# A serotonergic mechanism is involved in the pro-cognitive effect of AM6545 treatment in a mouse model of fragile X syndrome

Lucía de los Reyes-Ramírez[#1,2], Araceli Bergadà-Martínez[#1], Sara Martínez-Torres[1], Irene Martínez-Gallego[3], Pilar Losada-Ruiz[3], Silvia Arboleya[4], Miguel Gueimonde[4], Rafael Maldonado[1,5], Antonio Rodríguez-Moreno[3], Andrés Ozaita[*1,2]

**Affiliations**

[1]Laboratory of Neuropharmacology-NeuroPhar, Department of Medicine and Life Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

[2]Research group in Biology of Cognition, Department of Medicine and Life Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

[3]Laboratory of Cellular Neuroscience and Plasticity, Department of Physiology, Anatomy and Cell Biology, University Pablo de Olavide, Seville, Spain.

[4]Department of Microbiology and Biochemistry of Dairy Products, Instituto de Productos Lácteos de Asturias, Spanish Research Council (IPLA-CSIC), Oviedo, Spain.

[5]IMIM Hospital del Mar Research Institute, Barcelona, Spain.

[#]Equal contribution

*Corresponding author: Andrés Ozaita, Grup de Recerca en Biologia de la Cognició, Facultat de Medicina i Ciències de la Vida, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, C/Doctor Aiguader 88, 08003 Barcelona, Spain. Phone: +34-93-3160823; Fax: + 34-93-3160901; e-mail: andres.ozaita@upf.edu.

**Abstract**

Fragile X syndrome (FXS) is the principal monogenic syndrome leading to inherited intellectual disability and autism. It is caused by the silencing of *FMR1* gene that leads to the loss in the expression of its encoded protein, the fragile X messenger ribonucleoprotein 1 (FMRP). In synapses, FMRP has a key role in local mRNA modulation to maintain synaptic plasticity. The *Fmr1* KO (FX) mouse model shows cognitive impairment and some of the synaptic traits present in individuals with FXS together with alterations in gut microbiota. Previous studies revealed that pharmacological and genetic cannabinoid type-1 receptor (CB1R) inhibition significantly prevented central key alterations in FX mice. Here, we aimed to evaluate the effect of a sub-chronic treatment with the largely peripherally-restricted CB1R antagonist AM6545. We found that AM6545 reduced memory deficits and restored enhanced hippocampal mGluR5-dependent long-term depression and aberrant dendritic spine density in FX mice. At the peripheral level, AM6545 modified altered FX mice fecal microbiota composition, while in the hippocampus AM6545 treatment upregulated hippocampal 5-HT4 receptor (5-HT4R). 5-HT4R upregulation positively correlated with memory performance. Notably, acute pharmacological blockade of 5-HT4R abolished the pro-cognitive effect produced by AM6545. Together, our results suggest that peripheral CB1R inhibition ameliorates key alterations in FX mouse model and modifies the expression of serotonergic receptors important for cognitive performance.

## Introduction

Fragile X syndrome (FXS) is the most common monogenic origin of inherited intellectual disability and autism spectrum disorder (ASD) [1]. It is caused by a trinucleotide CGG expansion in the 5' UTR of the *FMR1* gene, which leads to the loss of expression of the fragile X messenger ribonucleoprotein 1 (FMRP) [2]. People with FXS present heterogeneous symptoms, including memory impairment, hyperactivity, impulsivity or autistic-like behavior [3].

The *Fmr1* KO (FX) mouse model recapitulates the main traits of the disorder [4]. Notably, an enhanced uncontrolled activity of the metabotropic receptor mGluR5, revealed by an enhanced mGluR5-dependent long-term depression (LTD) has been described in FX mice [5,6], as well as alterations in dendritic spine density and morphology with an increase in the immature dendritic spines [7,8]. In addition to these neuronal traits, the FX mice show gut microbiota dysbiosis [9].

The endocannabinoid system is a key modulator of synaptic plasticity and cognitive function [10], two factors that are affected in FXS. It was previously reported that systemic cannabinoid type-1 receptor (CB1R) inhibition using genetic or pharmacological approaches ameliorated cognitive deficits in FX mice, as well as the aberrant mGluR5-LTD and the altered CA1 spine density in the hippocampus [7,11]. However, systemic CB1R inhibition was found to induce psychiatric side effects when used as an anti-obesity treatment [12]. To avoid these hypothetical central adverse effects of CB1R inhibition, diverse strategies have been developed including CB1R peripherally-restricted antagonists and allosteric modulators. We recently demonstrated that AM6545, a peripherally-restricted CB1R antagonist, enhances memory performance in naïve mice

[13,14]. Therefore, we aimed to explore the potential beneficial effects of AM6545 in the alleviation of key alterations of FX mice.

The present study shows that AM6545 treatment ameliorates object-recognition memory and improves cellular and structural alterations in FX mice. Furthermore, we described a decrease in *Akkermansia* and *Lactobacillus* genera in FX that increase after AM6545 treatment. Interestingly, we found that the upregulation of serotonin receptor 4 in FX mice after the treatment is needed for AM6545 mnemonic effect. Together, this evidence undisclosed the inhibition of peripheral CB1R as an approach to alleviate the cognitive deficits in a mouse model for FXS through peripheral noradrenergic and serotonergic mechanisms.

## Materials and methods

### *Ethics*

All animal procedures were conducted following "Animals in Research: Reporting Experiments" (ARRIVE) guidelines and standard ethical guidelines [15,16] (European Directive 2010/63/EU), and were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB).

### *Animals*

FX mice in Friend Virus B background (*Fmr1* KO, FVB.129P2-Pde6b+Tyrc-chFmr1tm1Cgr/J) and wild-type mice (WT, FVB.129P2-Pde6b+Tyrc-ch/AntJ) were purchased from The Jackson Laboratory and crossed to obtain outbred FX and WT littermates. FX mice in C57BL/6J congenic background (B6.129P2-Fmr1tm1Cgr/J) were obtained from the Baylor College of Medicine Mouse Facility and crossed to generate inbred FX and WT littermates. The transgenic line Tg(Thy1-EGFP) MJrs/J (Thy1-EGFP mice) (Stock #007788, The Jackson Laboratories, USA) crossed to FX mice in C57BL/6J background was used to study the density and morphology of dendritic spines. Young adult male mice (12-14 weeks) used in this study were housed in Plexiglas cages (2-4 mice per cage) and maintained in temperature (21 ± 1°C) humidity (55 ± 10%) and light (light on at 8 am; light off at 8 pm) controlled environment. Food and water were available *ad libitum*. All the experiments were performed during the light phase after animals were handled for 1 week and habituated to the experimental room. All behavioral experiments were conducted by an observer blind to experimental conditions.

### *Drugs and treatments*

AM6545 (1 mg/kg) from Tocris-Bio-Techne was dissolved in 0.26% DMSO, 4.74% ethanol, 5% cremophor-EL and 90% saline. Sotalol (10 mg/kg) from Sigma-Aldrich was

dissolved in saline [13]. GR125487 (10 mg/kg) from Tocris-Bio-Techne was dissolved in saline. All drugs were injected intraperitoneally (i.p.) in a volume of 10 ml/kg of body weight for acute or sub-chronic (7d) treatment.

***Novel object-recognition test***

NORT was performed as previously described [13]. For sub-chronic treatment, the last AM6545 administration was performed after the familiarization phase (Supplementary Fig. 1A). Sotalol was administered acutely after the familiarization phase and, when required, 20 min before AM6545 acute administration (Supplementary Fig. 1B). GR125487 was administered acutely after the familiarization phase and, when required, 20 min before the last AM6545 administration (Supplementary Fig. 1C).

***Slice preparation for electrophysiological recordings***

Twenty-four hours after the last administration of AM6545 (1 mg/kg, 7d, i.p.), WT and FX mice were anesthetized with isoflurane (5%) and decapitated for slice preparation. Hippocampal slices were prepared as described in detail elsewhere [17–19].

***Electrophysiological recordings***

Whole-cell patch-clamp recordings were made from pyramidal cells located in the CA1 field of the hippocampus. CA1 pyramidal cells were patched under visual guidance by infrared differential interference contrast microscopy and verified to be pyramidal neurons by their characteristic voltage response to a current step protocol. Neurons were recorded in either voltage-clamp configuration with a patchclamp amplifier (Multiclamp 700B), and data were acquired using pCLAMP 10.2 software (Molecular Devices). Patch electrodes were pulled from borosilicate glass tubing and had a resistance of 4–7 MΩ when filled with (in mM) CsCl, 120; HEPES, 10; NaCl, 8; MgCl2, 1; CaCl2, 0.2; EGTA, 2; and QX-314, 20 (290 mOs/ml, pH 7.2–7.3). Cells were excluded from analysis if the

series resistance changed by more than 15% during the recording. Recordings were low-pass filtered at 3 kHz and acquired at 10 kHz. mGluR5-LTD was induced by treating slices with 3,5-dihydroxyphenylglycerine (DHPG) (100 μM) for 10 min. LTD was quantified by comparing the mean EPSC amplitude over the 30 min post-drug period with the mean EPSC amplitude during the baseline period and calculating the percentage change from 5 last min.

***Brain slice preparation for dendritic spine analysis***

Twenty-four hours after the last administration of AM6545 (1 mg/kg, 7d, i.p.), mice were deeply anesthetized by i.p. injection (0.2 ml/10 g of body weight) of a mixture of ketamine/xylazine (100 mg/kg and 20 mg/kg, respectively) prior to intracardiac perfusion of cold 4% paraformaldehyde. Brains were removed and stored in a solution of 30% sucrose at 4°C. Coronal frozen sections were made at 60 μm on a freezing microtome (Leica) and mounted for image acquisition.

***Dendritic spine analysis***

Secondary and tertiary apical dendrites of pyramidal neurons from the *stratum radiatum* of CA1 region of the dorsal hippocampus were analyzed. Images were acquired with a confocal microscope (TCS SP5 STED Leica) using a glycerol immersion lens plus 3 times magnification (63X/3) with a sequential line scan at 1024 × 1024 pixel resolution. Serial optical sections were acquired with a 0.13 μm step size. A minimum of 8 dendrites per animal were selected from 4 different slices. A maximum of 2 dendrites were selected from the same neuron. Confocal images were deconvoluted using Huygens Essential software and dendritic spines were analyzed and classified using the semiautomated software NeuronStudio with the described parameters (Supplementary Table 1). Spine density was calculated by expressing the average number of spines in a 10 μm portion of

the dendrite. Each spine was checked manually by an observer blind to experimental conditions for unbiased classification

***Sample preparation and 16S rRNA gene profiling of fecal microbiota analysis***

Fresh fecal samples were collected from each experimental group (Supplementary Table 2), and stored at -80°C until used. Fecal pellets were subsequently diluted 1/10 (w/v) in phosphate buffer saline and vigorously homogenized by vortexing. Cell pellet were isolated by centrifugation at 10,000 rpm for 15 min and, used for bacterial DNA isolation by using QIAamp DNA stool kit (Qiagen, GmbH, Germany) following manufacturer's instructions, including a bead-beating step in a Fastprep (MP Biomedicals, Thermo Fisher Scientific Inc.). The isolated DNA was used as template for partial sequencing (hypervariable V3-V4 region) of the *16S* rRNA gene on the Illumina platform following in-house protocols (Novogene Co.) [20]. Data from *16S* profiling gut microbiota were total sum scaling and cumulative sum scaling normalization to account for the non-normal distribution of taxonomic count data for diversity and abundance analyses, respectively [21]. α-diversity was studied by Chao 1 and Shannon indexes calculated with *QIIME*.

***Tissue preparation for mRNA and biochemical analysis***

Mice were treated with AM6545 (1 mg/kg, 7d, i.p.) or vehicle. Twenty-four hours after the last administration, mice were euthanized by cervical dislocation and hippocampal tissues were dissected on ice, frozen on dry ice and stored at -80°C until used.

***Synaptoneurosome preparation for RNA extraction***

Synaptoneurosome enriched fractions were obtained as previously described [22]. RNA extraction from synaptoneurosomes was performed using RNeasy Mini kit (74140 QIAGEN) according to the manufacturer's instructions. RNA concentration and integrity was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies).

***RNA-seq analysis***

RNA libraries from hippocampal synaptoneurosomes were obtained and processed as described in detail elsewhere [14]. Raw-data is available at GSE278037.

Differential gene expression (DGE) between experimental groups (Supplementary Table 2) was analyzed using *DESeq2* (v1.30) [23] with the cut-off set on |log2fold change| > 0.2 and *p-value* < 0.1 after Wald test correction procedure.

***Reverse transcription and quantitative real-time PCR analysis***

Reverse transcription and quantitative real-time PCR, were performed for hippocampal synaptoneurosomes RNA as previously described [14].

Quantification was performed using the comparative CT Method, relative expression was normalized to *18S* and the fold change was calculated using the 2(–ΔΔCt) formula [24]. The following primers were used: *Htr4*F - TGGAACAACATCGGCATAGTTGA and *Htr4*R - GTGATGGCATAGGGCTTGTTGAC [25], and *18S*F - GTAACCCGTTGAACCCCATT and *18S*R - CCATCCAATCGGTAGTAGCG [26].

***Immunoblot analysis***

For immunoblot of bulk hippocampal homogenates, frozen hippocampal tissues were prepared as described before [27]. The primary antibodies used were anti-5HT4R (rabbit, 1:1,000, ab60359, Abcam) and anti-GAPDH (mouse, 1:20,000, sc-32233, Santa Cruz Biotechnology). Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit (1:15,000, 7074S, Cell Signaling Technology) or anti-mouse (1:15,000, 7076S, Cell Signaling Technology) and visualized by enhanced chemiluminescence detection (Luminata Forte, Merck). Optical densities were quantified by *The Quantity One* software (v4.6.9) (Bio-Rad). after acquisition on ChemiDoc XRS System (Bio-Rad) and were normalized to GAPDH values in the same sample, expressed as a percentage of treatment control (FX-VEH).

***Statistical analysis***

Data analyses were performed using GraphPad Prism 8, RStudio (v4.0), MicrobiomeAnalyst and IBM SPSS (v26.0) software. Statistical comparisons were evaluated using unpaired Student's t-test for two groups comparison, Mann-Whitney U test for two groups comparisons in microbiota experiments (non-normally distributed data) or two-way ANOVA for multiple comparisons. Subsequent Bonferroni *post hoc* was used when required (significant interaction between factors). All results were expressed as mean ± s.e.m. Comparisons were considered statistically significant when $p < 0.05$.

## Results

### *AM6545 treatment ameliorate memory deficits in FX mice through a peripheral noradrenergic mechanism*

First, we tested whether sub-chronic AM6545 treatment could improve memory deficits in FX mice. WT and FX mice received the last administration of a sub-chronic treatment with AM6545 (1 mg/kg, 7d) immediately after the familiarization phase in the NORT and the test phase was performed 24h later. AM6545 ameliorated cognitive impairment in FX mice both in the FVB.129 (two-way ANOVA, interaction: $F(1,38) = 4.723$, $p = 0.036$; *post hoc* Bonferroni, WT-VEH *vs.* FX-VEH $p = 0.005$; FX-VEH *vs.* FX-AM6545 $p = 0.001$) (Fig. 1A) and the C57BL/6J genetic background (two-way ANOVA, interaction: $F(1,40) = 10.49$, $p = 0.002$; *post hoc* Bonferroni, WT-VEH *vs.* FX-VEH $p = 0.0004$; FX-VEH *vs.* FX-AM6545 $p = 0.0004$) (Fig. 1B), reinforcing the significance of the pro-cognitive effect in both backgrounds.

Since we previously demonstrated that AM6545 improves memory persistence through a peripheral adrenergic mechanism in naïve mice [13], we explored whether such a mechanism could also be involved in AM6545-treated FX mice. We found that the acute mnemonic effect of AM6545 was inhibited by acute pre-treatment with the peripherally-restricted β-adrenergic antagonist sotalol (10 mg/kg) (two-way ANOVA, interaction: $F(1,28) = 34.05$, $p = 0.000003$; *post hoc* Bonferroni, FX-saline-AM6545 *vs.* FX-sotalol-AM6545 $p < 0.0001$) (Fig. 1C), pointing to a peripheral adrenergic mechanism in the pro-cognitive effect of AM6545 in FX mice. No differences in total exploration time were observed between pharmacological treatments or genotypes (Supplementary Fig. 2) discarding confounding effects on exploratory behavior between conditions. Thus, sub-chronic AM6545 treatment prevents the cognitive deficits observed in FX mice through a peripheral noradrenergic mechanism.

***Sub-chronic AM6545 treatment restores cellular alterations in FX hippocampus***

The functional and structural synaptic alterations in FX mice that contribute to impairments in synaptic plasticity include aberrant mGluR5-LTD [5] and alterations in dendritic spine structure [28]. First, to test whether AM6545 treatment would restore the elevated hippocampal mGluR5-LTD, WT and FX mice were treated with AM6545 (1 mg/kg, 7d) or vehicle. The last AM6545 administration was performed 24h before brain extraction and hippocampal slice preparation for electrophysiological recording. After 10 min of baseline recording, bath application of DHPG (100 μM, 10 min) induced a transient acute depression, which is considered an electrophysiological readout of group I mGluR activation, followed by a small LTD in WT mice (Fig. 2A). However, we observed that the LTD induced by DHPG was significantly enhanced in vehicle-treated FX mice, which is consistent with previous reports on functional alterations in FXS [29]. Interestingly, we found that sub-chronic AM6545 treatment significantly ameliorated the enhanced mGluR5-LTD in hippocampal slices of FX mice (two-way ANOVA, interaction: $F(1,23) = 4.769$ $p = 0.043$; *post hoc* Bonferroni, WT-VEH *vs.* FX-VEH $p = 0.002$; FX-VEH *vs.* FX-AM6545 $p = 0.003$) (Fig. 2A-B).

Then, taking advantage of FX transgenic mice expressing the EGFP fluorescent protein under neuron-specific Thy1 promoter (Thy1-EGFP mice), we analyzed dendritic spine density and morphology of apical dendrites from CA1 24h after the last administration of sub-chronic AM6545 treatment. Vehicle-treated FX mice showed an increase in dendritic spine density compared to their WT littermates, that was normalized after sub-chronic AM6545 treatment (two-way ANOVA, interaction: $F(1,120) = 7.728$ $p = 0.0063$; *post hoc* Bonferroni, WT-VEH *vs.* FX-VEH $p < 0.000001$; FX-VEH *vs.* FX-AM6545 $p = 0.00034$) (Fig. 2C). Finally, we further classified the spines based on their morphology. We found that vehicle-treated FX mice presented an elevated density of thin (immature) spines that

was restored after sub-chronic AM6545 treatment (two-way ANOVA, interaction: $F(6,452) = 5.553$ $p = 0.000014$; *post hoc* Bonferroni, WT-VEH *vs.* FX-VEH $p < 0.0001$; FX-VEH *vs.* FX-AM6545 $p = 0.0010$) (Fig. 2D). Therefore, sub-chronic AM6545 treatment normalizes the aberrant mGluR5-dependent LTD and dendritic spine density and morphology of CA1 pyramidal neurons in FX mice.

***Fecal microbiome of FX mice is sensitive to AM6545 treatment***

To explore whether AM6545 could modify the gut microbiome, we investigated fecal microbiota in FX mice, and whether sub-chronic AM6545 treatment would modify fecal microbiota composition. To this end, we performed *16S* rRNA gene amplicon sequencing of feces samples in the same cohort of mice before treatment and after vehicle or AM6545 administration and analyzed the changes related either to the genotype or the treatment by paired comparison (Supplementary Table 2). In terms of structural differences, no major changes were observed in α-diversity between any of the groups analyzed (Supplementary Fig. 3).

We observed differences in fecal microbiome composition between WT and FX mice in basal conditions, such as the reduction in *Lactobacillus* genus (Fig. 3A, Supplementary Table 3). Then, after behavioral experiments and sub-chronic vehicle administration were performed, we found more changes between genotypes than in basal conditions. These changes included the reduction in the relative abundance of *Akkermansia* genus in vehicle-treated FX mice (Fig. 3B, Supplementary Table 4) compared to vehicle-treated WT mice.

Interestingly, sub-chronic AM6545 treatment results in an increase in bacteria belonging to both *Akkermansia* and *Lactobacillus* genera (Fig. 3C, Supplementary Table 5);

pointing to the ability of AM6545 treatment to restore microbiota alterations observed in FX mice.

***Serotonin receptor 4 is upregulated in FX hippocampus after sub-chronic AM6545 treatment***

To explore more in depth the synaptic impact of AM6545 in the context of FXS, we performed mRNA transcriptome analysis of hippocampal synaptoneurosomes. We treated FX mice for 7d with AM6545 or vehicle and obtained hippocampal samples 24h after the last AM6545 administration, just after the test phase of the NORT. DGE analysis revealed 7 genes significantly upregulated in FX mice treated with AM6545, whereas only *Dcp2*, a key component of mRNA decapping complex found in P-bodies [30], was downregulated (Fig. 4A, Supplementary Table 6).

Among the upregulated genes, we focused on *Htr4* for further molecular validation at both, mRNA and protein level, since *Htr4*/5-HT4R expression/function is associated with hippocampal plasticity and processing of information [31]. We used an independent cohort of FX mice sub-chronically treated with AM6545 or vehicle, to validate the *in silico* result. We found, using qRT-PCR, that *Htr4* was upregulated in hippocampal synaptoneurosomes from AM6545-treated FX mice (Student's t-test: $p = 0.048$) (Fig. 4B). Such upregulation in FX mice treated with AM6545 was also observed at protein level in immunoblots of bulk hippocampus (Student's t-test: $p = 0.022$) (Fig. 4C).

Interestingly, we found a positive correlation between discrimination index and *Htr4* levels from both RNA-sequencing (Pearson's correlation coefficient = 0.858, $p = 0.003$) (Supplementary Fig. 4A) and qRT-PCR data (Pearson's correlation coefficient = 0.485, $p = 0.048$) (Supplementary Fig. 4B). We also explored the relation between protein detection and discrimination index, although we found a non-significant correlation

(Supplementary Fig. 4C). This indicates that serotonin receptor 4 protein and mRNA levels increase in FX mice after AM6545 administration in a way that relates to the performance in NORT.

***Cognitive rescue of AM6545 in FX mice is dependent of a serotonergic mechanism***

Based on *Htr4*/5-HT4R correlation with memory in hippocampus, we hypothesized a possible implication of 5-HT4R in the alleviation of the cognitive impairment in FXS. To assess whether sub-chronic AM6545 treatment improves memory with the participation of a serotonergic signaling mechanism, FX mice were treated during 7d with vehicle or AM6545 and on the last day of treatment, they were pre-treated with the 5-HT4R antagonist GR125487 (10 mg/kg) or saline. Thus, immediately after the familiarization phase in the NORT, FX mice received an acute administration of GR125487 or saline, 20 min before the las AM6545 administration. When the test phase was performed 24h later, we observed that the pre-treatment with GR125487 abolished the mnemonic effect of sub-chronic AM6545 treatment in FX mice (two-way ANOVA, interaction: $F(1,34) = 6.221$, $p = 0.018$; *post hoc* Bonferroni, FX-saline-VEH *vs.* FX-saline-AM6545 $p = 0.013$; FX-saline-AM6545 *vs.* FX-GR125487-AM6545 $p = 0.037$) (Fig. 5). This effect was not related to modifications in the exploratory behavior since total exploration time did not significantly change (Supplementary Fig. 5). Together this indicates that the effect that AM6545 has on FX mice memory performance may involve 5-HT4R modulation.

## Discussion

In the present study we described that AM6545, a CB1R antagonist largely restricted to the periphery, rescues memory impairment, synaptic abnormalities and gut microbiota composition in FX mice. Moreover, we decipher that the memory rescue depends on peripheral β-adrenergic and 5-HTR4 signaling in this FXS mouse model.

Previous research in our group has pointed to the endocannabinoid system as a potential therapeutic target in the treatment of FXS-associated cognitive deficits. Specifically, low doses of the systemic CB1R antagonist rimonabant, and the neutral CB1R antagonist NESS0327 showed improvements in several traits of FX mice such as the memory impairment, the elevated mGluR5-LTD, the alterations in dendritic spines or the susceptibility to audiogenic epileptic seizures [7,11]. Here, we aimed to target the peripheral CB1R in the FXS mouse model, as we previously reported that the memory-enhancing effects of systemic CB1R inhibition with rimonabant in naïve mice can be reproduced by peripheral CB1R antagonists such as AM6545 [13,14].

First, we assessed memory performance in the NORT 24h after the familiarization phase in mice that were sub-chronically treated with AM6545. Under these conditions, we found a significant amelioration of the memory deficits after the 7d treatment in two different genetic backgrounds, one outbred (FVB.129) and another inbred (C57BL/6J). This finding indicates a genuine overall effect of AM6545 that is not specific of the genetic background similarly as it was described for two different strains in naïve mice [14].

To further characterize the mnemonic effect of AM6545, we addressed whether peripheral β-adrenergic receptors could be involved, as it happens in naïve mice [13]. Acute pre-treatment with the β-adrenergic peripheral antagonist sotalol bluntly prevented the memory improvement of the acute peripheral treatment with AM6545 in FX mice.

Thus, sotalol effect in FX mice after AM6545 treatment is reminiscent of its effect in preventing the memory improvement with AM6545 in naïve mice [13,14]. Since this goes in accordance with the modulation of cognitive performance by the activation of peripheral β-adrenergic receptors and/or afferent fibers from the vagus nerve [32,33], AM6545 effect in FX mice may also imply the participation of these fibers as in naïve mice [13]. Notably, vagus nerve electrostimulation increases noradrenaline levels in the hippocampus, and a reduction in noradrenergic transmission blocks the effects of vagus nerve stimulation in the central nervous system [34,35]. Moreover, our group also reported that auricular transcutaneous vagus nerve electrostimulation enhanced memory performance in FX mice [36], which reinforces the implication of vagal fibers afferents in memory improvement in FXS.

Interestingly, AM6545 normalizes the enhanced hippocampal mGluR5-LTD of FX mice and also restores the increased density of immature spines in the hippocampus. These cellular alterations have been well-described in FX mice [5,28] and have been typically associated to cognitive impairment in FXS [37]. The changes in synaptic function and structure of AM6545 are somehow reminiscent to the effect of sub-chronic treatment with rimonabant in which mGluR5-LTD and the number of immature spines in the hippocampus of FX mice were normalized [7,11]. Overall, these effects on synaptic plasticity in FX mice are consistent with the unanticipated hypothesis that rimonabant systemic effects can also be obtained targeting peripheral CB1R.

Given that AM6545 is a largely peripheral antagonist [38] and that alterations in gut microbiota have been described in FX mice [9], we next analyzed fecal microbiome composition. Here we reported a significant decrease in *Lactobacillus* and *Akkermansia* population in feces from FX mice compared to WT littermates. Notably, we also found that both reductions in abundance were restored after AM6545 treatment. Both genera

have been recently pinpointed by others in a similar context. Treatment with *Lactobacillus* in mouse models of ASD restored social cognitive deficits and synaptic functions [39,40]. Indeed, a clinical trial is ongoing to evaluate the beneficial effects of increasing *Lactobacillus* species in children with FXS (NCT06279858). Moreover, it is described the implication of the vagus nerve in the behavioral and molecular changes induced by *Lactobacillus* in naïve mice [41], which agrees with the proposed mechanism of action of AM6545 [13]. Consistent with our results, *Akkermansia* levels were described to be low in FX mice [9] and when their levels increase, cognitive deficits and autistic-like behaviors are restored [42,43]. Furthermore, treatment with *Akkermansia muciniphila* in a nonalcoholic steatohepatitis rat model reverse cognitive impairment [44]. Although further research is needed to elucidate the role of these genera in FXS, some of the effects of AM6545 administration could be attained through the microbiota-gut-brain axis [45].

Our transcriptomic analysis of hippocampal synaptoneurosomes revealed few changes in gene expression. *Dcp2* downregulation, could indicate a higher concentration of this protein in P-bodies, facilitating its interaction with excessive mRNAs found in FX synapses. Interestingly, we found the upregulation in *Htr4* in the hippocampus of FX mice that received AM6545. In addition, this increase in mRNA levels was further accompanied by a significant increase at protein level, strongly indicating a possible role of 5-HT4R in AM6545-mediated hippocampal changes. Importantly, 5-HT4R plays a relevant role in hippocampus-dependent learning and memory and the activation of these receptors has been related with cognitive enhancement [31]. The administration of 5-HT4R agonists enhance memory in naïve mice [46,47] and ameliorate cognitive deficits in Alzheimer´s Disease mouse models [48,49] while 5-HT4R decreased density has been

associated to cognitive impairments [50,51]. In this regard, this receptor subclass has been suggested as neuronal marker for learning and memory [52].

Moreover, dysregulation in serotonin modulation has also been reported in ASD and FXS [53,54]. Indeed, recent studies reported a reduced expression of 5-HT$1_B$R in subjects with FXS [55] and lower binding activity of 5HT$1_A$R in FX mice [56]. Interestingly, the modulation of specific serotonin receptors (5-HT1R, 5-HT2R, 5-HT5R and 5-HT7R) have been described to ameliorate behavior and synaptic plasticity in FXS models [57–61]. However, changes in 5-HT4R have not been previously associated with FXS.

Finally, to further understand the role of 5-HT4R in AM6545 effect in FXS, we addressed whether the activation of 5-HT4R may interfere with memory performance in FX mice. Acute pre-treatment with the 5-HT4R antagonist GR125487, abolished the pro-cognitive effect of the sub-chronic AM6545 treatment in FX mice. In this regard, previous studies demonstrated a significant interaction between serotonergic and endocannabinoid systems [62]. Genetic or pharmacological CB1R inhibition has been shown to enhance serotonin levels and control the function and expression of serotonin receptors [63–65]. Specifically, low doses of 5-HT4R agonists reverse CB1R-induced memory impairment [66]. Moreover, activation of 5-HT4Rs has been described to increase intracellular cAMP levels, induce LTP and regulate neurotransmitter release in hippocampus [67,68]; which may explain why its activation is needed for the mnemonic effect of AM6545 in FX mice.

Altogether our results suggest that the pharmacological inhibition of CB1R with the largely peripheral compound AM6545 alleviates significant central and peripheral alterations in the FX mouse and pinpoint to the 5-HT4R as a central effector in the amelioration of cognitive dysfunction.

**Acknowledgements**

We are grateful to Raquel Martín, Marta Linares, Dulce Real and Francisco Porrón for expert technical assistance and Lorena Galera-López and Laura Ciaran-Alfano for helpful discussion.

**Author contribution**

L.R.-R., Behavioral and biochemical experiments, transcriptomic study, statistical analysis, graphs and writing of the manuscript; A.B.-M., Behavioral and biochemical experiments, statistical analysis and writing of the manuscript; S.M.-T., Behavioral experiments. I.M.-G., P.L.-R. and A.R.-M., Electrophysiological experiments; S.A. and M.G., Microbiota experiments. R.M., Supervision and funding of the study. A.O., Conceptualization, supervision, funding of the study and writing of the manuscript. All authors revised the final version of the manuscript.

**Funding and disclosure**

L.R.-R. was supported by a predoctoral fellowship from Spanish Ministry of Science and Innovation (PRE2019-087644). A.B.-M. was supported by a predoctoral fellowship from Spanish Ministry of Universities (FPU20/02061). I.M-G. was supported by a predoctoral fellowship from Spanish Ministry of Science, Innovation and Universities (FPU17/04283). This work was supported by the Spanish Ministry of Science and Innovation (PID2021-123482OB-I00) and La Marató de TV3 Foundation (202224-30-31) to A.O., the Spanish Agencia Estatal de Investigación (PID2022-136597NB-I00) and the Junta de Andalucía (P20-0881) to A.R.-M, the MICIU/AEI/10.13039/501100011033 and European Union NextGenerationEU/PRTR (Grant CNS2023-145550) to S.A. and M.G. and Generalitat de Catalunya, AGAUR (2021 SGR 00912) to R.M.

**Data availability**

The behavioral and biochemical data that support the findings of this study are available from the corresponding author upon reasonable request. Transcriptomic data in the form of raw fastq files have been deposited on GEO database (GSE278037).

**Conflict of interest**

R.M. and A.O. declare intellectual property of the patent PCT/EP2013/055728. The remaining authors declare no conflict of interest.

## References


1. Hagerman, Berry-Kravis E, Hazlett HC, Bailey DB, Moine H, Kooy RF, et al. Fragile X syndrome. Nature Reviews Disease Primers 2017 3:1. 2017;3:1–19.

2. Verkerk AJMH, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DPA, Pizzuti A, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell. 1991;65:905–914.

3. Salcedo-Arellano MJ, Dufour B, McLennan Y, Martinez-Cerdeno V, Hagerman R. Fragile X syndrome and associated disorders: Clinical aspects and pathology. Neurobiol Dis. 2020;136:104740.

4. The Dutch-Belgian Fragile X Consortium, Bakker CE, Verheij C, Willemsen R, van der Helm R, Oerlemans F, et al. Fmr1 knockout mice: A model to study fragile X mental retardation. Cell. 1994;78:23–33.

5. Bear MF, Huber KM, Warren ST. The mGluR theory of fragile X mental retardation. Trends Neurosci. 2004;27:370–377.

6. Michalon A, Sidorov M, Ballard TM, Ozmen L, Spooren W, Wettstein JG, et al. Chronic pharmacological mGlu5 inhibition corrects fragile X in adult mice. Neuron. 2012;74:49–56.

7. Busquets-Garcia A, Gomis-González M, Guegan T, Agustín-Pavón C, Pastor A, Mato S, et al. Targeting the endocannabinoid system in the treatment of fragile X syndrome. Nat Med. 2013;19:603–607.

8. Levenga J, de Vrij FMS, Buijsen RAM, Li T, Nieuwenhuizen IM, Pop A, et al. Subregion-specific dendritic spine abnormalities in the hippocampus of Fmr1 KO mice. Neurobiol Learn Mem. 2011;95:467–472.

9. Altimiras F, Garcia JA, Palacios-García I, Hurley MJ, Deacon R, González B, et al. Altered Gut Microbiota in a Fragile X Syndrome Mouse Model. Front Neurosci. 2021;15.

10. Zanettini C, Panlilio L V., Alicki M, Goldberg SR, Haller J, Yasar S. Effects of endocannabinoid system modulation on cognitive and emotional behavior. Front Behav Neurosci. 2011;5.

11. Gomis-González M, Matute C, Maldonado R, Mato S, Ozaita A. Possible Therapeutic Doses of Cannabinoid Type 1 Receptor Antagonist Reverses Key Alterations in Fragile X Syndrome Mouse Model. Genes 2016, Vol 7, Page 56. 2016;7:56.

12. Christensen R, Kristensen PK, Bartels EM, Bliddal H, Astrup A. Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. Lancet. 2007;370:1706–1713.

13. Martínez-Torres S, Bergadà-Martínez A, Ortega JE, Galera-López L, Hervera A, de los Reyes-Ramírez L, et al. Peripheral CB1 receptor blockade acts as a memory enhancer through a noradrenergic mechanism. Neuropsychopharmacology 2022 48:2. 2023;48:341–350.

14. Bergadà-Martínez A, Reyes-Ramírez L de los, Martínez-Torres S, Gallego IM-, Maldonado R, Rodríguez-Moreno A, et al. Sub-chronic peripheral CB1R inhibition enhances cognitive performance and induces hippocampal synaptic plasticity changes in naïve mice. BioRxiv. 2024:2023.11.21.567995.

15. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. J Pharmacol Pharmacother. 2010;1:94–99.

16. du Sert NP, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol. 2020;18.

17. Pérez-Rodríguez M, Arroyo-García LE, Prius-Mengual J, Andrade-Talavera Y, Armengol JA, Pérez-Villegas EM, et al. Adenosine Receptor-Mediated Developmental Loss of Spike Timing-Dependent Depression in the Hippocampus. Cerebral Cortex (New York, NY). 2019;29:3266.

18. Lyon L, Borel M, Carrión M, Kew JNC, Corti C, Harrison PJ, et al. Hippocampal mossy fiber long-term depression in Grm2/3 double knockout mice. Synapse. 2011;65:945–954.

19. Falcón-Moya R, Martínez-Gallego I, Rodríguez-Moreno A. Kainate receptor modulation of glutamatergic synaptic transmission in the CA2 region of the hippocampus. J Neurochem. 2021;158:1083–1093.

20. Díaz-Marugan L, Gallizioli M, Márquez-Kisinousky L, Arboleya S, Mastrangelo A, Ruiz-Jaén F, et al. Poststroke Lung Infection by Opportunistic Commensal Bacteria Is Not Mediated by Their Expansion in the Gut Microbiota. Stroke. 2023;54:1875–1887.

21. Paulson JN, Colin Stine O, Bravo HC, Pop M. Differential abundance analysis for microbial marker-gene surveys. Nature Methods 2013 10:12. 2013;10:1200–1202.

22. Salgado-Mendialdúa V, Aguirre-Plans J, Guney E, Reig-Viader R, Maldonado R, Bayés, et al. Δ9-tetrahydrocannabinol modulates the proteasome system in the brain. Biochem Pharmacol. 2018;157:159–168.

23. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15.

24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402–408.

25. Bai M, Zhu XZ, Zhang Y, Zhang S, Zhang L, Xue L, et al. Anhedonia was associated with the dysregulation of hippocampal HTR4 and microRNA Let-7a in rats. Physiol Behav. 2014;129:135–141.

26. Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: Validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods. 2000;46:69–81.

27. Ozaita A, Puighermanal E, Maldonado R. Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain. J Neurochem. 2007;102:1105–1114.

28. Irwin SA, Galvez R, Greenough WT. Dendritic Spine Structural Anomalies in Fragile-X Mental Retardation Syndrome. Cerebral Cortex. 2000;10:1038–1044.

29. Huber KM, Gallagher SM, Warren ST, Bear MF. Altered synaptic plasticity in a mouse model of fragile X mental retardation. Proc Natl Acad Sci U S A. 2002;99:7746–7750.

30. Balagopal V, Parker R. Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. Curr Opin Cell Biol. 2009;21:403–408.

31. Hagena H, Manahan-Vaughan D. The serotonergic 5-HT4 receptor: A unique modulator of hippocampal synaptic information processing and cognition. Neurobiol Learn Mem. 2017;138:145–153.

32. Gold PE, Korol DL. Making Memories Matter. Front Integr Neurosci. 2012;6.

33. Miyashita T, Williams CL. Epinephrine administration increases neural impulses propagated along the vagus nerve: Role of peripheral beta-adrenergic receptors. Neurobiol Learn Mem. 2006;85:116–124.

34. Follesa P, Biggio F, Gorini G, Caria S, Talani G, Dazzi L, et al. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. Brain Res. 2007;1179:28–34.

35. Hassert DL, Miyashita T, Williams CL. The effects of peripheral vagal nerve stimulation at a memory-modulating intensity on norepinephrine output in the basolateral amygdala. Behavioral Neuroscience. 2004;118:79–88.

36. Vázquez-Oliver A, Brambilla-Pisoni C, Domingo-Gainza M, Maldonado R, Ivorra A, Ozaita A. Auricular transcutaneous vagus nerve stimulation improves memory persistence in naïve mice and in an intellectual disability mouse model. Brain Stimul. 2020;13:494–498.

37. Vanderklish PW, Edelman GM. Dendritic spines elongate after stimulation of group 1 metabotropic glutamate receptors in cultured hippocampal neurons. Proc Natl Acad Sci U S A. 2002;99:1639–1644.

38. Tam J, Vemuri VK, Liu J, Bátkai S, Mukhopadhyay B, Godlewski G, et al. Peripheral CB1 cannabinoid receptor blockade improves cardiometabolic risk in mouse models of obesity. J Clin Invest. 2010;120:2953–2966.

39. Olivito I, Avolio E, Minervini D, Soda T, Rocca C, Angelone T, et al. Ketogenic diet ameliorates autism spectrum disorders-like behaviors via reduced inflammatory factors and microbiota remodeling in BTBR T+ Itpr3tf/J mice. Exp Neurol. 2023;366.

40. Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA, et al. Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. Neuron. 2019;101:246-259.e6.

41. Bravo JA, Forsythe P, Chew M V., Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A. 2011;108:16050–16055.

42. Goo N, Bae HJ, Park K, Kim J, Jeong Y, Cai M, et al. The effect of fecal microbiota transplantation on autistic-like behaviors in Fmr1 KO mice. Life Sci. 2020;262:118497.

43. Guo P, Yang X, Guo X, Yang H, Pan J, Li Y. Dietary fish oil improves autistic behaviors and gut homeostasis by altering the gut microbial composition in a mouse model of fragile X syndrome. Brain Behav Immun. 2023;110:140–151.

44. Higarza SG, Arboleya S, Arias JL, Gueimonde M, Arias N. Akkermansia muciniphila and environmental enrichment reverse cognitive impairment associated with high-fat high-cholesterol consumption in rats. Gut Microbes. 2021;13:1–20.

45. Décarie-Spain L, Hayes AMR, Lauer LT, Kanoski SE. The gut-brain axis and cognitive control: A role for the vagus nerve. Semin Cell Dev Biol. 2024;156:201–209.

46. Freret T, Bouet V, Quiedeville A, Nee G, Dallemagne P, Rochais C, et al. Synergistic effect of acetylcholinesterase inhibition (donepezil) and 5-HT4 receptor activation (RS67333) on object recognition in mice. Behavioural Brain Research. 2012;230:304–308.

47. Quiedeville A, Boulouard M, Hamidouche K, Da Silva Costa-Aze V, Nee G, Rochais C, et al. Chronic activation of 5-HT4 receptors or blockade of 5-HT6 receptors improve memory performances. Behavioural Brain Research. 2015;293:10–17.

48. Claeysen S, Bockaert J, Giannoni P. Serotonin: A New Hope in Alzheimer's Disease? ACS Chem Neurosci. 2015;6:940–943.

49. Werner F-M, Covenas R. Serotonergic Drugs: Agonists/Antagonists at Specific Serotonergic Subreceptors for the Treatment of Cognitive, Depressant and Psychotic Symptoms in Alzheimer's Disease. Curr Pharm Des. 2016;22:2064–2071.

50. Lalut J, Karila D, Dallemagne P, Rochais C. Modulating 5-HT4 and 5-HT6 receptors in Alzheimer's disease treatment. Future Med Chem. 2017;9:781–795.

51. Murphy SE, De Cates AN, Gillespie AL, Godlewska BR, Scaife JC, Wright LC, et al. Translating the promise of 5HT4 receptor agonists for the treatment of depression. Psychol Med. 2021;51:1111–1120.

52. Meneses A. Serotonin, neural markers, and memory. Front Pharmacol. 2015;6:143.

53. Hanson AC, Hagerman RJ. Serotonin dysregulation in Fragile X Syndrome: implications for treatment. Intractable Rare Dis Res. 2014;3:110–117.

54. Hu Z, Ying X, Huang L, Zhao Y, Zhou D, Liu J, et al. Association of human serotonin receptor 4 promoter methylation with autism spectrum disorder. Medicine. 2020;99.

55. Kang Y, Zhou Y, Li Y, Han Y, Xu J, Niu W, et al. A human forebrain organoid model of fragile X syndrome exhibits altered neurogenesis and highlights new treatment strategies. Nature Neuroscience 2021 24:10. 2021;24:1377–1391.

56. Saraf TS, Chen Y, Tyagi R, Canal CE. Altered brain serotonin 5-HT1A receptor expression and function in juvenile Fmr1 knockout mice. Neuropharmacology. 2024;245.

57. Saraf TS, McGlynn RP, Bhatavdekar OM, Booth RG, Canal CE. FPT, a 2-Aminotetralin, Is a Potent Serotonin 5-HT1A, 5-HT1B, and 5-HT1D Receptor Agonist That Modulates Cortical Electroencephalogram Activity in Adult Fmr1 Knockout Mice. ACS Chem Neurosci. 2022;13:3629–3640.

58. Costa L, Spatuzza M, D'Antoni S, Bonaccorso CM, Trovato C, Musumeci SA, et al. Activation of 5-HT7 serotonin receptors reverses metabotropic glutamate receptor-mediated synaptic plasticity in wild-type and Fmr1 knockout mice, a model of Fragile X syndrome. Biol Psychiatry. 2012;72:924–933.

59. Costa L, Sardone LM, Lacivita E, Leopoldo M, Ciranna L. Novel agonists for serotonin 5-HT7 receptors reverse metabotropic glutamate receptor-mediated long-term depression in the hippocampus of wild-type and Fmr1 KO mice, a model of Fragile X Syndrome. Front Behav Neurosci. 2015;9.

60. Lim CS, Hoang ET, Viar KE, Stornetta RL, Scott MM, Zhu JJ. Pharmacological rescue of Ras signaling, GluA1-dependent synaptic plasticity, and learning deficits in a fragile X model. Genes Dev. 2014;28:273–289.

61. Yamazaki M, Arai T, Yarimizu J, Matsumoto M. 5-HT5A Receptor Antagonist ASP5736 Ameliorates Several Abnormal Behaviors in an Fmr1-Targeted Transgenic Male Rat Model of Fragile X Syndrome. Int J Neuropsychopharmacol. 2022;25:786–793.

62. Peters KZ, Cheer JF, Tonini R. Modulating the Neuromodulators: Dopamine, Serotonin, and the Endocannabinoid System. Trends Neurosci. 2021;44:464–477.

63. Aso E, Renoir T, Mengod G, Ledent C, Hamon M, Maldonado R, et al. Lack of CB1 receptor activity impairs serotonergic negative feedback. J Neurochem. 2009;109:935–944.

64. Darmani NA, Janoyan JJ, Kumar N, Crim JL. Behaviorally active doses of the CB1 receptor antagonist SR 141716A increase brain serotonin and dopamine levels and turnover. Pharmacol Biochem Behav. 2003;75:777–787.

65. Mato S, Aso E, Castro E, Martín M, Valverde O, Maldonado R, et al. CB1 knockout mice display impaired functionality of 5-HT1A and 5-HT2A/C receptors. J Neurochem. 2007;103:2111–2120.

66. Nasehi M, Rostam-Nezhad E, Ebrahimi-Ghiri M, Zarrindast MR. Interaction between hippocampal serotonin and cannabinoid systems in reactivity to spatial and object novelty detection. Behavioural Brain Research. 2017;317:272–278.

67. de Cates AN, Wright LC, Martens MAG, Gibson D, Türkmen C, Filippini N, et al. Déjà-vu? Neural and behavioural effects of the 5-HT4 receptor agonist, prucalopride, in a hippocampal-dependent memory task. Translational Psychiatry 2021 11:1. 2021;11:1–9.

68. Ciranna L. Serotonin as a modulator of glutamate- and GABA-mediated neurotransmission: implications in physiological functions and in pathology. Curr Neuropharmacol. 2006;4:101–114.

## Figures

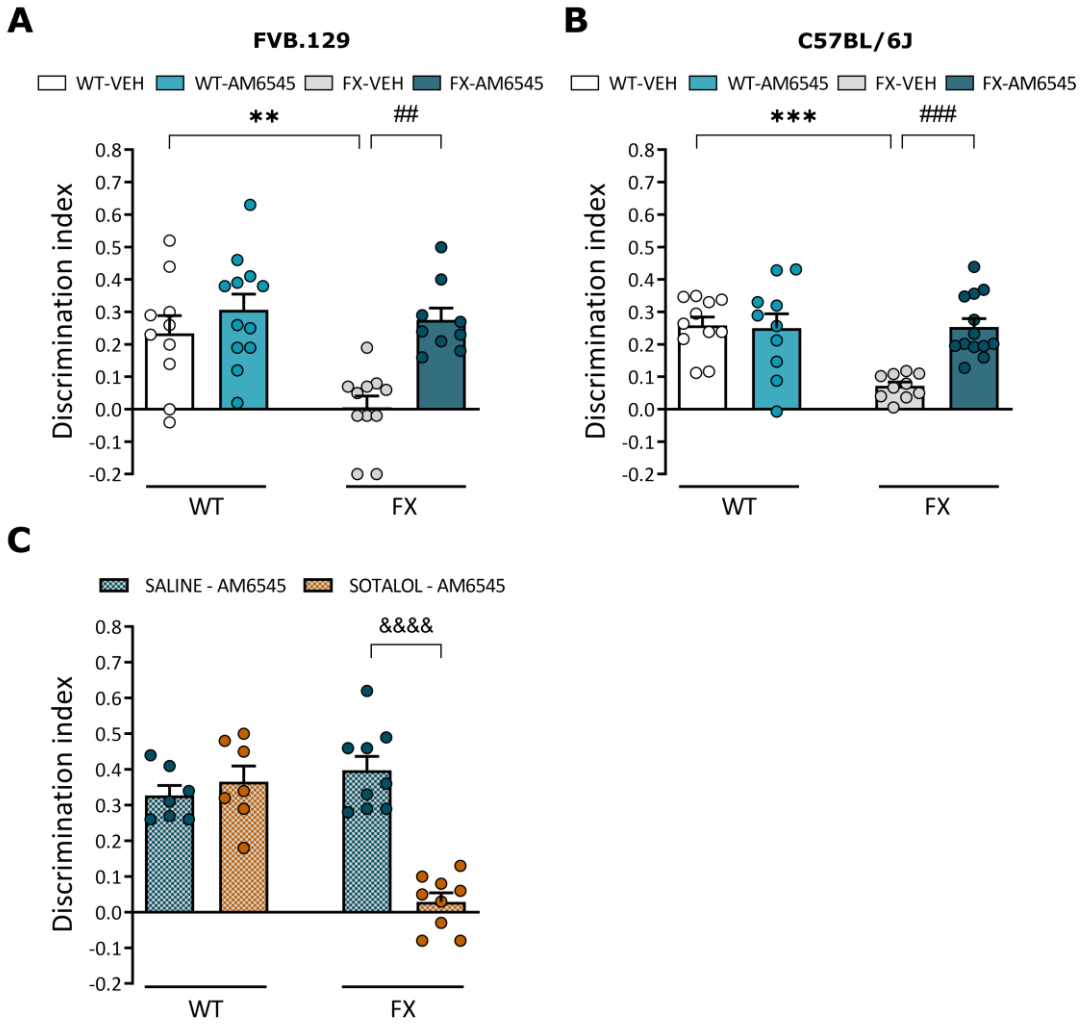


**Figure 1: Memory rescue in FX mice by AM6545 treatment is mediated by peripheral noradrenergic signaling. (A,B)** Discrimination index of WT and FX mice after sub-chronic administration of vehicle (VEH) or AM6545 (1 mg/kg, 7d) in **(A)** FVB.129 ($n$ = 9-12) and **(B)** C57BL/6J ($n$ = 10-13) genetic background. **(C)** Discrimination index of FVB.129 WT and FX mice pre-treated with saline or sotalol (10 mg/kg) before acute AM6545 (1 mg/kg) treatment ($n$ = 7-9). Data are expressed as mean ± s.e.m. ** $p < 0.01$, *** $p < 0.001$ (genotype effect); ## $p < 0.01$, ### $p < 0.001$ (treatment effect); &&&& $p < 0.0001$ (pre-treatment effect) by two-way ANOVA followed by Bonferroni *post hoc*.

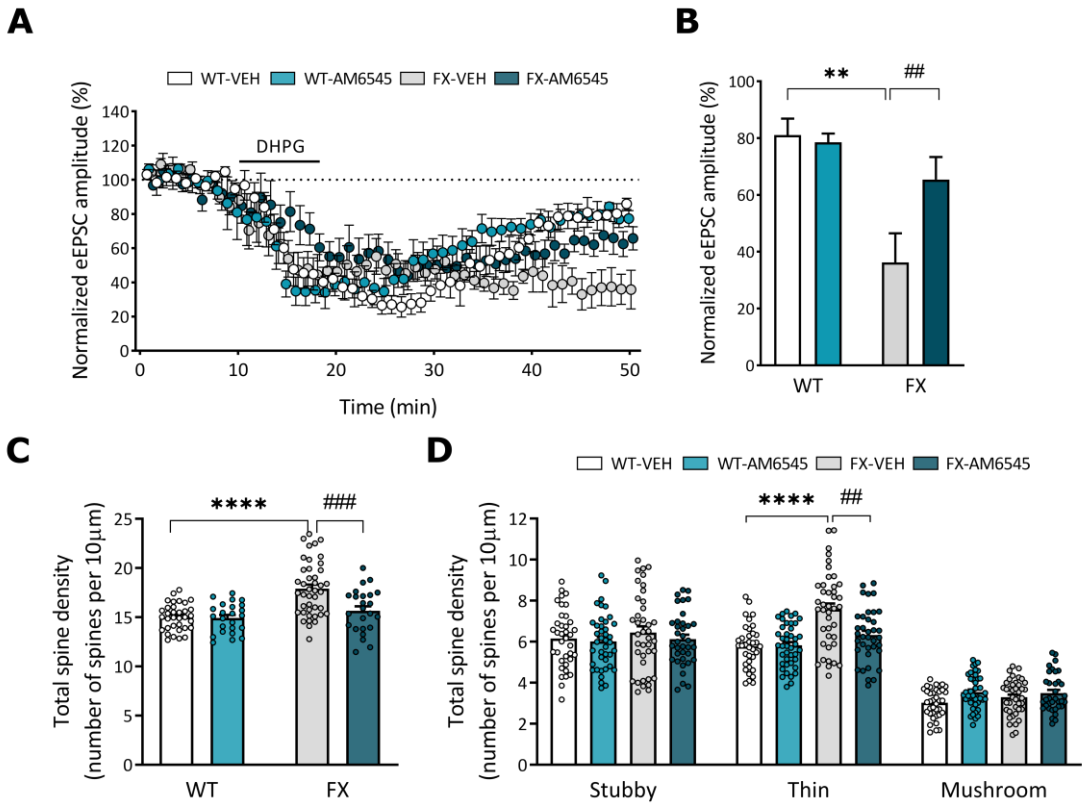


**Figure 2: Sub-chronic AM6545 treatment ameliorates aberrant mGluR5-dependent LTD and normalizes dendritic spines alterations in FX mice. (A)** Average time courses of the change in the amplitude of evoked excitatory postsynaptic currents (eEPSC) in hippocampal slices from FVB.129 mice treated for 7d with vehicle (VEH) or AM6545 (1 mg/kg, 7d) after bath application of DHPG ($n$ = 6-7 mice). **(B)** Mean EPSC amplitude values of the last 5 min of recordings in **A**. **(C)** Quantification of the total number of spines in 10 µm of CA1 pyramidal dendrites of C57BL/6J WT and FX mice treated for 7d with VEH or AM6545 ($n$ = 4-5 mice). **(D)** Morphological analysis of dendritic spines in the dendrites of pyramidal CA1 neurons in C57BL/6J WT and FX mice after pharmacological treatments ($n$ = 4-5 mice). Each dot represents a hippocampal slice. Data are expressed as mean ± s.e.m. ** $p < 0.01$, **** $p < 0.0001$ (genotype effect); ## $p < 0.01$, ### $p < 0.001$ (treatment effect); by two-way ANOVA followed by Bonferroni *post hoc*.

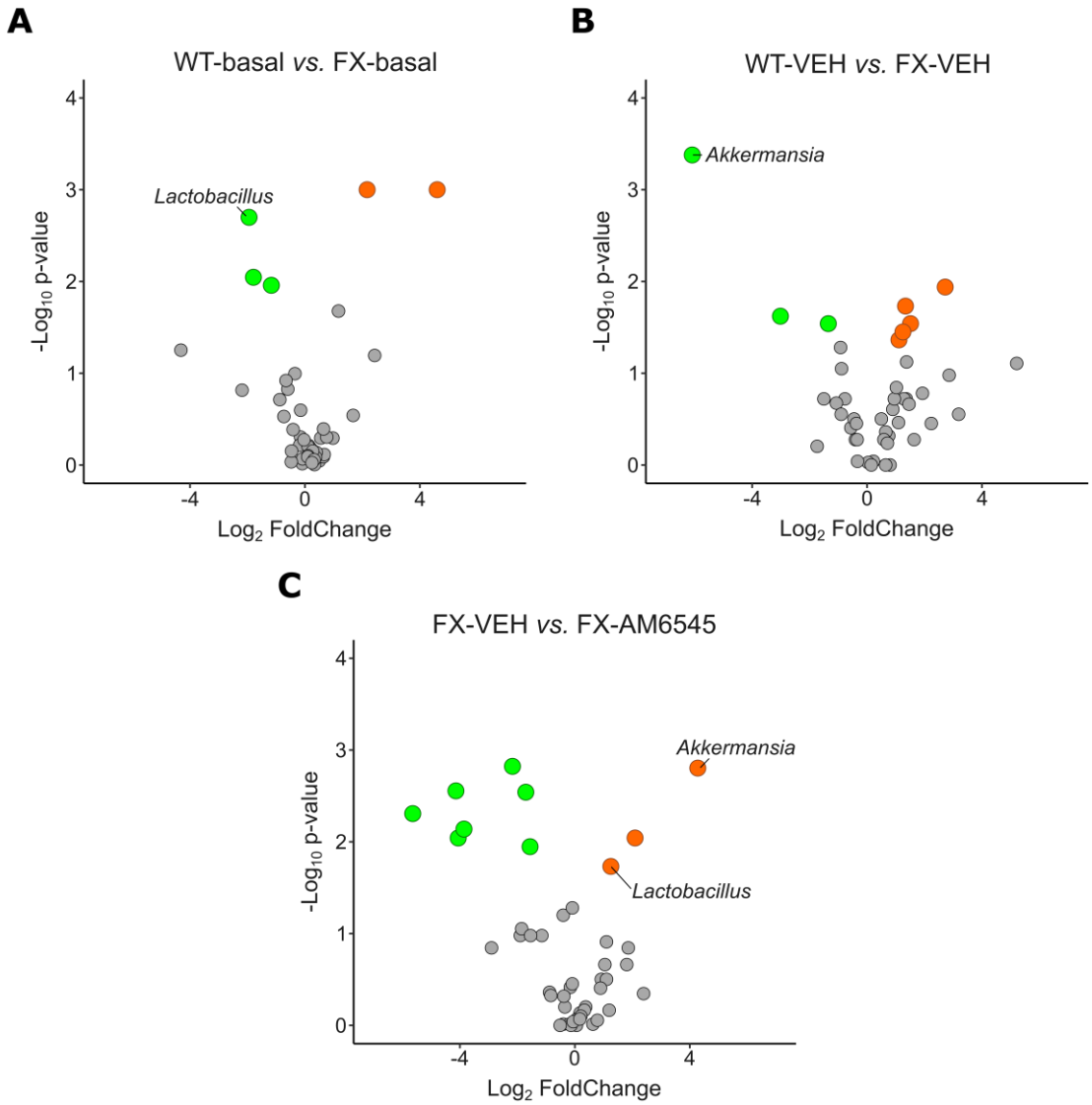


**Figure 3: Fecal microbiome changes in FX mice after sub-chronic AM6545 treatment.** Volcano plot showing differential abundance of microbiome composition in **(A)** C57BL/6J FX non-manipulated mice compared to WT non-manipulated mice (WT-basal *vs.* FX-basal), **(B)** C57BL/6J vehicle-treated FX mice compared to vehicle-treated WT mice (WT-VEH *vs.* FX-VEH) and **(C)** C57BL/6J FX mice sub-chronically treated with AM6545 (1mg/kg, 7d) compared to vehicle-treated FX mice (FX-VEH *vs.* FX-AM6545). Orange and green dots indicate enriched or depleted species in each comparison in terms of relative abundance at *genus* level. (WT-basal, $n$ = 19; FX-basal, $n$ = 20; WT-VEH, $n$ = 10; FX-VEH, $n$ = 10; FX-AM6545, $n$ = 10). Significance is set at $p < 0.05$

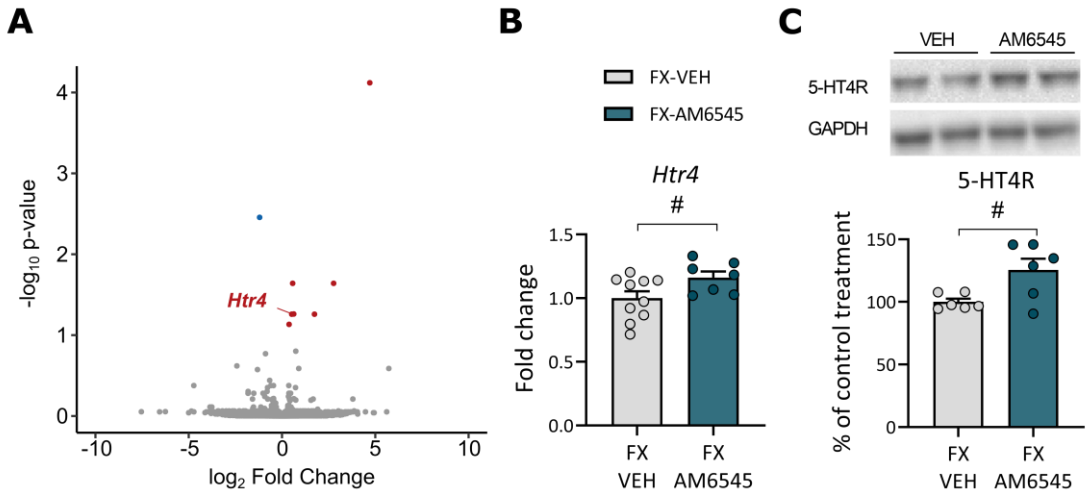


**Figure 4: Sub-chronic AM6545 treatment upregulates serotonin 4 receptor in the hippocampus of FX mice. (A)** Volcano plot showing differentially expressed genes in C57BL/6J FX mice treated with vehicle (VEH) or AM6545 (1 mg/kg, 7d). Red and blue dots represent significantly up or downregulated genes, respectively. (FX-VEH, $n$ = 5; FX-AM6545, $n$ = 4). Cut-off was set at |log2FC| > 0.2 and adjusted *p-value* < 0.1. **(B)** Synaptic hippocampal mRNA levels of *Htr4* in C57BL/6J FX mice treated for 7d with vehicle (VEH) or AM6545 (1 mg/kg, 7d) ($n$ = 7-10). **(C)** Quantification and representative immunoblot of 5-HT4R in bulk hippocampus of C57BL/6J FX mice after VEH or AM6545 administration ($n$ = 6). Data are expressed as mean ± s.e.m. # $p < 0.05$ (treatment effect) by Student's t-test

**Figure 5**

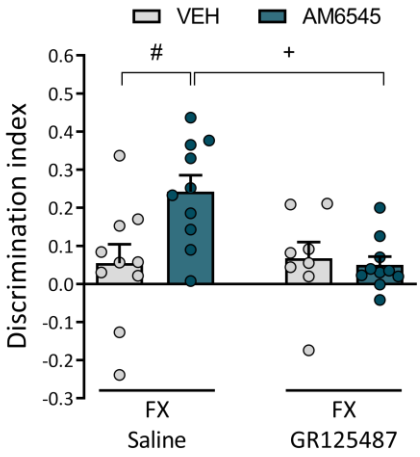


**Figure 5: A serotonergic mechanism mediates the cognitive improvement of FX mice after sub-chronic AM6545 treatment.** Discrimination index of C57BL/6J FX mice after sub-chronic vehicle (VEH) or AM6545 (1 mg/kg, 7d) administration and pre-treatment with saline or GR125487 (10 mg/kg) after the familiarization phase of the NORT ($n$ = 8-10). Data are expressed as mean ± s.e.m. + $p < 0.05$ (pre-treatment effect); # $p < 0.05$ (treatment effect) by two-way ANOVA followed by Bonferroni *post hoc.*