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Palmitoylethanolamide counteracts autistic-like behaviours in BTBR T + tf/J mice: Contribution of central and peripheral mechanisms

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ABSTRACT

Autism spectrum disorders (ASD) are a group of heterogeneous neurodevelopmental conditions characterized by impaired social interaction, and repetitive stereotyped behaviours. Interestingly, functional and inflammatory gastrointestinal diseases are often reported as a comorbidity in ASDs, indicating gut-brain axis as a novel emerging approach. Recently, a central role for peroxisome-proliferator activated receptor (PPAR)-α has been addressed in neurological functions, associated with the behaviour. Among endogenous lipids, palmitoylethanolamide (PEA), a PPAR-α agonist, has been extensively studied for its anti-inflammatory effects both at central and peripheral level.

Based on this background, the aim of this study was to investigate the pharmacological effects of PEA on autistic-like behaviour of BTBR T + tf/J mice and to shed light on the contributing mechanisms.

Our results showed that PEA reverted the altered behavioural phenotype of BTBR mice, and this effect was contingent to PPAR-α activation. Moreover, PEA was able to restore hippocampal BDNF signalling pathway, and improve mitochondrial dysfunction, both pathological aspects, known to be consistently associated with ASDs. Furthermore, PEA reduced the overall inflammatory state of BTBR mice, reducing the expression of pro-inflammatory cytokines at hippocampal, serum, and colonic level. The analysis of gut permeability and the expression of colonic tight junctions showed a reduction of leaky gut in PEA-treated BTBR mice. This finding together with PEA effect on gut microbiota composition suggests an involvement of microbiota-gut-brain axis.

In conclusion, our results demonstrated a therapeutic potential of PEA in limiting ASD symptoms, through its pleiotropic mechanism of action, supporting neuroprotection, anti-inflammatory effects, and the modulation of gut-brain axis.

1. Introduction

Autism spectrum disorders (ASD) are a range of complex neurodevelopmental conditions characterized by impaired social interaction, isolated areas of interest, and repetitive stereotyped behaviours (Maresca and de Magistris, 2015). The aetiology of ASD is likely multifactorial and based on genetic, epigenetic and environmental risk factors, although the precise molecular and cellular links remain ill-defined (Waye and Cheng, 2018). Previously, we have demonstrated that genetic inactivation of peroxisome-proliferator activated receptor (PPAR)-α, particularly abundant in the CNS (Moreno et al., 2004), leads to a behavioural and cognitive phenotype reminiscent of that of preclinical models of ASD, including the mouse model BTBR T + tf/J (BTBR), which displays an improved repetitive behaviour, when autistic mice were treated with a synthetic PPAR-α agonist (D'Agostino et al., 2015). These results not only indicated a central role for this receptor in neurological functions associated with the behaviour, but more interestingly highlighted PPAR-α as a potential pharmacological target to lessen ASD symptoms. Notably, in sodium valproate (VPA)-induced model of ASD it has been shown a reduction in PPAR-α expression in frontal cortex accompanied by an increase of several fatty acid ethanolamides at hippocampal levels following exposure to the

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sociability test, including palmitoylethanolamide (PEA), and oleylethanolamide (OEA), identified as PPAR-α endogenous agonists (Kerr et al., 2013). Moreover, it has been demonstrated that the administration of PEA in combination with luteolin, was effective in reducing social and behavioural defects shown by sodium VPA-induced autistic mice (Bertolino et al., 2017).

ASDs have been associated with several comorbidities, including immune dysregulation and gastrointestinal ailments (Ashwood et al., 2011; Buie, 2013; Coury et al., 2012; Li et al., 2009; Morgan et al., 2010; Vargas et al., 2005). Indeed, in recent years a focus on the role of the bidirectional communication between the GI tract and the CNS, namely gut-brain axis, has been addressed in the pathogenesis of several brain disorders (Mayer et al., 2014; Wang and Kasper, 2014), including ASDs, whose therapeutic strategies are overlooked (Cristiano et al., 2018). The aetiology of GI abnormalities in ASDs is unclear, though the association between GI dysfunction and ASD-associated behaviours suggests that overlapping developmental defects in the brain and the intestine could be responsible for the observed phenotypes. Actually, GI alterations in terms of inflammation and leaky gut can contribute to the pathogenesis or lead to the progression of brain disorders. We have recently shown that marked intestinal dysbiosis, and damage were shown in BTBR mice, together with an impairment of gut integrity and an increase of inflammatory cytokines expression in colonic tissue (Coretti et al., 2017), indicating an increased gut permeability and translocation of pro-inflammatory factors across the intestinal epithelium into the bloodstream.

Gut inflammation and impairment of gut integrity can be associated with microbial dysbiosis. In BTBR mice alteration of gut microbiota composition has been recently describes: in particular, in BTBR mice gut microbiota alterations have been found to be associate with impaired behaviour, immune system dysregulation and GI alteration (Coretti et al., 2017; Klein et al., 2016). BTBR mice showed a phenotype that mirror many ASD pathological aspects found in children patients, including alterations in both innate and adaptive arms of the immune system (Onore et al., 2013).

Here, we have studied the effect of PEA treatment on autistic-like phenotype of BTBR mice, exploring the underpinning molecular mechanisms involved at hippocampal level. In particular, we investigated the contingency of PPAR-α activation/function in PEA beneficial effects, the involvement of brain derived neurotrophic factor (BDNF) signalling pathway and mitochondrial function and efficiency. Furthermore, the modulating effect of PEA on gut permeability and inflammation was assessed, in order to investigate the involvement of gut-brain axis, evaluating systemic cytokines and gut microbiota composition.

2. Materials and methods

2.1. Animals

Male C57Bl/6J (B6), *B6.129S4-SvJae-Pparatm1Gonz* PPAR-alpha null (KO) and BTBR T + tf/J (BTBR) (3–4 months old) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and colonies maintained in our animal facility genotyped according to supplier webpage (http://jaxmice.jax.org), using the RedExtract kit (Sigma–Aldrich, Italy).

All animals were housed in groups in a room maintained at 22 °C, on a 12 h:12 h light:dark cycle, with *ad libitum* access to water and standard laboratory chow diet. To minimalized litter effects, we used mice from at least 5 different litters in each experiment. All experimental procedures were carried out in compliance with the international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept) and approved by Italian Ministry of Health.

2.2. Drugs and treatment

Ultra-micronized PEA (PEA-um®; Epitech Group, Italy) and GW6471 (N-((2S)-2-(((1Z)-1-Methyl-3-oxo-3-(4-(trifluoromethyl) phenyl)prop-1-enyl) amino)-3-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy)phenyl)propyl) propanamide), a PPAR-α antagonist (GW; Tocris, Bristol, UK) were dissolved in PEG400, Tween 80 and sterile saline (Sigma-Aldrich, Milan, Italy) to obtain a final concentration of PEG400 and Tween 80 of 20 and 10% v/v, respectively and injected at a volume of 10 ml/kg body weight.

BTBR, KO and control B6 mice were daily intraperitoneal-injected (i.p.) for 10 days with Vehicle, PEA (10 or 30 mg/kg), and/or GW (1 mg/kg). When BTBR mice were co-treated with PEA and GW, the antagonist was injected 30 min before PEA administration. Testing began at ages 3–4 months, on day 9th, one hour after the last injection. In details, behavioural tasks described below were conducted in the same mice from 5 different litters, in a battery on two separate days (9th-10th) according to Paylor et al. (2006), with sufficient intervals between tests and in a sequence that begins with the least stressful quick observational tests followed by the more stressful complex tasks, except for the valuation of locomotor activity and anxiety behaviour (elevated plus maze and open filed test) that were conducted on a separate cohort of mice. All experiments and data analyses were performed by operators blinded to genotype, when possible, and treatment.

2.3. Marble burying assay

20 marbles were arranged in a 5x4 grid in a Plexiglas cage filled with 5 cm of clean bedding. Each mouse was individually placed into this cage and allow to freely explore for 30 min. At the end of the session, the mouse was gently removed and the number of buried marbles (< 2/3 visible above bedding) were counted by the tester.

2.4. Self-Grooming test

Mice were placed in an empty Plexiglas cage (30 x 40 cm) and left undisturbed freely to explore the arena. Following 10 min of habituation, the self-grooming activity was manual scored by a trained observer for other 20 min. Grooming behaviour included head washing, body grooming, genital/tail grooming and paw and leg licking.

2.5. Three-chambered social test

Social interaction was examined using the three-chambered apparatus as previously described (Crawley, 2004). Briefly, this test consists of 3 phases; the first one is a 5-minute habituation to the empty arena. In the following 10 min session of social preference, the experimental mouse is exposed to an inanimate object in one side of the chamber and a stimulus mouse in the other chamber. Then, the third final 10-minute phase of social novelty consists to evaluate mouse preference between the known mouse and a novel stimulus mouse placed in the previously inanimate object chamber. Time spent in sniffing the object or mouse and time spent in each chamber were automatically detected by a videocamera coupled with a video-tracking software (Any-maze, Stoelting).

2.6. In vivo intestinal permeability assay

In vivo intestinal permeability assay was performed for a subset of mice using fluorescein isothiocyanate-labeled dextran (FITC-dextran) method, as previously described (Cani et al., 2009). Briefly, food and water were withdrawn for 6 h and mice (n = 5, each group) were administrated by gavage with FITC labeled dextran 4000 (Sigma-Aldrich, Milan, Italy), as permeability tracer (60 mg/100 g body weight). After 24 h blood of all animals was collected by intracardiac puncture and

centrifuged (3000 rpm for 15 min at RT). Then plasma FITC-dextran concentration was determined (excitation, 485 nm; emission, 535 nm; HTS-7000 Plus-plate-reader; Perkin Elmer, Wellesley, MA, USA), using a standard curve generated by serial dilution of the tracer.

2.7. Western blotting

Hippocampus was homogenized in lysis buffer (50 mM Tris–HCl, pH 7.4; 1 mM EDTA; 100 mM NaCl; 20 mM NaF; 3 mM Na3VO4; 1 mM PMSF with 1% (v/v) Nonidet P-40; and protease inhibitor cocktail). Lysates were centrifuged at 20,000*g* for 15 min at 4 °C. Protein concentrations were estimated using bovine serum albumin as a standard in a Bradford reagent assay. Proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes, and incubated with the following primary antibodies: PPAR-α (cat no P0369, Sigma Aldrich), 1: 500; Bdnf (cat no sc-546, Santa Cruz), 1: 200; pCREB (cat no 9196, Cell Signaling Technologies (CST), MA, USA), 1:1000; CREB (cat no 4820, CST) 1: 2000; β-actin (Sigma Aldrich), 1:1000. The signals were visualized with the ECL system (Pierce) by Image Quant (GE Healtcare, Milan, Italy). The protein bands were densitometrically analyzed with the Quantity One software (Bio-Rad Laboratories).

2.8. Real-time PCR analysis

Total RNA was extracted from hippocampus and colon using Trizol (Ambion). Two micrograms of total RNA were used in first-strand cDNA synthesis (Promega, Madison, WI) according the manufacturer instructions. PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories). The PCR conditions were 15 min at 95 °C followed by 40 cycles of two-step PCR denaturation at 94 °C for 15 s, annealing extension at 55 °C for 30 s and extension at 72 °C for 30 s. Each sample contained 500 ng cDNA in 2X QuantiTech SYBRGreen PCR Master Mix and primers *Ppara, Bdnf, Ntrk2*, *Tnfa*, *Il1b*, *Il6, Ocln* and *Tjp1* purchased from Qiagen (Hilden, Germany). The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

2.9. Serum parameters

Blood was collected and serum obtained by centrifugation. Serum Tnf-α, Il-1β and Il-6 were measured using commercially available ELISA kits (Thermo Fisher Scientific, Rockford, IL), following the manufacturer's instructions.

2.10. Mitochondrial parameters

Hippocampal mitochondrial isolation and oxygen consumption were performed as previously reported (Lama et al., 2017). Oxygen consumption was polarographically measured using a Clark-type electrode in the presence of substrates and ADP (state 3) or with substrates alone (state 4), and the respiratory control ratio was calculated. The degree of coupling was determined by applying equation by Cairns et al. (1998): *degree of coupling* = $\sqrt{1-(Jo)_{sh}/(Jo)_{unc}}$ where $(Jo)_{sh}$ represents the oxygen consumption rate in the presence of oligomycin that inhibits ATP synthase, and $(Jo)_{unc}$ is the uncoupled rate of oxygen consumption induced by carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which dissipates the transmitochondrial proton gradient. $(Jo)_{sh}$ and $(Jo)_{unc}$ were measured as above using succinate (10 mmol/l) rotenone (3.75 μmol/l) in the presence of oligomycin (2 μg/ml) or FCCP (1 μmol/L), respectively. The specific activity of superoxide dismutase (SOD) was measured spectrophotometrically, as previously reported (Flohe and Otting, 1984).

2.11. Oxidative stress assay

The levels of ROS were determined by the procedure of Montoliu et al. (1994). Briefly, fresh hippocampal homogenate was diluted in 100 mM potassium phosphate buffer (pH 7.4) and incubated with a final concentration of 5 μM dichlorofluorescein diacetate (Sigma–Aldrich) in dimethyl sulfoxide for 15 min at 37 °C. The dye-loaded samples were centrifuged and the pellet suspended in 100 mM potassium phosphate buffer and incubated for 60 min at 37 °C. The fluorescence measurements were performed with a HTS-7000 Plus-plate-reader spectrofluorometer (Perkin Elmer, Wellesley, Massachusetts, USA) at 488 nm for excitation and 525 nm for emission wavelengths. Reactive oxygen species (ROS) stress were quantified from the dichlorofluoresce in standard curve in dimethyl sulfoxide (0–1 mM).

2.12. 16S metagenomic sequencing and data analysis

Fecal samples were collected from a subset of six mice randomly selected from each group and quickly stored at −80 °C. Fecal microbiota was studied by performing V3-V4 16S rDNA amplicon sequencing in order to obtain the operational taxonomic units (OTUs) defining the bacterial communities. Sequencing samples from frozen fecal pellets were prepared, sequenced and subsequently processed as previously described (Coretti et al., 2017). To avoid sample size biases in subsequent analyses, samples were normalized to 10,595 sequences/sample using a sequence rarefaction procedure. To assess sampling depth coverage and species heterogeneity in each sample, alpha diversity metrics were employed on rarefied OTU tables using Good's coverage, Observed species and Shannon's diversity index. A two-sample permutation *t*-test, using 999 Monte Carlo permutations to compute p-value, was performed to compare the alpha diversities between sample groups. OTUs diversity among sample communities (beta diversity) was assessed by applying unweighted and weighted UniFrac distances. Statistical significance of beta diversities was assessed on phylogenetic distances matrixes using ANOSIM method with 999 permutations. Statistical differences in OTUs frequencies among groups across different taxonomic levels were assessed through one-way ANOVA followed by Tukey multiple comparison post-hoc tests.

2.13. Statistical analysis

Data are presented as mean ± SEM. All the experiments were analyzed using analysis of variance (ANOVA) for multiple comparisons followed by Bonferroni's *post hoc* test, using GraphPad Prism (GraphPad software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$ in all the statistical analyses.

3. Results

3.1. PEA reduces repetitive behaviour and increased sociability in BTBR mice

The effect of PEA on ASD-like behaviour was assessed by marble burying, self-grooming, and 3-chambered social test. To examine the repetitive/perseverative phenotype, mice of all groups were scored for the number of marbles they bury from the top of the bedding and for the time spent in home cage self-grooming. We observed that Vehicle treated BTBR mice buried a greater number of marbles (Fig. 1A; $p < 0.0001$) and displayed higher self-grooming scores (Fig. 1B; $p < 0.0001$) compared with control Vehicle treated B6. PEA (10 mg/kg) induced a slight improvement in the repetitive behaviour, whereas Bonferroni's post hoc analysis indicated that at the highest dose (30 mg/kg) PEA significantly decreased stereotyped behaviours in both marble buried assay (Fig. 1A; $F(3,36) = 23.78$, $p < 0.0001$) and self-grooming scores (Fig. 1B; $F(3,36) = 18.40$, $p < 0.05$).

To assess PPAR-α involvement in PEA effect, BTBR mice were

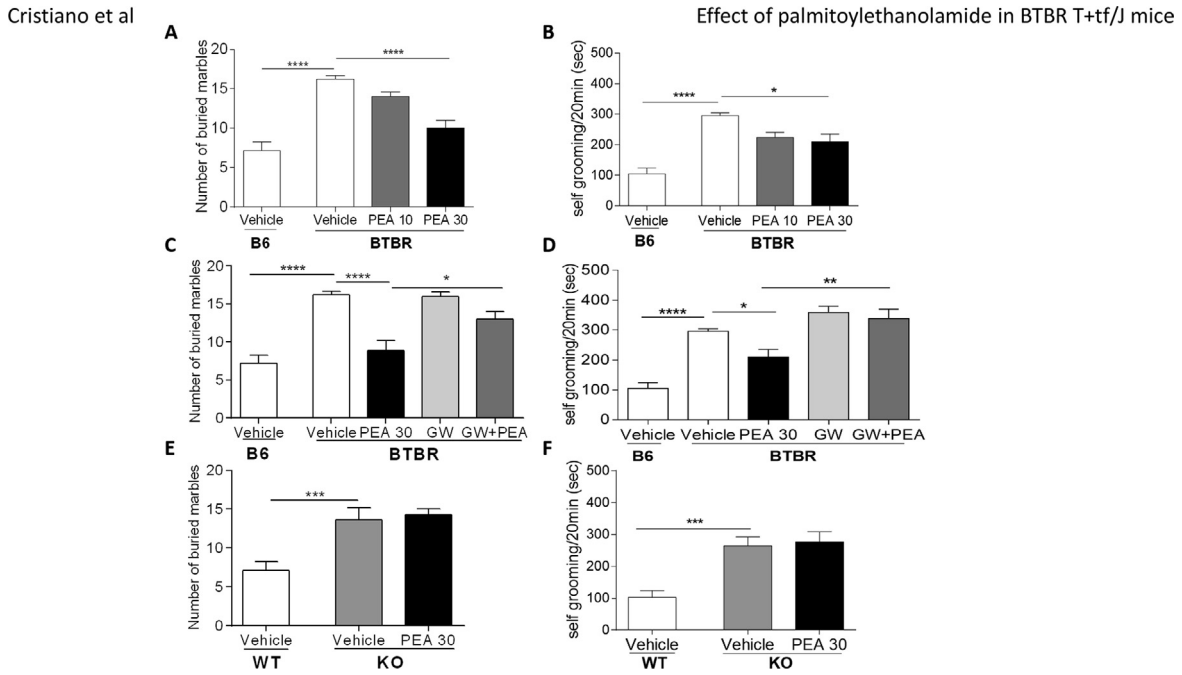

Fig. 1. Effect of PEA on repetitive stereotyped autist-like behaviour of BTBR mice and involvement of PPAR-α. (A-B) Number of buried marble and self-grooming time of control B6 and BTBR mice following i.p. daily administration of Vehicle, PEA (10–30 mg/kg) for 10 days (n = 10 each group). (C-D) number of buried marble and self-grooming time of control B6 and BTBR mice following daily GW 6471 (GW, 1 mg/kg i.p.) treatment alone or 1 hr before PEA (30 mg/kg) for 10 days (n = 10 each group). (E-F) Number of buried marble and self-grooming time of WT (n = 9) and PPAR-α null mice (KO) daily i.p. treated with Vehicle (n = 10) or PEA 30 mg/kg (n = 10) for 10 days. All data are presented as means ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

treated with the PPAR-α antagonist GW6471 before PEA administration. Bonferroni's post hoc analysis revealed that the highest dose of PEA failed in exerting its effect on both repetitive behaviours (Fig. 1C; $F(4,45) = 19.51$, $p < 0.05$. Fig. 1D; $F(4,45) = 22.01$, $p < 0.01$). The antagonist alone had no effect on BTBR mice in both behavioural paradigms. Consistently, PEA treatment did not modify the autistic-like traits showed by PPAR-α null mice (D'Agostino et al., 2015) in both behavioural tests (Fig. 1E; $F(2,26) = 10.42$, $p < 0.001$. Fig. 1F; $F(2,24) = 12.39$, $p < 0.001$), indicating the obligatory involvement of PPAR-α.

In addition, we investigated the possible effect of PEA on social preference, using the three-chambered social test, evaluating the time spent in the chamber with the object or with a mouse and the time spent in sniffing the object or the mouse. Vehicle treated BTBR mice failed to display significant sociability both on time in the zone (Fig. 2A; $p < 0.01$) and on time in social sniffing (Fig. 2B; $p < 0.01$) during the second phase of the automated three-chambered social approach task compared with Vehicle treated B6. PEA treatment improved sociability of BTBR mice: at the dose of 10 mg/kg, mice spent equal time in the zone and in sniffing the novel mouse and the novel object, while, at 30 mg/kg, they significantly spent more time in the mouse chamber and in sniffing the mouse compared to the object (Fig. 2A; $F(5,54) = 11.43$, $p < 0.001$; 2B; $F(5,54) = 9.280$, $p < 0.001$).

Then, PPAR-α involvement was assess in social behaviour of BTBR mice. Results showed that PEA failed to improve sociability both in time in the zone and in sniffing time of BTBR mice, when pre-treated with GW (Fig. 2C; $F(9,90) = 14.20p < 0.0001$; Fig. 2D; $F(9,90) = 8.701p < 0.001$). The antagonist alone had no effect on BTBR mice. PEA treated PPAR-α null mice showed equal time spent in the mouse zone and in sniffing time with the mouse and the object (Fig. 2E; $F(5,52) = 2.780$, $p = 0.1019$). Vehicle treated PPAR-α null mice showed a not significant preference for the object side (Fig. 2F $F(5,54) = 4.088$, $t = 1.436$).

During the last phase of the test, we analysed the time spent in area and the time spent in sniffing familiar or new mouse (Supplementary Fig. S1). All BTBR and B6 groups spent more time in the chamber with the novel mouse than the familiar mouse, as well as in sniffing time, indicating no behavioural impairment in this phase of the test in Vehicle- or treated BTBR mice. Instead, Vehicle- or PEA-treated PPAR-α null mice showed a significant preference for the familiar mouse, consistently with D'Agostino et al. (2015).

A single PEA administration (30 mg/kg i.p.) in BTBR mice was also tested, revealing 1 h after no effect on all behavioural tests (Supplementary Fig. S2) and then no early fast effect of PEA administration. Moreover, all behavioural skills were not affected by PEA in B6, indicating that this drug had no behavioural effects *per se* in control mice (Supplementary Fig. S3).

In order to exclude the induction of anxiety and/or locomotor deficits by drugs, we tested all mice in open field and elevated plus maze test, revealing no difference due to treatments, but only an overall difference between strains (Supplementary Fig. S4).

3.2. PEA induces PPAR-α expression and BDNF/TrkB system at hippocampal level

To strengthen PPAR-α role in autism, we subsequently evaluated PPAR-α expression in the hippocampus. BTBR mice displayed a significant reduction in PPAR-α mRNA ($p < 0.01$) and protein ($p < 0.001$) expression, that were significantly reconstituted by PEA treatment (30 mg/kg) at both mRNA ($p < 0.05$) and protein ($p < 0.01$) level (Fig. 3A; $F(2,25) = 5.754$. Fig. 3B; $F(2,5) = 12.84$).

Since impairment of BDNF/tropomyosin-related kinase B (TrkB) pathway can contribute to behavioural deficits related to autism, this pathway was also investigated. Indeed, Bonferroni's post doc analysis showed significant lower hippocampal level of BDNF and a not significant decreasing trend of TrkB in both mRNA ($p < 0.05$ and NS) and protein expression ($p < 0.05$ and NS) in BTBR mice compared with B6 control mice (Fig. 3C; $F(2,23) = 4,300$. Fig. 3D; $F(2,12) = 22.84$. Fig. 3E; $F(2,18) = 15.114$. Fig. 3F; $F(2,9) = 11,78$). PEA treatment (30 mg/kg) restored both BDNF (Fig. 3C; mRNA level $p < 0.05$.

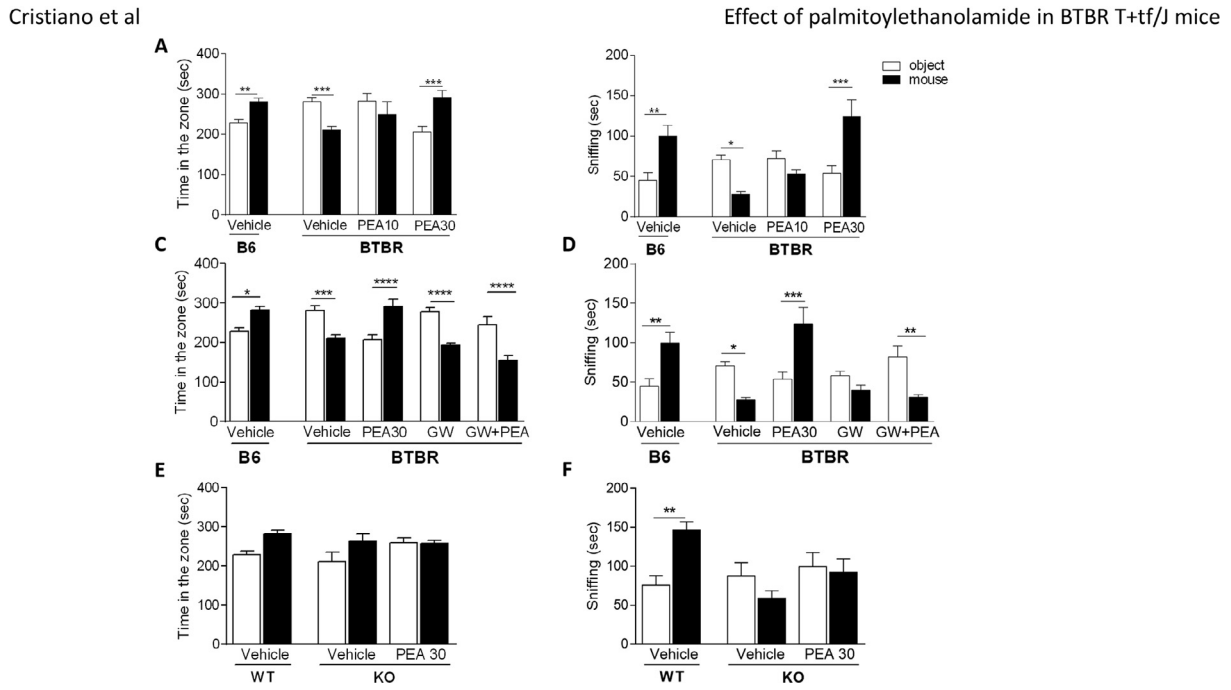

Fig. 2. Effect of PEA on social behaviour of BTBR mice and involvement of PPAR-α. (A) Time in the zone (B) and sniffing time during the second social preference phase of 3-chambered social test of control B6 and BTBR mice following i.p. daily administration of Vehicle, PEA (10–30 mg/kg) for 10 days (n = 10 each group). (C) Time in the zone (D) and sniffing time during the sociability phase of 3-chambered social test of control B6 and BTBR mice following daily GW 6471 (GW, 1 mg/kg i.p.) treatment alone or 1 hr before PEA (30 mg/kg) for 10 days (n = 10 each group). (E) Time in the zone (F) and sniffing time during the sociability phase of 3-chambered social test of WT (n = 9) and PPAR-α null mice (KO) daily i.p. treated with Vehicle (n = 10) or PEA 30 mg/kg (n = 10) for 10 days. All data are presented as means ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

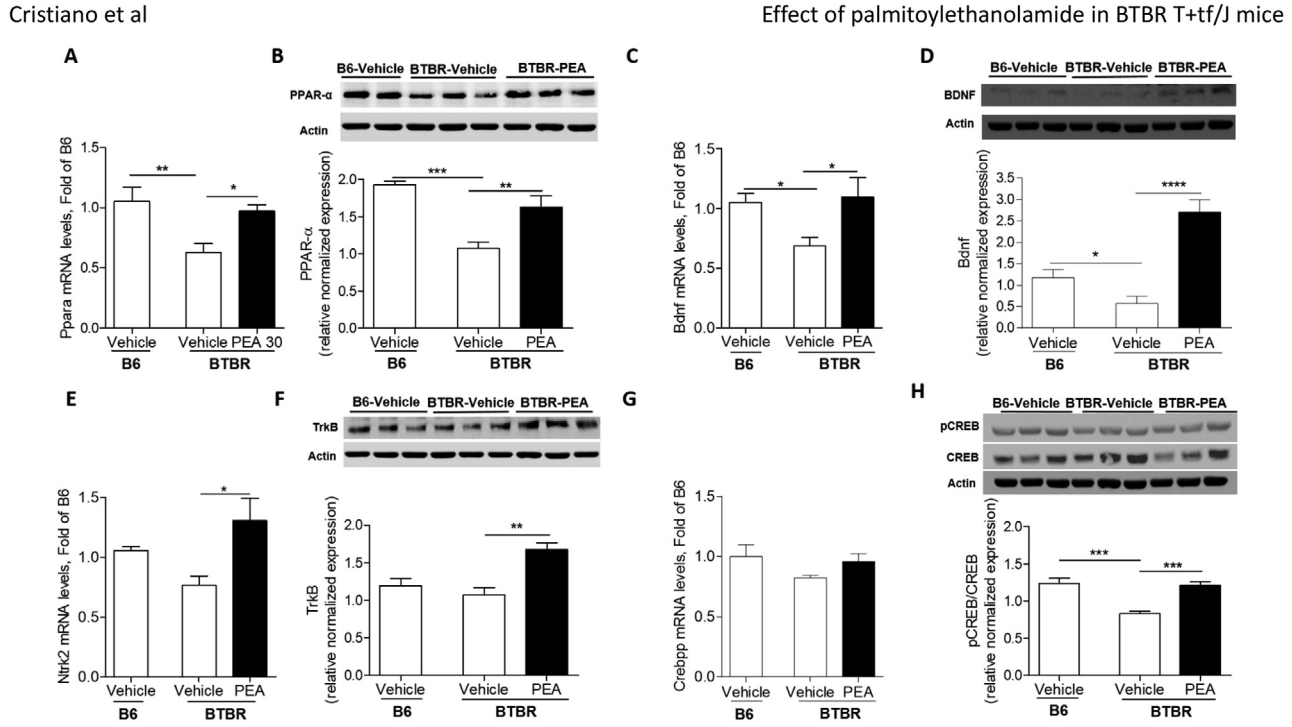

Fig. 3. PEA effect on hippocampal gene transcription and expression. RT-PCR and western blot detection of PPAR-α (A-B), Bdnf (C-D) and TrkB mRNA and protein (E-F) expression in the hippocampus from B6 and BTBR mice treated with Vehicle or PEA 30 mg/kg (n = 7–11 per group). RT-PCR of CREB (G) and western blot detection of p-CREB and CREB (H) protein expression in the hippocampus from B6 and BTBR mice following daily i.p. administration of Vehicle or PEA 30 mg/kg for 10 days (n = 6–8 per group). Representative western blot from a 2–3 animals each group following the above treatments are shown. Signals from each animal quantified and peptide expression shown as a ratio of housekeeping gene β-Actin (Actin) and with total CREB. Results are expressed as mean ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.001$.

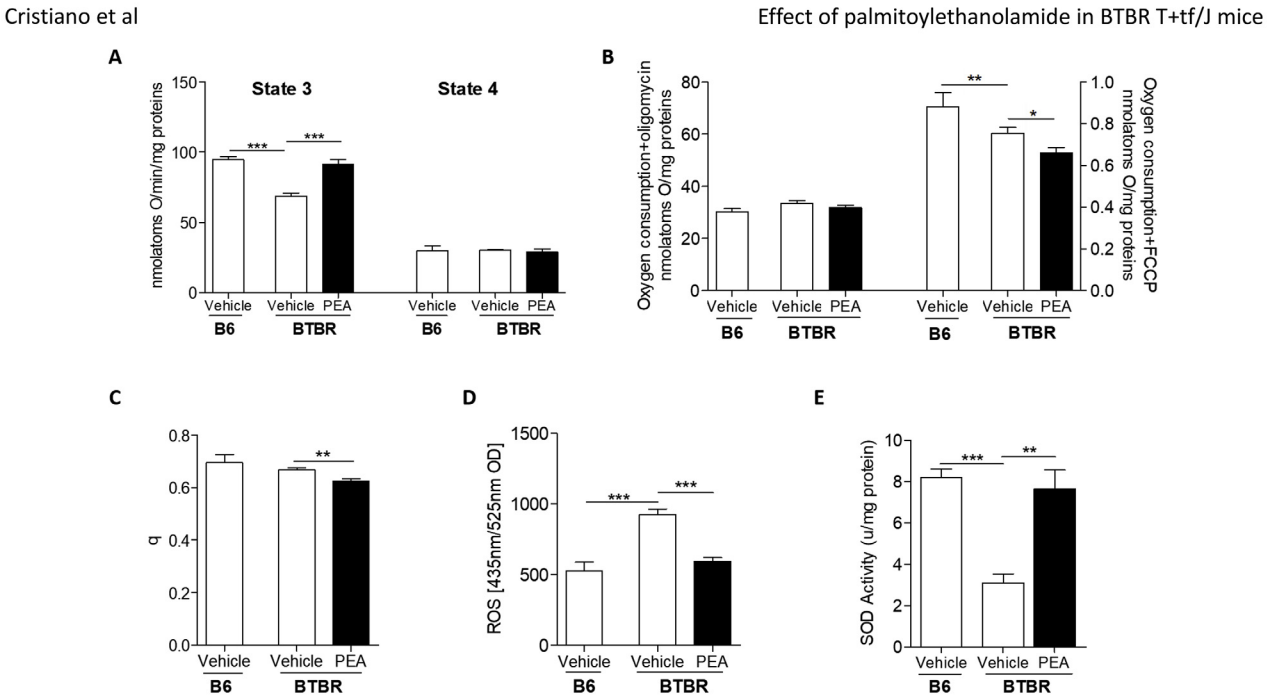

Fig. 4. Effect of PEA on mitochondrial and oxidative stress. (A) Mitochondrial respiration in the presence of succinate as substrate, (B) oxygen consumption in the presence of oligomycin (left side), or uncoupled by FCCP (right side) and (C) degree of coupling values calculated from oxygen consumption in the presence of oligomycin and uncoupled by FCCP in the hippocampus from B6 and BTBR mice following daily i.p. administration of Vehicle or PEA 30 mg/kg for 10 days (n = 12 per group). (D) ROS production in the hippocampus from B6 and BTBR mice following daily i.p. administration of Vehicle or PEA 30 mg/kg for 10 days (n = 12 per group). (E) SOD activity in the hippocampus from B6 and BTBR mice following daily i.p. administration of Vehicle or PEA 30 mg/kg for 10 days (n = 5) All data are presented as means ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Fig. 3D; protein level $p < 0.0001$) and TrkB expression (Fig. 3E; mRNA level $p < 0.05$. Fig. 3F; protein level $p < 0.01$) in BTBR mice. Therefore, activation of cAMP response element-binding protein (CREB) downstream this pathway was investigated. Results showed CREB mRNA level did not significant changed by PEA treatment (30 mg/kg) in BTBR mice (Fig. 3G; F(2,20) = 1,558), while, at protein level, the significant reduced phosphorylated state of CREB in BTBR mice ($p < 0.01$) was restored by PEA treatment ($p < 0.001$) (Fig. 3H; F(2,12) = 19.85).

3.3. PEA treatment improves hippocampal mitochondrial function and reduces oxidative stress

Mitochondrial state 3 respiration using succinate as substrate was decreased in BTBR mice compared with B6. Repeated treatments of PEA restored mitochondrial respiration (Fig. 4A left side; F(2,13) = 30.22, $p < 0.001$), whereas no variation was observed in mitochondrial state 4 respiration (Fig. 3A right side). No variation was found in oligomycin state 4 respiration, while a significant decrease was found in maximal FCCP-stimulated respiration in PEA treated mice compared BTBR mice (Fig. 4B). As a consequence, hippocampal mitochondrial energetic efficiency, assessed as degree of coupling, was significantly lower in PEA treated mice compared to BTBR mice (Fig. 4C F(2,33) = 3.700, $p < 0.01$).

ROS production was significantly increased in hippocampus from BTBR mice ($p < 0.01$) and blunted by PEA treatment (Fig. 4D; F(2,30) = 18.00, $p < 0.001$), similarly, BTBR mice showed a significant lower SOD activity ($p < 0.001$), which was restored by PEA (Fig. 4E; F(2,8) = 25.50, $p < 0.01$).

3.4. PEA reduces systemic and tissue cytokines

Brain inflammation has been associated with the pathogenesis and progression of ASD: in particular, pro-inflammatory cytokines correlate with impaired sociability and aberrant behaviour linked to ASD in both humans and mice (Heo et al., 2011; Theoharides et al., 2016). BTBR mice showed a significant increased level of TNFα ($p < 0.01$), IL-1β ($p < 0.01$), and IL-6 ($p < 0.01$) in the hippocampus compared to B6 mice and significant reductions of all cytokines TNFα ($p < 0.01$), IL-1β ($p < 0.05$), and IL-6 (NS) were found in BTBR PEA mice compared to BTBR group (Fig. 5A; F(2,18) = 11.84. Fig. 5B; F(2,14) = 9.711. Fig. 5C; F(2,18) = 9.096). In parallel, inflammatory cytokines were elevated also in colon ($p < 0.001$, $p < 0.001$, $p < 0.01$ showed in Fig. 5D F(2,17) = 72.61. Fig. 5E F(2,16) = 11.03. Fig. 5F; F(2,15) = 12.21) and serum (all $p < 0.001$. Fig. 5G; F(2,15) = 43.61. Fig. 5H; F(2,15) = 57.35. Fig. 5I; F(2,15) = 18.74) in BTBR mice compared to B6 control mice. Bonferroni's post doc analysis showed that all cytokine, TNFα ($p < 0.001$), IL-1β ($p < 0.05$), and IL-6 ($p < 0.01$) at colonic level, TNFα ($p < 0.001$), IL-1β ($p < 0.001$), and IL-6 ($p < 0.01$) as well as at serum level, were downregulated by PEA treatment (Fig. 5D-I). These results revealed both peripheral and central effects of PEA in autistic mice, opening a new scenario on the mechanism of action of PEA.

3.5. PEA effects on gut permeability and microbiota

BTBR mice are characterized by a compromised epithelial barrier integrity: a significant increase in gut permeability was shown in vivo in BTBR mice evaluating the plasmatic levels of FITC-labelled dextran at 24 h (Fig. 6A; F(2,16) = 9.311, $p < 0.01$). PEA improved epithelial barrier integrity ($p < 0.05$) of BTBR mice, increasing the transcription of *Tjp1* and *Ocln* mRNA transcripts (all $p < 0.001$) in colonic tissues found significantly reduced in BTBR mice compared to B6 control mice (all $p < 0.001$; Fig. 6B; F(2,20) = 37.03. Fig. 6C; F(2,26) = 11.32).

Faecal microbiota composition was analysed by next-generation sequencing method to evaluate the effect of PEA administration on gut

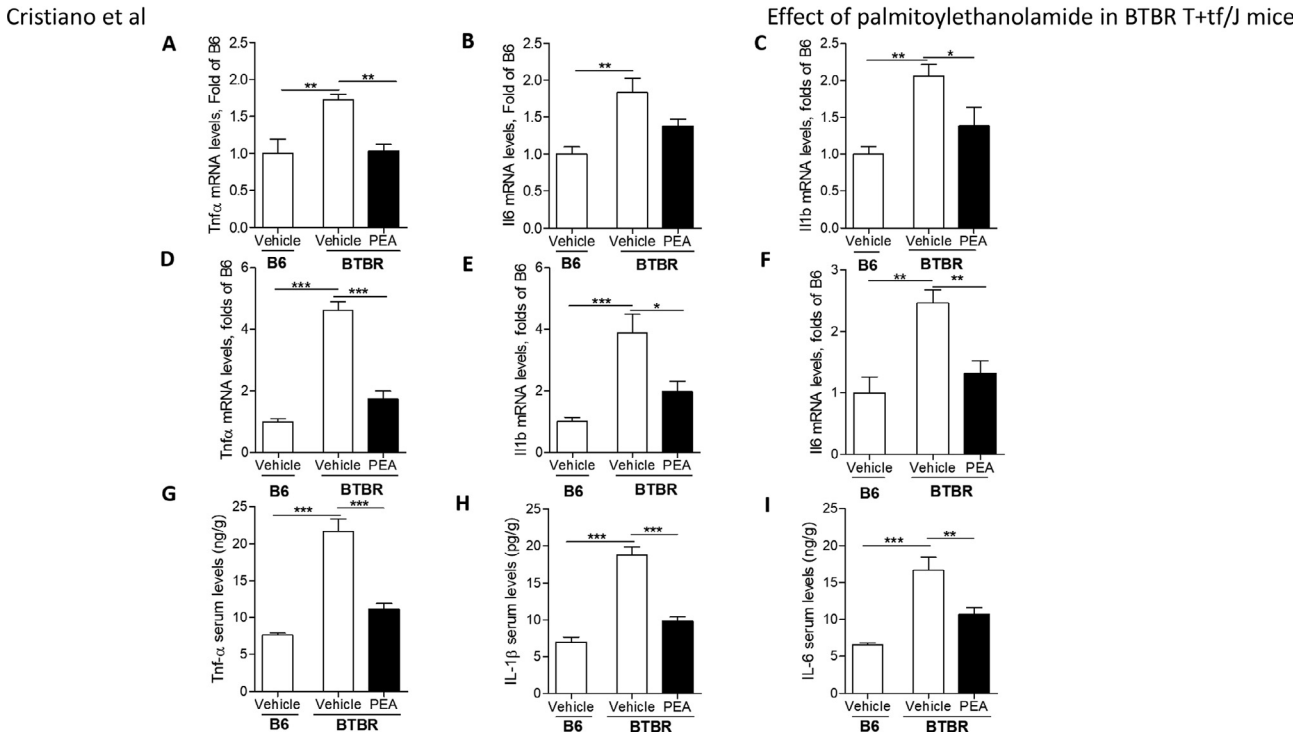

Fig. 5. PEA modulation of central and peripheral inflammatory cytokines. Changes in Tnf-α, IL-1β, and IL-6 mRNA levels in hippocampus (A-C), and colon (D-F), and cytokine analysis in serum (G-I) of B6 or BTBR mice following daily i.p. administration of Vehicle or PEA 30 mg/kg for 10 days (n = 6 per group). Results are showed as mean ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

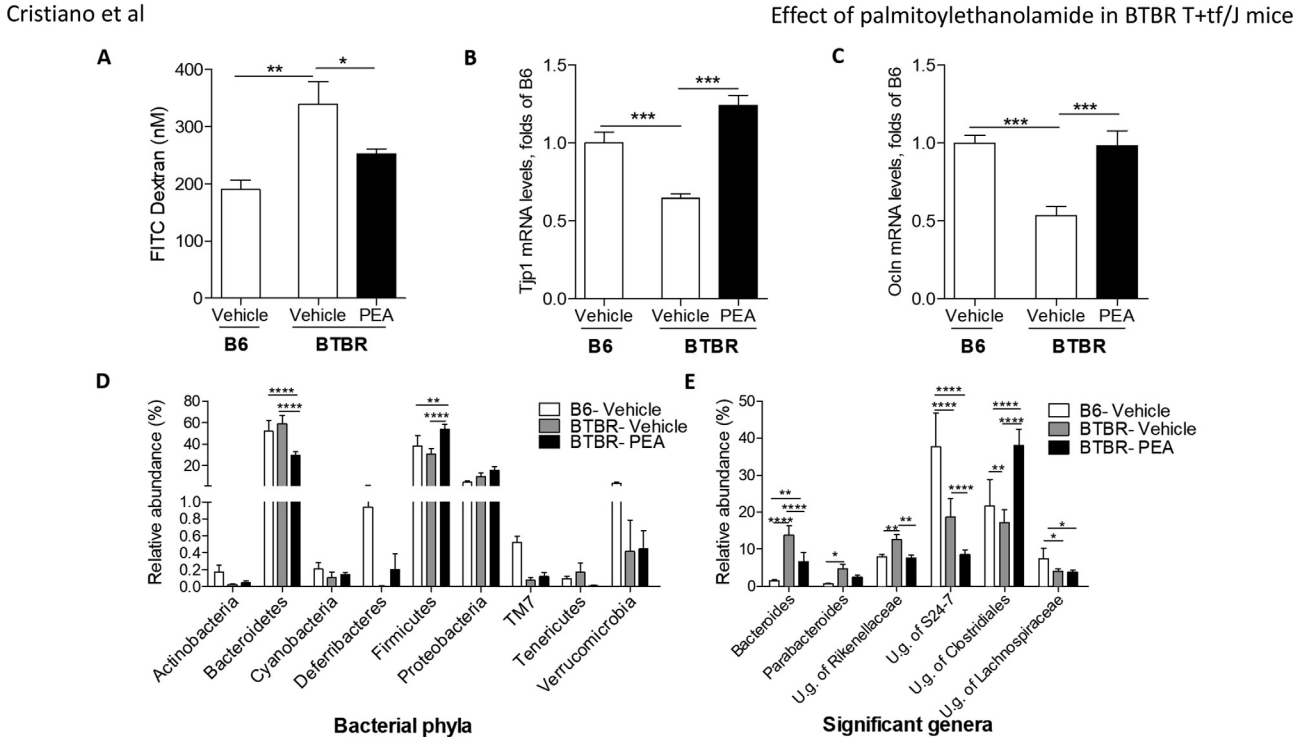

Fig. 6. PEA effect on intestinal permeability and faecal microbiota composition. (A) in vivo permeability detected by plasmatic levels of FITC-labelled dextran from B6 or BTBR mice following daily i.p. administration of Vehicle or PEA 30 mg/kg for 10 days (n = 6 per group). (B-C) changes in mRNA expression of *Tjp1* and *Ocln* in colon tissue from B6 or BTBR mice following daily i.p. administration of Vehicle or PEA 30 mg/kg for 10 days (n = 6 per group). (D) Phylum level composition of fecal microbiota from B6 and BTBR mice following daily i.p. administration of Vehicle or PEA 30 mg/kg for 10 days (n = 6 per group). (E) Bacterial genera belonging to Bacteroidetes and Firmicutes phyla found to be significantly different among groups (n = 6 per group). Results are showed as mean ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ $^{****}p < 0.0001$.

microbial communities of BTBR mice with respect to similar ages of cases of untreated BTBR and B6 control mice. Alpha diversity analyses did not show significant alteration in bacterial species richness and diversity within groups. On the contrary, measuring phylogenetic distances by beta diversity analyses, we found significant differences in microbial species assortment (unweighted beta diversity) among groups (see Table S1, supplemental materials and data). Interestingly, R statistic ANOSIM, computed on phylogenetic distances among samples, revealed that upon PEA treatment the overall microbial community of BTBR mice was restructured and resulted significantly different from that of untreated BTBR mice (Supplementary Table S1; BTBR vs BTBR-PEA, $R = 0.0522$; $p < 0.01$). PEA treatment strongly impacted on levels of definite identified phyla. Among the 9 phyla detected, Bacteroidetes and Firmicutes were the most abundant and primarily affected by PEA treatment (Fig. 6D; $F(16,135) = 4.049$); Firmicutes/Bacteroidetes ratio was considerably higher in BTBR mice treated with PEA due to significant increase in Firmicutes and decrease in Bacteroidetes (Fig. 5D; both $p < 0.0001$). At genus level, the increase of Firmicutes/Bacteroidetes ratio upon PEA administration was mainly a consequence of diminution of *Bacteroides* ($p < 0.0001$), *U. g. of Rikenellaceae* ($p < 0.01$), *U.g. of S24-7* ($p < 0.0001$) (phylum Bacteroidetes) and increase of *U.g. of Clostridiales* ($p < 0.0001$) (phylum Firmicutes) (Fig. 6E; $F(162,1230) = 5.216$). Thus, PEA treatment establishes a new microbiota profile, marking differences with microbiota composition found in untreated BTBR mice and recovering similarities for some aspects with microbial assortment of B6 control mice.

4. Discussion

In this study, we highlight the beneficial and dose-dependent activity of PEA on repetitive and stereotypic impairment, and social behaviour in BTBR autism mouse model, and shed light on the contributing central and peripheral mechanisms, that may be involved in this effect.

Here, we have demonstrated that the positive effect exerted by PEA is contingent to PPAR-α activation, since receptor blockade by its antagonist blunted the beneficial behavioural effect evoked by PEA in BTBR mice. According to behavioural results, we found significant lower PPAR-α mRNA and protein expression in the hippocampus of BTBR compared to B6 mice that was increased after PEA treatment of BTBR mice. Moreover, this acylethanolamide failed in inducing its effect in ameliorating stereotyped behaviours in PPAR-α KO mice, which exhibit a similar phenotypic-like autism behaviour in these tasks, differently from social interaction that was not significantly altered.

In BTBR mice, the higher dose of PEA significantly improved repetitive behaviour in two behavioural tests and sociability in the three-chambered assay. We excluded possible drug-induced confounding hyper- or hypo- activity during behavioural assays by open field and elevated plus maze tests in all strains. Moreover, a single PEA administration had no effect on all behavioural tests, indicating no early rapid effect of PEA administration, whereas pharmacological effect appears only after repeated administrations, most likely due to the consolidation of PEA-induced mechanisms for a delayed effect.

Only recently, limited literature shows the possible therapeutic potential of PEA in ASDs. Specifically, Bertolino et al. (2017) found that PEA combined with Luteolin ameliorates social and non-social impairment in VPA-induced ASD in mice and reported a reduction of behavioural alterations of an autistic child. Indeed, previously Antonucci et al. (2015) had already published the ameliorating effect of PEA treatment in two teen-age boys, displayed as an improvement in cognitive and behavioural skills. Very recently, PEA has been proposed as an adjunctive therapy in autistic children receiving risperidone (Khalaj et al., 2018), since the co-treatment was more effective than risperidone plus placebo, on irritability and hyperactivity symptoms, suggesting once again the potential of PEA in counteracting the core symptoms of autism.

Autism is a multifactorial disorder, where, many genes converge, through multiple molecular mechanisms, onto common processes involved in the development and function of neural circuits, whose alteration leads and sustains the core symptoms of ASDs (Geschwind, 2008). Evidence based on environmental factors, such as infection, gut microbiome alteration, and immunological features are also growing, suggesting the involvement of many scenarios worth investigating. Obviously, even if targeted specific treatments would clarify the relevance of the altered pathways or functions in ASDs, on the other hand the complex heterogeneity in ASDs children population could require a multi-targeted approach. Here, we analysed the modulatory effect of PEA on many of the pathways and factors consistently associated to ASDs pathology and symptoms.

Up to date, PEA has proven to be a multi-target compound: the main pharmacological effects of PEA are mediated by the activation of PPAR-α (Lo Verme et al., 2005; LoVerme et al., 2006), a transcription factor involved in the regulation of gene networks, which control pain and inflammation (D'Agostino et al., 2009). This mechanism is supported by the inhibition of neuronal firing (Hansen, 2010) and by the stimulation of neurosteroid synthesis, indicating that multiple mechanisms contribute to the central effect of PEA (Sasso et al., 2010). Indeed, PEA levels seems to elevate in injured brain under pathophysiological stimuli, as shown in glutamate-treated neocortical neurons ex vivo (Hansen et al., 1997) and in cortex after CNS injury (Berger et al., 2004; Franklin et al., 2003), suggesting its key physiological role in maintaining cellular homeostasis when faced with external stressors and neuroinflammation. Consistently, tissue levels of PEA, as well as of other N-acylethanolamines, were higher in the hippocampus of VPA-exposed rats immediately following social exposure, indicating the involvement of fatty acid amide alteration in behavioral abnormalities observed in ASDs (Kerr, Downey, Conboy, Finn and Roche, 2013).

A recent study showed that PPAR-α activation prevents cognitive deficit (Greene-Schloesser et al., 2014) and increased hippocampal neurogenesis after cerebral ischemia (Xuan et al., 2015). Indeed, this receptor at hippocampal level modulates the expression of various plasticity-related molecules and their functions via direct transcriptional control of the master regulator CREB (Rivera et al., 2014; Roy et al., 2013). Interestingly, an alteration of BDNF/TrkB signalling pathway has been correlated with autism (Stephenson et al., 2011). Consistently, a significant reduction of BDNF-induced potentiation of synaptic transmission was found in hippocampus from BTBR (Daimon et al., 2015; Scattoni et al., 2013). Here, we found lower BDNF level and no significant changes of TrkB expression between BTBR and B6 mice; while repeated administrations of PEA increased both mRNA and protein levels of BDNF and TrkB compared with BTBR mice. The involvement of BDNF signalling pathway in the molecular mechanisms contributing to PEA effect, was confirmed by the increase in the phosphorylated state of CREB transcription factor, where several pathways downstream TrkB activation were shown to converge, in order to upregulate gene expression to exert pro-survival effects and synapse-enhancing function.

Apart from factors involved in synapse development and plasticity, ASD pathogenesis also comprises other disparate alterations, including mitochondrial dysfunction, dysregulated immune response and chronic neuroinflammation. Mitochondria, the primary cellular energy-generating system, produce key factors during inflammation and oxidation and constitute the main source of free radicals. Therefore, mitochondrial dysfunctions are related to inflammation and other energy-dependent disturbances, where the generation of ROS exceeds the physiological antioxidant activity, causing cellular oxidative damage (Chan, 2017). Due to the higher mitochondrial energy demand of the brain compared to other tissues, subtle changes in mitochondrial energy production preferentially affect the brain. Therefore, mitochondrial dysfunction may be a key feature common to a wide spectrum of neurological and neurodevelopmental diseases, including ASDs (Pei and Wallace, 2018). For example, altered expression of electron

transport chain genes have been observed in autism (Anitha et al., 2013). Accordingly, hippocampal mitochondria in BTBR mice exhibited a reduced respiratory capacity, as indicated by the decrease in succinate State 3 oxygen consumption, which would partially block electron flow within the respiratory chain and consequently increase oxidative stress. Further, the decreased SOD activity could contribute to excessive ROS formation in BTBR mice. Notably, PEA treatment restored mitochondrial function and decreased oxidative stress partially due to the increase in SOD activity. A concomitant decline in mitochondrial energy efficiency, as evidenced by the decreased degree of coupling in PEA treatment, may also contribute to counteract excessive ROS formation in PEA-treated BTRB mice. In fact, one of the postulated roles of uncoupling is known to be the maintenance of mitochondrial membrane potential below the critical threshold for ROS production (Korshunov et al., 1997). Together, these findings indicate that PEA counteracts mitochondrial dysfunction and helps rescue brain energy metabolism during pathological states, balancing ROS production/antioxidant defences and limiting oxidative stress. Therefore, PEA could limit all those events related to excessive ROS, such as the destruction of cellular components including lipids, protein, and DNA, and ultimately cell death via apoptosis or necrosis. Moreover, oxidative stress could trigger redox-sensitive transcription factors and pro-inflammatory gene expression, sustaining the ongoing inflammatory state.

Many psychiatric disorders, as well as ASDs, are associated with distinct inflammatory mechanisms in the periphery and in the CNS. Neuroinflammation is driven by glial cells, in particular, activated microglial cells acquire a M1 phenotype, synthetizing pro-inflammatory cytokines, such as TNF-α IL-1β, and IL-6. Indeed, all these cytokines were found increased in the brain and blood from BTBR and VPA-treated mice (Cipriani et al., 2018), a pro-inflammatory transcription profile related with the autistic traits in both mouse models. These alterations were also found in ASDs patients, where cytokines were elevated both at CNS and serum level (Ahmad et al., 2018; Masi et al., 2015; Vargas et al., 2005), strengthening the overall inflammation as a typical feature shown in ASDs more than in other central disorders. It has been underlined that the inflammatory state is also mediated by the gastrointestinal system, due to increased permeability, synthesis of cytokines and alteration of gut microbiota composition (Coretti et al., 2017). Furthermore, BTBR mice show a number of immune abnormalities, many of which are also observed in children with ASDs (Onore et al., 2012). Here, in BTBR mice PEA was able to reduce cytokine production, decreasing the inflammatory state at colonic, central and systemic levels, and improved intestinal permeability. Therefore, it is conceivable to hypothesise that systemic administration of PEA could directly impact neuroinflammation, being able to cross the blood brain barrier, but also indirectly reduce peripheral inflammatory input to the brain, through its anti-inflammatory properties at colonic and systemic level. Therefore, our hypothesis is that PEA would reduce the trafficking of inappropriate gut-derived detrimental factors into the portal circulation and the crossing through a permissive blood–brain barrier, leading to a reduction in systemic and ultimately central inflammation. Actually, it was already proposed by de Theije et al. (2014) an involvement of gut-brain axis in ASD by multiple ways, including cytokines deriving from colonic inflammation, which could elicit and/or sustain an immune response influencing at central level the altered behaviour in ASDs.

Finally, PEA peripheral effect could be also mediated by immune alteration in the gastrointestinal tract, in turn possibly modulated by gut microbiota changes. In fact, we observed a global alteration of faecal microbial communities and, in particular, a variation in proportion of Firmicutes and Bacteroidetes in BTBR mice upon PEA treatment. A robust augment of Firmicutes, mainly due to the increase of Unclassified genus of Clostridiales, to the expense of Bacteroidetes, is the most evident alteration induced by PEA administration. Clostridia include the majority of species able to produce butyrate as metabolic product of anaerobic fermentation. Indeed, butyrate has a prominent and beneficial role in the maintenance of gut physiology in terms of energy source for colonic epithelial cells, regulation of gut barrier permeability, suppression of inflammation, modulation of immune functions (Canani et al., 2011). Even though further analyses are needed to identify definite bacterial species modulated by PEA treatment, it is conceivable that its positive effect on gut homeostasis may be also associated to the remodelling of gut microbiota composition.

In conclusion, PEA showed to be a multifunctional compound, worthy to be considered against complex diseases, such as autism and its comorbidities, due to its pleiotropic mechanism of action, supporting neuroprotection, anti-inflammatory effects, and the modulation of gut-brain axis, through restoring gut integrity and remodelling gut microbiota composition.

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Conflict of interest statement

All authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbi.2018.09.003.

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