in FXS patients [7, 73–76]. We found that increasing AEA levels via systemic administration of the selective FAAH inhibitor URB597 corrected the impaired short-term memory in the novel object recognition task in juvenile and adult *Fmr1*<sup>Δexon 8</sup> rats, as well as their impaired social discrimination in the social discrimination test. These tasks allow to assess the discriminative abilities of rodents respectively referred to object and social recognition and are based on the innate preference of rodents for exploring objects or congeners instead of re-exploring something they have already been exposed to, referred to as familiar. The ability to recognize familiar objects and social conspecifics in rodents relies on hippocampal integrity [77–80], thus suggesting the critical role of this brain region in recognition memory. Notably, in the social discrimination experiments, we

tested adult rats after 7 days of individual housing, which is much shorter than the prolonged social isolation periods causing a dysregulation of the ECS [81–84]. Compared to its beneficial effects on short-term object and social recognition deficits, we found that URB597 only attenuated the impaired sociability displayed by juvenile *Fmr1*<sup>-Δ</sup>*exon 8* rats in the three-chamber task. For the first time, these results suggest that AEA is specifically involved in the short-term memory (for either a novel object or a social partner) deficits displayed by *Fmr1*<sup>-Δ</sup>*exon 8* rats, thus suggesting the potential of AEA signaling as an endogenous cannabinoid target for the short-term recognition memory deficits observed in this genetic rat model of ASD. Since juvenile *Fmr1*<sup>-Δ</sup>*exon 8* rats displayed reduced AEA levels in the hippocampus, together with increased FAAH activity, we hypothesized that intra-hippocampal infusion of URB597 would restore AEA levels and in turn rescue the short-term cognitive deficits shown by these animals. In line with this, hippocampal infusion of URB597 rescued the impaired short-term memory of *Fmr1*<sup>-Δ</sup>*exon 8* rats in the novel object and social discrimination tasks, thus supporting the idea that their alteration of object and social recognition involves hippocampal AEA signaling. Although we did not directly measure the levels of AEA following URB597 administration, extensive literature evidence shows that pharmacological inhibition of FAAH enhances AEA levels and reverses the behavioral deficits observed in different animal models of ASD [67, 85–91]. Thus, it is reasonable to hypothesize that URB597 administration had beneficial effects in *Fmr1*<sup>-Δ</sup>*exon 8* rats by increasing AEA levels. The hypothesis that dysregulated AEA signaling in the hippocampus could account for the altered object and social discrimination displayed by *Fmr1*<sup>-Δ</sup>*exon 8* rats is supported by several studies indicating that AEA exerts an overall modulatory effect in hippocampal-mediated cognitive processes in both physiological and pathological conditions [50, 61, 92–96]. It should be noted, however, that we here investigated only a short-term form of memory (i.e., with a 30 min inter-trial interval); therefore, testing the effects of URB597 on potential long-term memory deficits in *Fmr1*<sup>-Δ</sup>*exon 8* rats remains an interesting point that deserves further investigation.

As for the putative mechanisms involved, the positive effect of intra-hippocampal infusion of URB597 in *Fmr1*<sup>-Δ</sup>*exon 8* rats likely relates to an association between the ECS and FMRP loss, as it has previously been shown in the hippocampus of *Fmr1* KO mice [97]. Thus, it is tempting to speculate that the loss of FMRP function in *Fmr1*<sup>-Δ</sup>*exon 8* rats could produce diminished CB1-mediated signaling driven by compromised hippocampal AEA, resulting in short-term recognition memory impairment. Despite there is consistent evidence suggesting that CB1 cannabinoid receptors mediate the beneficial effects of FAAH inhibitors in animal models of ASD and FXS [67, 88, 91, 98, 99], the putative mechanisms involved still remain to be fully elucidated. For instance, our results did not show any significant difference in the expression of the main cannabinoid receptors (i.e., CB1, CB2, TRPV1, GPR55) at the juvenile age, except for TRPV1 expression in the adult hippocampus. Since AEA also activates TRPV1 receptors [100], one can argue that the effects of URB597 could be mediated by this receptor, at least at adulthood. However, this remains an intriguing issue that needs to be further addressed.

Persistent deficits in social communication and social interaction are common key features of both ASD and FXS [7] and we have recently shown (and here confirmed) that *Fmr1*<sup>-Δ</sup>*exon 8* rats display altered sociability in the three-chamber task [38]. Together with AEA, the other main endocannabinoid 2-AG has a significant role in social behavior [101, 102]. Interestingly, increasing 2-AG levels augments social play in rats [63, 103, 104], while reducing 2-AG levels decreases acquisition of social conditioned place preference in mice [105]. Previous work has demonstrated that activation of CB1 cannabinoid receptors by endocannabinoids, including 2-AG, has a functional role in social behavior via

modulation of both nucleus accumbens signaling and basolateral amygdala-nucleus accumbens activity [55, 63, 105, 106]. Based on the finding that juvenile *Fmr1*<sup>-Δ</sup>*exon 8* rats showed abnormal 2-AG levels in the amygdala, together with a reduction in the expression of MAGL enzyme, and on evidence showing that pharmacological or genetic blockade of CB1 cannabinoid receptors normalized some traits of FXS [31, 68], we hypothesized that blocking CB1 receptors into the amygdala could restore the impaired sociability displayed by *Fmr1*<sup>-Δ</sup>*exon 8* rats possibly interfering with 2-AG signaling. Interestingly, we found that infusion of the CB1 antagonist/inverse agonist SR141716A in the basolateral amygdala of *Fmr1*<sup>-Δ</sup>*exon 8* rats rescued their atypical performance in the three-chamber test. This supports the hypothesis that excessive 2-AG signaling in the amygdala, possibly due to the decreased expression of MAGL enzyme as revealed by the qPCR experiments, contributes to the impaired social function seen in these rats. In line with our findings, recent evidence showed that basolateral amygdala-nucleus accumbens circuit activation reduces sociability and it is highly regulated by 2-AG-mediated endocannabinoid signaling [107]: thus, optogenetic activation of the basolateral amygdala-nucleus accumbens glutamatergic circuit decreases sociability in the three-chamber task, increases social avoidance and reduces social interaction seeking in Shank3 mutant mice displaying ASD-like behaviors, via 2-AG signaling. These findings reinforce the idea that region-specific alterations in brain endocannabinoid signaling affect different facets of the behavioral profile of this genetic rat model of ASD, and further support FXS-related dysfunction in the amygdala [108–110], moving beyond the idea of the pathogenesis of FXS based primarily on studies focused in the hippocampus and neocortex.

Overall, our results indicate the multimodal role of AEA and 2-AG signaling in the autistic-like traits observed in a genetic model of autism based on FMR1 deletion in rats. We hypothesize that impairment in AEA signaling in the hippocampus, possibly as a consequence of an increased FAAH activity, mediates short-term novel object and social discrimination deficits in *Fmr1*<sup>-Δ</sup>*exon 8* rats, whereas exaggerated 2-AG signaling in the amygdala underlies their altered sociability through excessive activation of CB1 cannabinoid receptors. These findings not only provide clarification of the underlying molecular mechanisms of AEA and 2-AG in ASD-related socio-cognitive impairment, but also indicate that any assessment of potential pharmacological manipulations targeting endocannabinoid neurotransmission in ASD should consider that these compounds may have brain region- and behaviorally-specific effects.

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### AUTHOR CONTRIBUTIONS

SS and AM performed, analyzed, and contributed to the design of the behavioral experiments. MM and MNH performed and analyzed the biochemical experiments to measure endocannabinoid levels. EC, AR, AF, and FA performed the qPCR and Western blotting experiments. EC, VB, and PC contributed to the behavioral experiments. SS, AM, and VT wrote the paper. VT supervised the project, designed the experiments, and wrote, revised, and edited the paper. All authors contributed to the paper and approved the submitted version.

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### COMPETING INTERESTS

The authors declare no competing interests.

### ADDITIONAL INFORMATION

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