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Maternal treatment with sodium butyrate reduces the development of autism-like traits in mice offspring

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ABSTRACT

Several studies indicate a relationship between maternal gut microbiota alteration and increased risk of autism spectrum disorders (ASD) in offspring. The possibility of compensating for such metabolic dysfunction at a very early stage of disease via maternal treatment has not been enough explored. Here, we examined in BTBR mouse model of ASD the effect of maternal treatment with the gut microbial metabolite butyrate (BUT) on the behavioral and synaptic plasticity deficits in juvenile and adult offspring. We show that BUT treatment of BTBR dams rescues the social and partially the repetitive behavior deficits in the offspring. In addition, maternal BUT implementation prevents the cerebellar cortex hypertrophy as well as the Purkinje cells firing and long-term synaptic plasticity deficits in BTBR mice. Our results demonstrate, for the first time, that maternal BUT treatment can improve ASD-like symptoms in offspring thus providing new directions for the early treatment of neurodevelopmental disorders.

1. Introduction

Autism spectrum disorders (ASD) is a childhood neurodevelopmental condition, characterized by impaired social communication and social interaction as well as restricted and stereotyped behavioural patterns, interests, and activities [1,2]. It is considered a multifactorial disease resulting from a combination of genetic, epigenetic, and environmental risk factors [3,4].

Multiple studies indicate that complications during pregnancy such as infection, obesity, stress, and gastrointestinal (GI) disorders represent an important risk factor for ASD in offspring [5–14]. These risk factors are often associated with an imbalance of gut microbiota which may lead to long-term cognitive and behavioral deficits through the gut-brain axis [15]. Since a healthy state of the maternal intestinal microbiome impacts fetal neurodevelopment [12,13,16,17], microbiota-targeted therapies may be a strategy for reducing the severity of ASD symptoms [18–20].

Among the microbiota-derived metabolites, butyrate (BUT) is a short-chain fatty acid (SCFA) produced in the colon by bacterial fermentation of dietary fibers and resistant starch. In addition to exerting local effects in the gut, BUT has neuroactive properties influencing neurological and behavioral processes. In particular, at the intestinal level, BUT inhibits the histone deacetylases, reduces the expression of intestinal pro-inflammatory mediators [21], and regulates the gut permeability [22]. Due to its ability to cross the blood-brain barrier [23], BUT has pro-cognitive effects counteracting memory deficits in Alzheimer's Disease [24,25] and aging-related memory decline [26–28]. In addition, BUT has antidepressant effects [29,30], and may attenuate anxiety-like behavior [31].

A recent study indicates that microbial metabolites are altered in ASD subjects that show low levels of fecal acetic acid and butyrate and a high level of fecal valeric acid [32]. According to Kratzman and collaborators [33], treatment with BUT attenuates social deficits in an ASD mouse model by regulating gene transcription in the prefrontal cortex. Although several studies indicate that the dysregulation of microbial metabolites plays a role in the development of central nervous system (CNS) disorders leading to long-term cognitive and behavioral deficits [15], the beneficial effect of the maternal intake of BUT on ASD

Abbreviations: ASD, Autism spectrum disorders; BUT, butyrate; SCFA, short-chain fatty acid; BTBR, BTBR T + tf/J; B6, C57Bl/6 J; P, postnatal day; GL, granular layer; ML, molecular layer; LTP, long-term potentiation; LTD, long-term depression; PC, Purkinje cells; PF, parallel fiber; PFC, prefrontal cortex.

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behavioral and neuronal abnormalities in the offspring has never been investigated. To address these questions, we used an inbred BTBR T + Itpr3tf/J (BTBR) mouse strain which shows a range of ASD-like behavioural deficits, including low levels of sociability, altered communication, and repetitive/compulsive behaviors [34–36], associated with gastrointestinal dysfunction and altered microbiota composition [37,38]. In these mice, we examined whether the BUT treatment of BTBR dams, from mating to weaning, prevents the behavioural alterations in the offspring during juvenile and adult life. To determine the effects of BUT-treatment on the structural and functional neuronal alterations we focused our investigation on a specific brain region such as the cerebellum whose development extends from the early embryonic period until the first postnatal years, and therefore it is particularly sensitive to the effects of early-life manipulation [39]. Besides its role in balance and motor functions, the cerebellum is also involved in cognitive function and social interaction [40,41]. Cerebellar alterations have been observed in ASD patients and animal models in correlation with social and communication impairments as well as restricted interests and repetitive behaviors [42–48]. Therefore, the analysis of cerebellar alterations in BTBR offspring might provide new insights into how BUT treatment targets brain dysfunctions in ASD.

2. Materials and methods

2.1. Animals

BTBR T + tf/J (BTBR) mice and their control C57Bl/6 J mice (B6) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The colonies were maintained in our animal facility housed in a room provided with controlled temperature and humidity, 12 h:12 h light: dark cycle, ad libitum access to water, and a standard laboratory chow diet. 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 3 R concept) and approved by the Italian Ministry of Health under protocol no. 140/2016-PR.

2.2. Treatment

Breeding pairs of BTBR and B6 mice were randomly assigned to receive either standard drinking water or water supplemented with 30 mg/kg BUT (Sodium butyrate 98%, Sigma Aldrich, St. Louis, MO). BUT treated and untreated mice consumed the same daily volume of water. BUT was dissolved in daily drinking water considering that each mouse consumes 4 ML/day. The concentration of BUT in the water bottle was adjusted for body weight. The butyrate supplementation continued through mating, gestation, and lactation; after weaning (at P18) pups were moved to a new cage with ad libitum access to standard water and a standard laboratory chow diet (without BUT supplementation), see Fig. 1. The dose of BUT was selected based on our previous studies proving its tolerance and safety in mice [49,50].

2.3. Experimental design

Breeding pairs (3–4 months old) were randomly selected from the same cages of males and females, to ensure that there was no difference in the microbiome composition between the experimental and control groups at the beginning of the experiment. After spending 2 weeks in a cage with a male, each female was moved to an individual cage. The offspring coming from the same litter was weaned at P18 and separated by sex in groups of up to six per cage. Eight different mating pairs were used for each experimental condition: control B6, B6 from BUT-treated B6 dams, BTBR, and BTBR from BUT-treated BTBR dams. For the experiments we used all male offspring, while females were excluded to avoid gender bias and microbiota variability [37,51,52]. Moreover, to minimize the litter effects, mice from the cage were randomly assigned for behavioural, histological, or electrophysiological analysis at postnatal day 30 (P30) or 60 (P60).

2.4. Behavioural experiments

On P30 and P60, eight male mice from different litters were subjected to behavioural tests during the light period (between 10:00 and 14:00) [53–56]. To minimize the carryover effects, the tests were performed in the following order by all mice, three-chamber social approach, marble burying, self-grooming test. Mice were moved into the testing area at least 1 h prior to testing for acclimatization. Apparatuses were cleaned with 70% ethanol after each test (EtOH; Sigma-Aldrich, St. Louis, MO, USA).

The marble burying and self-grooming tests were performed to assess repetitive behaviour, while the three-chambered social test was performed to assess sociability. Manual scoring with a stopwatch (for marble burying and self-grooming tests) was performed by a trained observer blind to treatment, and automatic scoring (three-chamber social test) was performed using the AnyMaze video tracking system.

2.4.1. Marble burying test

Each mouse was individually placed in a plastic cage with 20 glass marbles (1.5 cm in diameter) placed on the woodchip bedding (3 cm high) and arranged in five rows of four. The mouse was allowed to freely explore the cage for 15 min. Then it was removed from the testing container and placed in its home cage. The number of marbles buried was counted considering as buried a marble covered by 75% with bedding. After the test, the marbles were thoroughly cleaned, and new bedding was used for each mouse [57].

2.4.2. Spontaneous self-grooming behaviour

Mice were individually placed in an empty plastic cage (28 cm wide × 17 cm long × 12 cm high) filled with 1-cm layer of bedding to reduce neophobia and prevent digging behavior and allowed to freely explore the testing cage for 20 min. The first 10 min was considered as a habituation period. Using the AnyMaze video tracking system we measured the total distance travelled and the time spent in centre defined as the central 50% of the cage. During the following 10 min, the time spent in grooming was manually scored from a video recorded by a

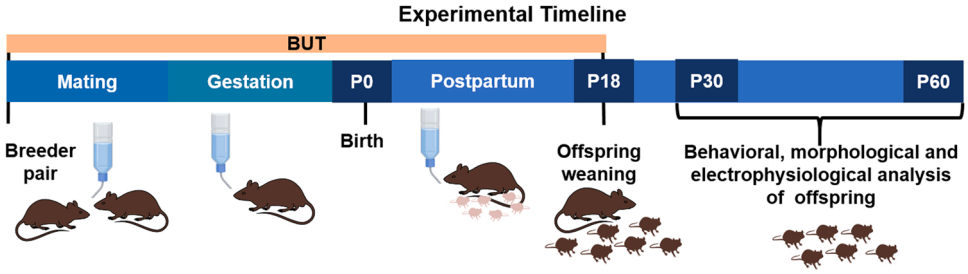

Fig. 1. Experimental timeline.

trained observer, so that that the observers were unable to identify the strains by the fur color. Grooming behaviour included head washing, body grooming, genital/tail grooming, and paw and leg licking [54]. After the test, the cage was thoroughly cleaned.

2.4.3. Social approach test

Social approach behaviour was tested in a three-chambered apparatus [Plexiglas box: each chamber measures 20 cm (length) × 40.5 cm (width) × 22 cm (height) with small opening doors (10-cm width × 5-cm height)], as previously described [54,58]. The test consisted of 5-min of habituation in the empty apparatus where the mouse was placed and allowed to explore the middle-chamber with the dividers closed. In the following 10-min session the dividers were raised allowing the test subject to move freely throughout all three chambers of the apparatus to assess the social behavior. A stranger mouse was placed in one of the two side-chambers under an enclosed grid cup which allowed visual, olfactory, auditory and some tactile contact between the mice, but prevented fighting; another identical empty cup was placed on the other side. The stranger mouse was of the same strain and sex but from different litters and cage (located in a different room) and was previously habituated to the cup for three 30-min sessions for 2 days. The stranger location in the left vs. right side chamber was systematically alternated between trials. A total number of 2 strangers were used for each strain and each stranger interacted with N. 8 subjects (4 BUT-treated and 4 untreated mice). A videocamera coupled with a video-tracking software (Any-maze, Stoelting) allowed us to measure the time spent in each chamber and sniffing, i.e. touching the cup with the nose, head and forelimbs. Lack of innate side preference was confirmed placing the subject mouse in an entirely empty apparatus, with no wire cups, for 5 min before starting the test. The measure of preference for social novelty was not reported, since this is not relevant to autism-like symptoms as sociability [38,59,60].

2.4.4. Maternal behaviour

Maternal behavior in postpartum females was assessed from postpartum days 2–7 on seven dams (modified from [61]). A video camera was mounted above the cage and the observations were conducted in the colony room three times per day (9.00 a.m., 3.30 p.m., and 6.30 p.m.) for 15 min without handling or perturbing the animals. We evaluated both maternal and nonmaternal behaviors. The maternal behavior (MB) included nursing (N) [dams were nursing in any position], licking the pups (L) [each time dams were liking their pups], physical contact with the pups (C) [dams were in the nest in contact with pups but not feeding or licking them]. The non-maternal behavior (NMB) included no interaction with pups (X) [any behavior without contacts with pups excepted eating or drinking] and eating/drinking (E). The maternal (MB) behavior and non-maternal behavior (NMB) were calculated using the sum scores [MB = $N + L + C$] and [NMB = $X + E$], respectively. The index of maternal behavior was then obtained using the formula [MB/(MB + NMB)].

2.4.5. Pup retrieval

On postpartum days 3, dams and pups were moved to a testing room and given 30 min to habituate to the new environment. The dam was sequestered on the nest using a cardboard barrier while the pups were scattered randomly around the home aquarium. The dams were then given up to 10 min to retrieve 6 pups to the nest. The latency to retrieve the first pup [the interval between removal of the cardboard barrier and return of the first pup back to the nest] and the latency to retrieve all pups [the total time between removal of the cardboard barrier and return of all pups to the nest] were scored in [62,63].

2.5. Plasma BUT extraction

The offspring BUT plasma level was measured at P1, P18 and P30 (n = 6 mice for group), while the maternal BUT plasma levels was measured on postpartum day 1 (n = 5 dams) and day 18 (n = 8 dams).

For BUT detection in the blood, plasma samples were acidified with 20 µl 85% (w/v) of H3PO4, mixed for 5 min and incubated on ice for other 5 min. The acidified samples were extracted by adding ethyl acetate (1:1, v/v), mixed for 5 min and then centrifuged for 20 min at 12,000g at RT. Finally, the organic extract (containing BUT) was carefully removed and transferred into a new glass tube for GC-MS analysis. A standard curve (1–50 µg/ML) was generated at the beginning of the run. A blank solvent (ethyl acetate) was injected between every sample to ensure no memory effects.

2.6. Gas-chromatography mass spectrometry (GC/MS) analysis

The GC column was an Agilent 122–7032ui (DB-WAX-U, Agilent Technologies, Santa Clara, California, USA) of 30 m, internal diameter of 0.25 mm, and film thickness of 0.25 µm. The GC was programmed to achieve the following run parameters: the initial column temperature was set at 90 °C, hold of 2 min, and then increased to 100 °C at a rate of 2 °C/min, hold of 10 min, finally ramp of 5 °C/min up to a final temperature of 110 °C for a total run time of 21 min, gas flow of 70 ML min− 1 splitless to maintain 12.67 p.s.i. column head pressure, and septum purge of 2.0 ML min− 1. Helium was the carrier gas (1.5 ML min−1 constant). The parameters of mass spectrometer were source at 230 °C and MS Quad at 150 °C.

2.7. Histological analyses

Histological analyses were performed as previously described [64] on control and BUT-treated BTBR offspring mice at both P30 and P60. Mice were anesthetized using a cocktail of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) via intraperitoneal injection. The mice were intracardially perfused with 4% paraformaldehyde in 0.12 M phosphate buffer, pH= 7.2–7.4. Following perfusion, the brains were removed and post-fixated in 4% paraformaldehyde at 4 °C overnight. The day after brains were transferred to a solution made of 30% sucrose in 0.12 M phosphate buffer. The cerebellum was separated and embedded in an optimal cutting temperature compound (Killik, O.C.T., Bio-Optica Milano SPA) and frozen in ice-cold isopentane. Cerebella were serially cut by a cryostat in 30 µm-thick sagittal slices and collected in phosphate-buffered saline (PBS).

For Cresyl Violet Staining (Nissl Staining) free-floating sections were washed twice in PBS. The series was mounted on gelatin-coated slides and let air dry. Mounted series were washed for 2 min in distilled water to remove any residual salts and then stained in 0.1% Cresyl violet solution for 15 min. Sections were then rinsed again in distilled water for 2 min and dehydrated using a series of alcohols: 50% (2 min), 70% (2 min), 95% (I) (2 min), 95% (II) (few seconds) and 100% (2 min). Next, the slides were immersed in xylene for 5 min and finally, a clear glass coverslip was applied using a permanent mounting medium.

Slides were scanned with Slide-Scanner Axiscan Z1 (ZEISS, Oberkochen, DE) both at low and high magnification (5x and 20x) then modified and adapted in colour, contrast, and brightness with Zen Software 2.1 (ZEISS, Oberkochen, DE) and analyzed using ImageJ software (1.52 t version). Only cerebellar vermis sections were taken into consideration for the morphological study. To evaluate cerebellar architecture, we measured for each slice the perimeter and the total area as well as the area of white matter, molecular and granular layers on the whole extent of each lobule. At least four vermis slices/animal of four animals were analyzed. All measurements were done blind relative to the mouse genotype and treatment.

2.8. Electrophysiological recordings

Cerebellar slices were prepared as previously described [65,66] from B6 and BTBR mice of both sexes at P30 and P60. Briefly, the mice were anesthetized with isoflurane, USP (Abbott Laboratories, Illinois, USA),

and decapitated. The cerebellar vermis was removed and placed in an ice-cold artificial cerebrospinal fluid (ACSF) containing (mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 20 glucose. The pH was maintained at 7.4 by bubbling with 95% O_2/5% CO_2. Parasagittal cerebellar slices (200 μm thick) were cut using a vibratome (Vibroslice 752, Campden Instruments, Loughborough, UK) and kept for at least 30 min at 32 °C for recovering and then at room temperature, at about 25 °C [67]. Each slice was transferred to a recording chamber mounted on an upright microscope (BX50WI, Olympus, Japan) and perfused with oxygenated ACSF.

Whole-cell patch-clamp recordings were done from Purkinje cells (PCs) using an EPC-8 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Pipettes of borosilicate glass with resistances between 2.5 and 3.0 MΩ were used for recordings.

PC firing activity was examined in the current-clamp mode. Patch pipettes were filled with a K-gluconate-based internal solution containing (in mM) 140 K-gluconate, 10 HEPES, 0.5 EGTA, 4 MgCl2, 4 Na2ATP, and 0.4 Na3GTP, and the pH was adjusted to 7.3 with KOH and filtered at 0.2 μm. Depolarizing current steps (500 ms duration each) ranging from + 100 to + 800 pA, in increments of 100 pA, were delivered to the PC. Bicuculline (20 μM) and Kynurenic acid (1 mM) were added to the saline solution to inhibit the $GABA_A$ and ionotropic glutamate receptors, respectively.

PCs were voltage clamped at − 60 mV to record the excitatory postsynaptic currents (EPSCs) at the parallel fiber to Purkinje cell synapses. PF-EPSCs were evoked via a stimulation electrode (made of sodalime glass with a tip diameter of 10–15 μm) filled with ACSF and placed into the molecular layer. Double pulses with an interpulse interval of 100 ms were applied every 20 s by an isolated stimulator (A/M Systems, Carlsborg, WA, USA). For the long-term potentiation experiments, the patch pipettes were filled with an internal solution containing (mM): 120 potassium gluconate, 9 KCl, 3,5 MgCl2, 10 Hepes, 4 NaCl, 15 sucrose, 4 Na2ATP, 0.4 Na3GTP, 10 EGTA; the pH was adjusted to 7.2 with KOH. For the long-term depression experiment, the internal solution contained (mM): 130 potassium gluconate, 2 NaCl, 4 MgCl 2, 4Na 2ATP, 0.4 NaGTP, 20 HEPES, 0.25 EGTA. Bicuculline (20 μM) was added to the perfusate to inhibit the $GABA_A$ receptors.

Signals were filtered at 3 kHz, digitized at 10 kHz, and stored using the Pulse software (HEKA Elektronik). Data were analyzed using the Igor Pro 6.3 software (Wavemetrics, Lake Oswego, OR, USA) applying the custom scripts Neuromatic v 3.0 C[68]. Each EPSC was normalized to the mean EPSC amplitude obtained during a 10-min recording immediately before the induction of LTP or LTD.

2.9. Statistical analysis

GraphPad Prism® 9 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis of the data and graph preparation. The sample size for each experiment was determined according to our experience and to achieve statistical significance. Shapiro-Wilk normality test was used to assess Gaussian distribution. When data followed a normal distribution, parametric tests were used, i.e., t-test for comparing two groups or ANOVA for more than two groups. The data that did not follow a normal distribution were analyzed using the non-parametric Mann-Whitney test for comparing two groups, while Kruskal-Wallis followed by Dunn's post-test was used to compare more than two groups. No sample, mice, or data points were excluded from the behavioural and histology analysis. Concerning the electrophysiological analysis, we excluded that data if the access resistance changed by > 20% over the recording or exceeded 25 MΩ. A p-value less than 0.05 was considered significant. Data are expressed as mean ± standard error of the mean (± SEM).

3. Results

3.1. BUT-treatment of dams affects BUT plasma level in offspring and their mother

To determine whether the BUT administered to mothers reached the offspring via the placenta or through breastmilk, we measured the butyric acid plasma level in the dams and their offspring.

The level of butyric acid in plasma was lower in BTBR dams compared to B6 on postpartum day 1 (PP1) (n = 5 mice; one-way ANOVA, effect of interaction $F(3,16) = 17.79$; $p = 0.0078$, Fig. 2A) and PP18 (n = 8 mice; one-way ANOVA, effect of interaction $F(3,28) = 26.06$; $p = 0.0019$, Fig. 2A). The treatment with BUT significantly increased the plasma level of butyric acid in both B6 (on PP1, n = 5 mice; one-way ANOVA, effect of interaction $F(3,16) = 17.79$; $p = 0.0145$ and PP18, n = 8 mice; one-way ANOVA, effect of interaction $F(3,28) = 26.06$; $p = 0.0003$, Fig. 2A) and BTBR dams (on PP1, n = 5 mice; one-way ANOVA, effect of interaction $F(3,16) = 17.79$; $p = 0.0266$, and PP18, n = 8 mice; one-way ANOVA, effect of interaction $F(3,28) = 26.06$; $p = 0.0006$, Fig. 2A).

Similarly, BTBR offspring showed lower plasma level of butyric acid compared to B6 mice at P1 (n = 6 mice; one-way ANOVA, effect of interaction $F(3,20) = 14.87$; $p = 0.0225$, Fig. 2B), P18 (n = 6 mice; one-way ANOVA, effect of interaction $F(3,20) = 30.94$; $p = 0.0013$, Fig. 2B) and P30 (n = 6 mice; one-way ANOVA, effect of interaction $F(3,20) = 25.31$; $p = 0.0010$, Fig. 2B).

BUT supplementation during pregnancy and lactation significantly increased plasma levels of butyric acid in B6 (n = 6 mice; at P1, one-way ANOVA, effect of interaction $F(3,20) = 14.87$; $p = 0.115$, at P18, $F(3,20) = 30.94$; $p = 0.0003$, and P30, $F(3,20) = 25.31$; $p = 0.0039$, Fig. 2B) and in BTBR offspring (n = 6 mice; at P1, one-way ANOVA, effect of interaction $F(3,20) = 14.87$; $p = 0.0348$, at P18, $F(3,20) = 30.94$; $p = 0.0047$, and P30, $F(3,20) = 25.31$; $p = 0.00389$, Fig. 2B). These results suggest that BUT is transferred through the placental during gestation and breastmilk during lactation from dams to offspring.

3.2. Effect of BUT-treatment of dams on maternal behavior

To determine whether BUT administration had an impact on maternal breeding care, we analyzed the relationship between maternal and non-maternal behaviors expressed as index of maternal behavior toward the offspring from PP2 to PP7. Overall, the behavioral analysis revealed that there was no significant difference in maternal care behavior between B6 and BTBR mice as well as between untreated and BUT-treated BTBR and B6 mice (n = 7 mice; two-way ANOVA, effect of interaction $F(15,120) = 0.5942$; $p = 0.8746$, Fig. 3A).

In addition, the pup retrieval test conducted on PP3 did not show any significant difference between treated and untreated B6 and BTBR dams in the latency to retrieve the first (n = 7 mice; one-way ANOVA, effect of interaction $F(3,24) = 2.036$; $p = 0.1356$, Fig. 3B) and all pups (n = 7 mice; one-way ANOVA, effect of interaction $F(3,24) = 0.468$; $p = 0.7083$, Fig. 3C).

3.3. BUT-treatment of dams reduces repetitive behaviour in BTBR offspring

To examine the effect of BUT on ASD-like behaviour, we tested the BTBR mice from BUT-treated dams on repetitive/perseverative tasks, such as marble burying and self-grooming tests (Fig. 4).

BTBR mice from untreated dams spent more time on self-grooming compared to B6 mice from untreated and BUT-treated dams at P30 (n = 8 mice; one-way ANOVA, effect of interaction $F(3,28) = 35.14$; $p < 0.0001$ BTBR vs B6, $p < 0.0001$ BTBR vs B6 +BUT, Fig. 4A) and P60 (n = 8 mice; one-way ANOVA, effect of interaction $F(3,28) = 23.26$, $p < 0.0001$ BTBR vs B6, $p < 0.0001$ BTBR vs B6 +BUT, Fig. 4B). The BTBR offspring from BUT-treated dams exhibited a reduced self-

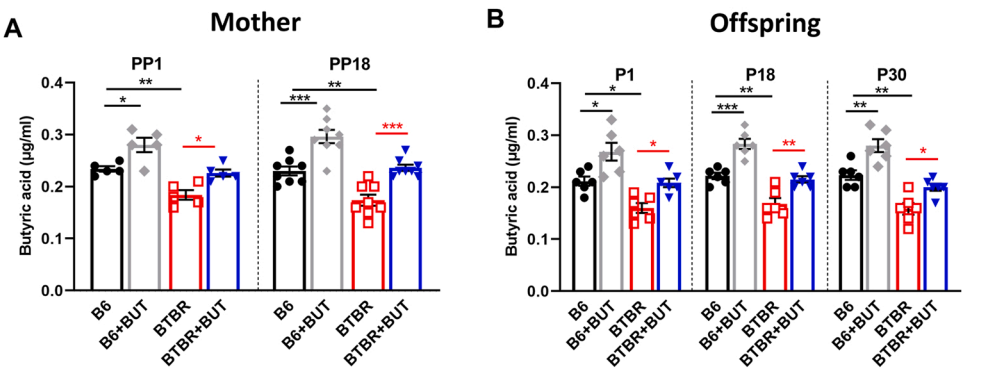

Fig. 2. Plasma levels of butyric acid following BUT treatment in BTBR dams and offspring. (A) Butyric acid plasma level in B6 and BTBR mothers on post-partum day 1 (PP1) and day 18 (PP18). (B) Butyric acid plasma level (ug/ML) in B6 and BTBR offspring born from BUT treated and untreated mothers at P1, P18, and P30. Statistical analysis was done using the one-way ANOVA followed by Tukey's post-test. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ vs control B6 (black), vs control BTBR (red). N = 5–8 animals/treatment.

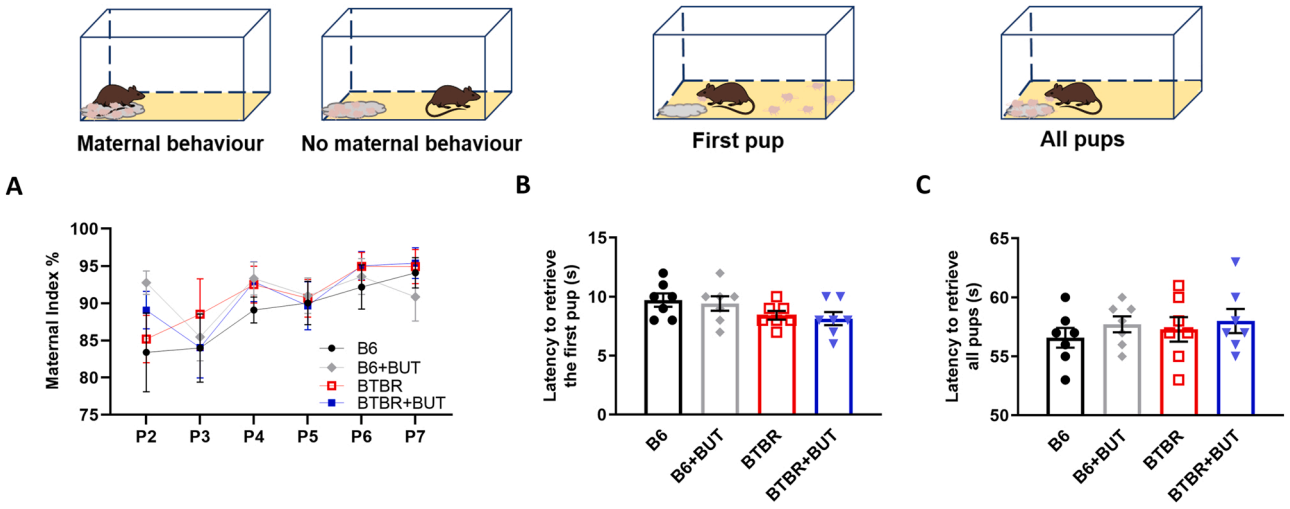

Fig. 3. BUT-treatment of dams did not affect maternal behaviour. (A) Index of maternal behaviour of BUT treated and untreated mothers from PP2 to PP7. (B) Latency to retrieve the first pup (expressed in seconds) by BUT treated and untreated mothers on PP3. (C) Latency to retrieve all pups (expressed in seconds) of BUT treated and untreated mothers on PP3. Statistical analysis was done using the two or one-way ANOVA followed by Tukey's post-test. N = 7 animals/treatment.

grooming behavior than BTBR from untreated mothers at both P30 (n = 8 mice; one-way ANOVA, effect of interaction F(3,28)= 35.14, p = 0.0029, Fig. 4A) and P60 (n = 8 mice; one-way ANOVA, effect of interaction F(3,28)= 23.26, p = 0.0003, Fig. 4B). BTBR-treated offspring reached the control level at P60 (n = 8 mice; one-way ANOVA, effect of interaction F(3,28)= 23.26, p = 0.42, Fig. 4B).

In addition, BTBR mice significantly buried more marbles than B6 mice born from untreated and BUT-treated dams at both P30 (n = 8 mice; one-way ANOVA, effect of interaction F(3,28)= 17,65; p < 0.0001 BTBR vs B6, p < 0.0001 BTBR vs B6 +BUT, Fig. 4C) and P60 (n = 8 mice; one-way ANOVA, effect of interaction F(3,28)= 16,63, p < 0.0001 BTBR vs B6, p < 0.0001 BTBR vs B6 +BUT, Fig. 4D). When the same test was performed in BTBR mice from BUT-treated dams, the number of buried marbles was significantly reduced with respect to BTBR mice from untreated dams at P30 (n = 8 mice; one-way ANOVA, effect of interaction F(3,28)= 17,65; p = 0.0331, Fig. 4C), while at P60 the number of buried marbles was similar to untreated BTBR mice (n = 8 mice; one-way ANOVA, effect of interaction F(3,28)= 16,63; p = 0.3157, Fig. 4D). These data suggest the positive effect of BUT on repetitive behaviours at P30 and for the self-grooming also at P60. BUT treatment did not alter the behavioural phenotypes in control B6 mice indicating that, at the dose and timing used, it doesn't have any effect per se.

3.4. Maternal BUT treatment improves sociability in BTBR mice

Next, we examined whether BUT treatment of dams also improved the social deficit of BTBR offspring evaluated using the three-chambered social test.

During the 5-min habituation, no innate side preference was present in all tested groups, as shown by the similar amount of time spent in the left and right side chambers (n = 8 mice; at P30 one-way ANOVA, effect of interaction F(7,56)= 0.7256, p = 0.6509; at P60 F(7,56)= 0.8069, p = 0.5851, Fig. 5A and B).

Under control conditions, B6 mice spent more time sniffing the mouse than the object at P30 (n = 8 mice; t-test, p < 0.001, Fig. 5C) and P60 (n = 8 mice; t-test, p < 0.0001, Fig. 5D). This social behaviour of B6 was not affected by BUT treatment of dams at P30 (n = 8 mice; t-test, p < 0.01, Fig. 5C) and P60 (n = 8 mice; t-test, p < 0.001, Fig. 5D). On the other hand, and in accordance with previous studies [34,56], BTBR spent significantly less time sniffing the mouse than the object at P30 (n = 8 mice; t-test, p < 0.001, Fig. 5C) and P60 (n = 8 mice; t-test, p < 0.0001, Fig. 5D). This deficit was rescued in BTBR mice born from BUT-treated dams (n = 8; t-test, p < 0.01 and P60 t-test, p < 0.01, Fig. 5C and D).

The analysis of the time spent in the chamber with the novel mouse recapitulated the sniffing time data. We observed a significantly higher preference for the chamber containing the mouse than the chamber containing the object in B6 mice (n = 8; at P30 t-test, p < 0.01 and at P60 t-test, p < 0.05, Fig. 5E and F) and B6 mice from BUT-treated dams (n = 8; at P30 t-test, p < 0.01 and P60 t-test, p < 0.05, Fig. 5E and F). On the other hand, BTBR spent more time in the chamber with the novel mouse than that with the object at P30 (n = 8; t-test, p < 0.05; Fig. 5E), and this result was also confirmed at P60 (n = 8; Wilcoxon-test,

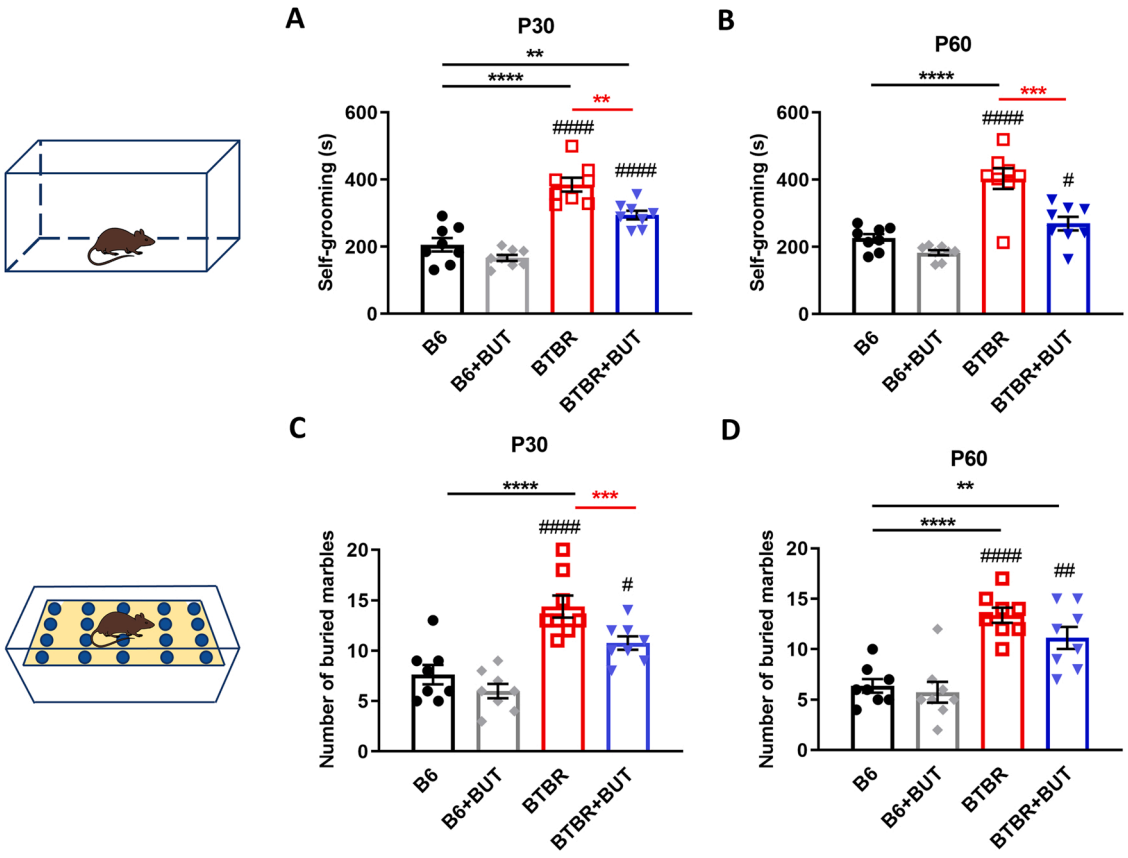

Fig. 4. BUT treatment of dams mitigates the repetitive behaviour in BTBR offspring. Self-grooming behaviour of BTBR and B6 mice born from BUT treated and untreated mothers at P30 (A) and P60 (B). Number of buried marbles by BTBR and B6 mice from BUT treated and untreated dams at P30 (C) and P60 (D). Statistical analysis was done using the one-way ANOVA followed by Tukey's post-test. * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$ vs control B6 (black), vs control BTBR (red); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ vs B6 +BUT. N = 8 animals/treatment.

$p < 0.05$; Fig. 5F). The social behavior was improved in BTBR mice from BUT-treated mothers (n = 8; at P30 t-test, $p < 0.0001$ and P60 t-test, $p < 0.05$, Fig. 5E and F).

Overall, these results suggest that maternal BUT treatment prevents social deficits in juvenile and adult offspring.

3.5. *Maternal BUT treatment reduces the cerebellar cortex expansion in the BTBR offspring*

BTBR mice exhibit abnormal development of the cerebellum affecting foliation and granule cell proliferation [69]. Indeed, at P30 and P60, the analysis of cerebellar Nissl-stained sagittal sections revealed that BTBR mice showed a significant increase of the whole cerebellar area (n = 4; at P30 Kruskal-Wallis test followed by Dunn's post-test, $p < 0.05$ and P60 one-way ANOVA, effect of interaction $F(2,9) = 26.42$, $p < 0.001$; Fig. 6A, B and E), and perimeter (n = 4; at P30 one-way ANOVA, effect of interaction $F(2,9) = 18.37$, $p < 0.001$ and P60 one-way ANOVA, effect of interaction $F(2,9) = 51.42$, $p < 0.0001$ Fig. 6A, C and F), compared to B6 mice.

In addition, at P30 and P60, BTBR mice showed a significant increase of the area of the molecular layer (ML) (n = 4; at P30 one-way ANOVA, effect of interaction $F(2,9) = 12.92$, $p < 0.01$ and P60 one-way ANOVA, effect of interaction $F(2,9) = 8.362$, $p < 0.05$; Fig. 6D and G), granular layer (GL) (n = 4; at P30 one-way ANOVA, effect of interaction $F(2,9) = 17.63$, $p < 0.001$ and P60 one-way ANOVA, effect of interaction $F(2,8) = 8.839$, $p < 0.05$; Fig. 6D and G) and, white matter (WM) (n = 4; at P30 one-way ANOVA, effect of interaction $F(2,9) = 25.48$, $p < 0.05$ and P60 one-way ANOVA, effect of interaction $F(2,8) = 5.001$, $p < 0.05$, Fig. 6D and G) respect to B6 mice.

We, therefore, examined the structure of the cerebellar cortex of BTBR mice born from untreated and BUT-treated dams at P30 and P60 to determine if BUT treatment prevents the abnormal enlargement of the cerebellar cortex. At P30, BTBR offspring from BUT-treated dams showed a significant reduction of the whole cerebellar area (n = 4; Kruskal-Wallis test followed by Dunn's post-test, $p < 0.05$; Fig. 6B) and perimeter (n = 4; one-way ANOVA, effect of interaction $F(2,9) = 18.37$, $p < 0.05$; Fig. 6C) compared to BTBR offspring from vehicle-treated mothers.

Moreover, at P30 BTBR offspring from BUT-treated mothers showed a significant reduction of the area of the ML (n = 4; one-way ANOVA, effect of interaction $F(2,9) = 12.92$, $p < 0.05$; Fig. 6D), GL (n = 4; one-way ANOVA, effect of interaction $F(2,9) = 17.63$, $p < 0.05$, Fig. 6D) and WM (n = 4; one-way ANOVA, effect of interaction $F(2,9) = 25.48$, $p < 0.001$, Fig. 6D) respect to BTBR offspring from vehicle-treated mothers. These results suggest that the BUT treatment prevents the abnormal enlargement of the cerebellar cortex of BTBR mice at a young age.

However, at P60 the total cerebellar area (n = 4; one-way ANOVA, effect of interaction $F(2,9) = 26.42$, $p = 0.7039$, Fig. 6E), perimeter (n = 4; one-way ANOVA, effect of interaction $F(2,9) = 51.42$, $p = 0.4945$; Fig. 6F), and the distinct ML (n = 4; one-way ANOVA, effect of interaction $F(2,9) = 8.362$, $p = 0.4945$, Fig. 6G), GL (n = 4; one-way ANOVA, effect of interaction $F(2,8) = 8.839$, $p = 0.681$, Fig. 6G), WM (n = 4; one-way ANOVA, effect of interaction $F(2,8) = 5.001$, $p = 0.5835$, Fig. 6G) areas in BTBR-treated mice were comparable to BTBR offspring from vehicle-treated dams, indicating that BUT treatment of dams up to weaning was not sufficient to counteract the abnormal development of the cerebellum in the adult BTBR mice.

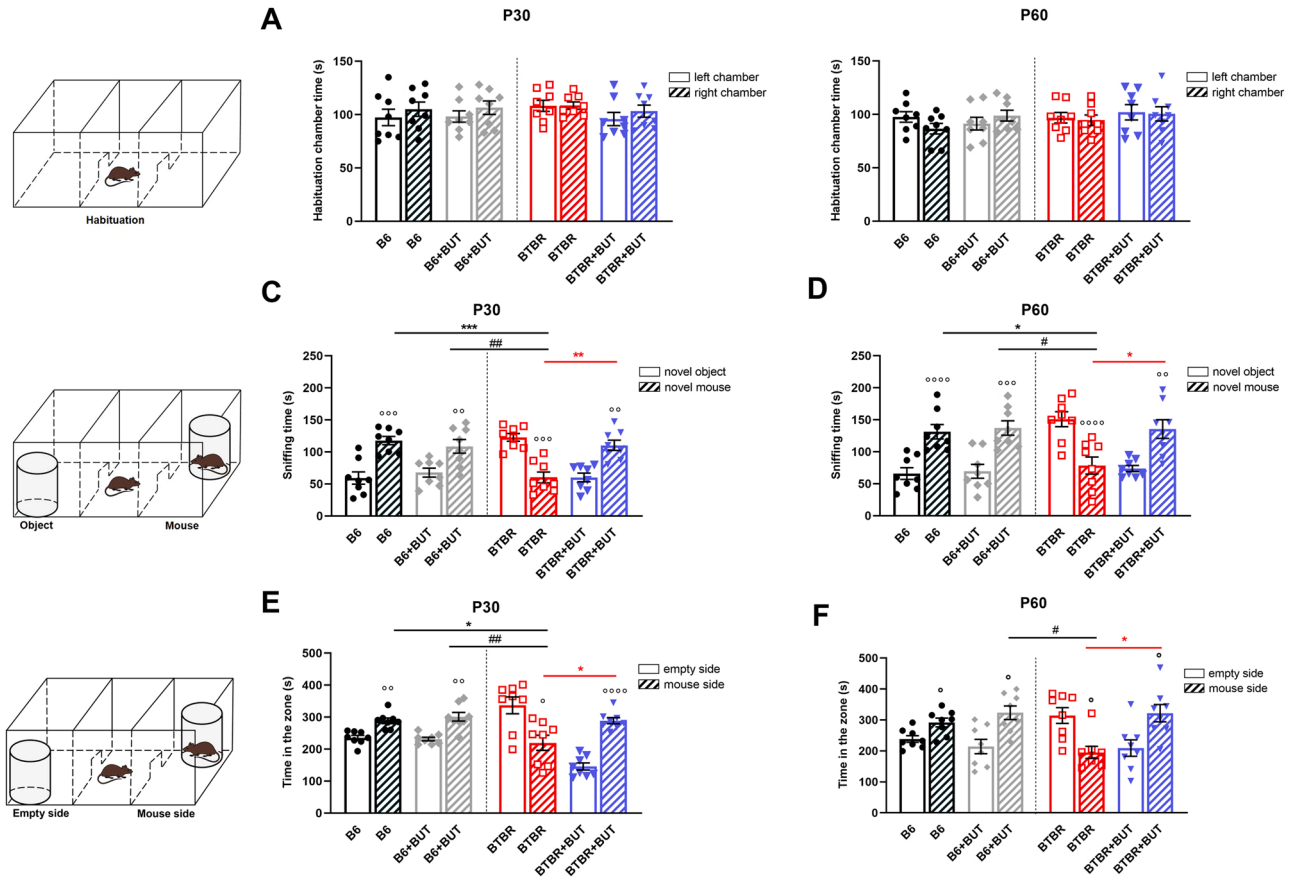

Fig. 5. Maternal BUT treatment improves the social behaviour in BTBR offspring. (A) At P30 and (B) at P60, innate chamber side bias during the 5-min habituation phase before the start of the sociability test of BTBR and B6 mice from BUT treated and untreated dams. (C) At P30 and (D) at P60, time spent in sniffing a novel mouse or a novel object of BTBR and B6 mice from BUT-treated and untreated dams. (E) At P30 and (F) at P60, time spent in mouse or empty side of the apparatus of BTBR and B6 mice from BUT treated and untreated dams. Statistical analysis was done using the Student t-test and one-way ANOVA followed by Tukey's post-test except for the sociability data shown in panel F where Kruskal-Wallis with Dunn's post-test was used. * $p < 0.05$, * ** $p < 0.001$ vs control B6 (black), vs control BTBR (red); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs B6 +BUT; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$, $^{\circ\circ\circ\circ}p < 0.0001$ vs empty side. N = 8 animals/treatment.

3.6. Effect of BUT treatment on BTBR Purkinje cell excitability and synaptic plasticity

To determine whether the abnormal cerebellar cortex structure was associated with its dysfunction, we examined the Purkinje cells (PC) electrophysiological properties via patch-clamp recordings (Fig. 7A-C). In accordance with previous studies carried out in different ASD-mouse models [70,71], BTBR PCs showed a decreased excitability with respect to control B6, displaying low-frequency bursts of action potentials in response to high-intensity stimulation (over 600 pA) at P60 (two-way ANOVA, effect of interaction F(14,80)= 1.640; p = 0.0063 at 700 pA, p = 0.0044 at 800 pA, Fig. 7C) but not a P30 (p = n.s, Fig. 7B). Interestingly, BUT treatment prevented the alterations in the BTBR PC membrane excitability, and the firing pattern was not significantly different from that of the B6 (two-way ANOVA, effect of interaction F (14,80)= 1.640; p = 0.00366 at 700 pA, p = 0.0043 at 800 pA, Fig. 7C).

Deficits in synaptic plasticity from different brain regions have been reported in mouse models of ASD and are considered causative factors underlying autistic-like functional and cognitive impairments [72,73]. We, therefore, examined the two main forms of cerebellar synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) at parallel fiber (PF) – PC synapses in cerebellar slices of BTBR born from untreated or BUT-treated mothers compared to control mice. To induce PF-LTP, we applied a PF burst protocol consisting of a train of 15 pulses at 100 Hz repeated every 3 s for 5 min [65,74]. This protocol triggered a potentiation in cerebellar slices prepared from control mice at P30 (49% ± 6%, n = 5; p < 0.0001 vs baseline, Fig. 8A) and P60 (21% ± 5%; n = 6; p = 0.0011 vs baseline, Fig. 8C). In contrast, the same protocol did not induce any PF-LTP in untreated BTBR mice at P30 (0% ± 4, n = 5; p = 0.7103 vs baseline, Fig. 8A and B) but produced a depression at P60 (by 22% ± 4%; n = 8; p < 0.0001 vs baseline, Fig. 8C and D). LTP was successfully induced in BTBR mice from BUT-treated dams both at P30 (46% ± 10%, n = 5; p = 0.0039 vs baseline; one-way ANOVA, effect of interaction F(2,10)= 11.32; p = 0.0027 BTBR vs B6, p = 0.0133 BTBR+BUT vs BTBR Fig. 8A and B) and P60 (26% ± 7%; n = 8; p = 0.0029 vs baseline; Kruskal-Wallis test followed by Dunn's post-test, p = 0.0052 BTBR vs B6, p = 0.0052 BTBR+BUT vs BTBR, Fig. 8C and D). For LTD induction, we applied the PF burst protocol (300 stimuli at 1 Hz) in conjunction with depolarizing steps to the PC soma (200 ms, −60 to +20 mV), mimicking the CF stimulation [65]. As shown in Fig. 8E and F, LTD was successfully induced in both B6 (by 27% ± 3%; n = 5; p < 0.01 vs baseline) and BTBR mice (24% ± 10%; n = 4; p < 0.05 vs baseline) at P30; no significant difference between the EPSC amplitude changes was observed in both groups (p = n.s.). These results suggest a selective impairment of LTP, but not LTD, in BTBR mice offspring, which was prevented by dam treatment with BUT.

4. Discussion

In this study, we demonstrated that maternal administration of BUT rescues the behavioral and synaptic plasticity deficits of BTBR offspring. In particular, the BUT treatment of dams was able to prevent the social deficit in juvenile and adult BTBR mice examined in the three-chamber

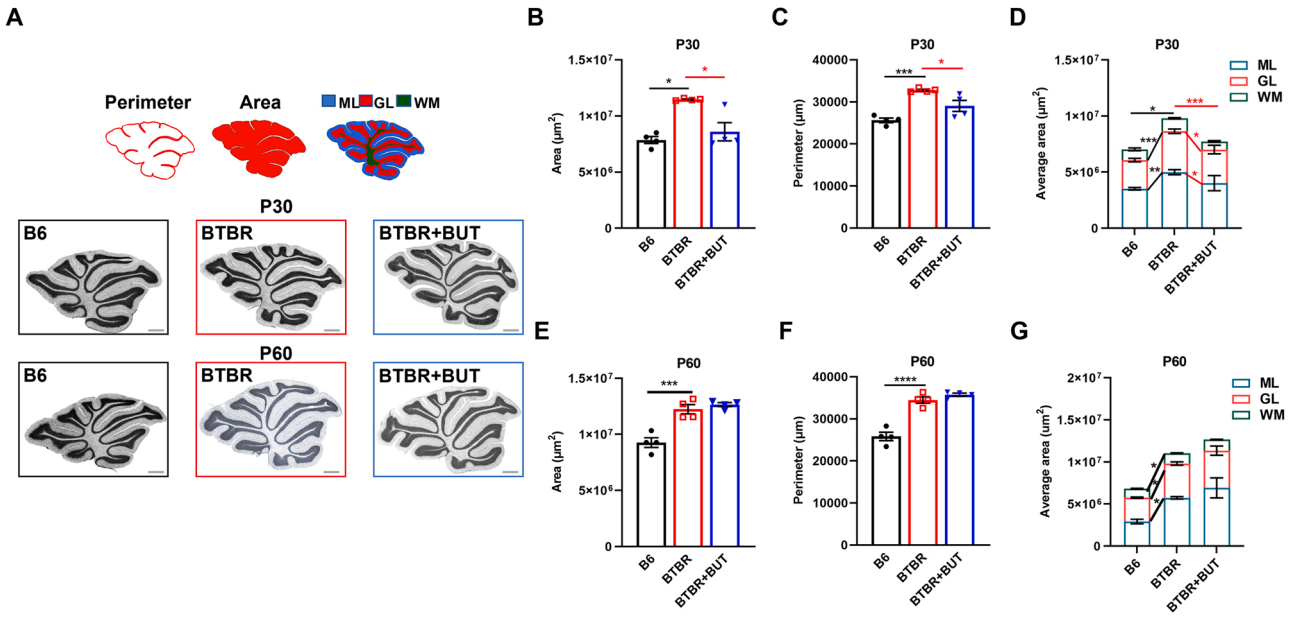

Fig. 6. Maternal BUT treatment prevents the hypertrophy of BTBR cerebellar cortex at P30. (A) Nissl-stained sagittal sections of the cerebellum at P30 and P60. (B) At P30 and (E) at P60, average sagittal cerebellar area of B6 and BTBR mice from BUT-treated versus untreated dams. (C) At P30 and (F) at P60, average sagittal cerebellar section perimeter of B6 and BTBR offspring from BUT-treated versus untreated mothers. (D) At P30 and (G) at P60 cerebellar cortex shows average areas of the molecular layer, (ML), granular layer (GL), and white matter (WM) of B6 and BTBR offspring from BUT-treated versus untreated mothers. Scale bar 500 μm. Statistical analysis was done using one-way ANOVA followed by Tukey's post-test except for the area shown in panel B where Kruskal-Wallis with Dunn's post-test was used. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ and * ** * $p < 0.0001$ vs control B6 (black), vs control BTBR (red). N = 4 animals/treatment.

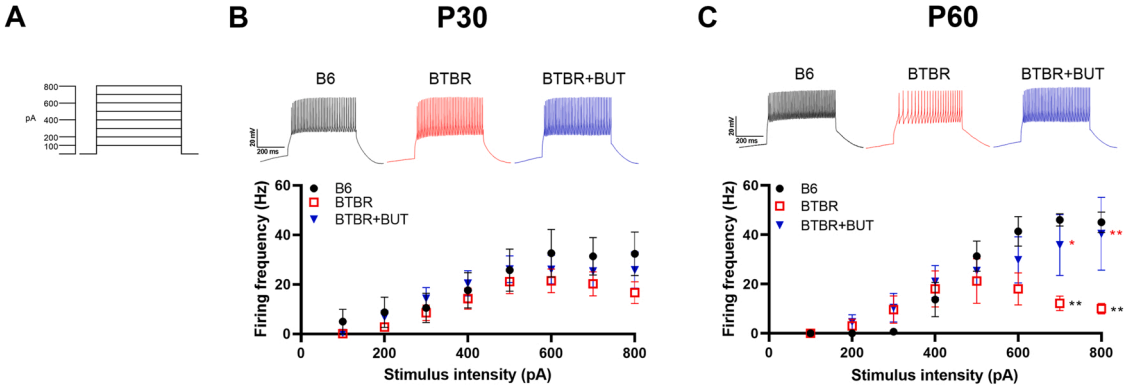

Fig. 7. BUT-treatment of dams improves the Purkinje cell firing activity in adult BTBR mice. (A) Depolarizing steps from + 100 to + 800 pA in 100 pA increments were delivered to the PC. (B) At P30 and (C) at P60, Purkinje cell firing frequency as a function of stimulation intensity in B6 and BTBR mice born from BUT-treated or untreated dams. The inserts on the top show representative traces of the evoked firing. Statistical analysis was done using the one-way ANOVA followed by Tukey's post-test. * $p < 0.05$ and * * $p < 0.01$ vs control B6 (black), vs control BTBR (red). N = 4 animals/treatment.

test. At P30, BTBR mice from BUT-treated dams also show a reduction of repetitive behavior in the self-grooming and marble-burying tests compared to untreated BTBR mice, while at P60 the repetitive behavior is reduced only for self-grooming. It is likely that the BUT treatment of BTBR offspring following weaning may improve behavioral performance over a long-term period.

Previous studies have demonstrated that the direct chronic administration of BUT alone or in combination with α-Lactalbumin in BTBR adult mice improves their social and repetitive behavior [33,75]. In this study, BUT is administered to the mother and it is likely transferred to offspring through the placenta during pregnancy and by breastfeeding after birth thus affecting the offspring's brain development and behavior, as the offspring's plasma BUT levels appeared to be high at birth and up to P30. The transfer into the fetus may occur through different mechanisms involving monocarboxylate transporters expressed in the placenta [76] but also amniotic fluid [77] and maternal microbiome [78]. Maternal BUT supplementation during pregnancy and lactation has several beneficial effects on offspring such as an increased rate of blastocyst formation [79] and pup survival [80]. In addition, BUT supplementation to pregnant mice determines a downregulation of the genes involved in inflammatory signaling in the offspring colon [81].

By examining the effect of maternal BUT treatment on the cerebellar structure, we found a significant reduction of molecular and granular layer hypertrophy, which was observed in BTBR mice. The increase of GL thickness in BTBR cerebella is likely due to the enhanced proliferation of granule cell precursors associated to the deficit in the migration of granule cells while the increased spine density of PCs may be responsible of the increased ML, as previously reported [69]. An enlargement of ML has been also observed in another mouse model of ASD, in which PTEN was specifically deleted in PCs, characterized by impaired sociability and repetitive behavior in association with abnormalities in PC dendrites and axons [70].

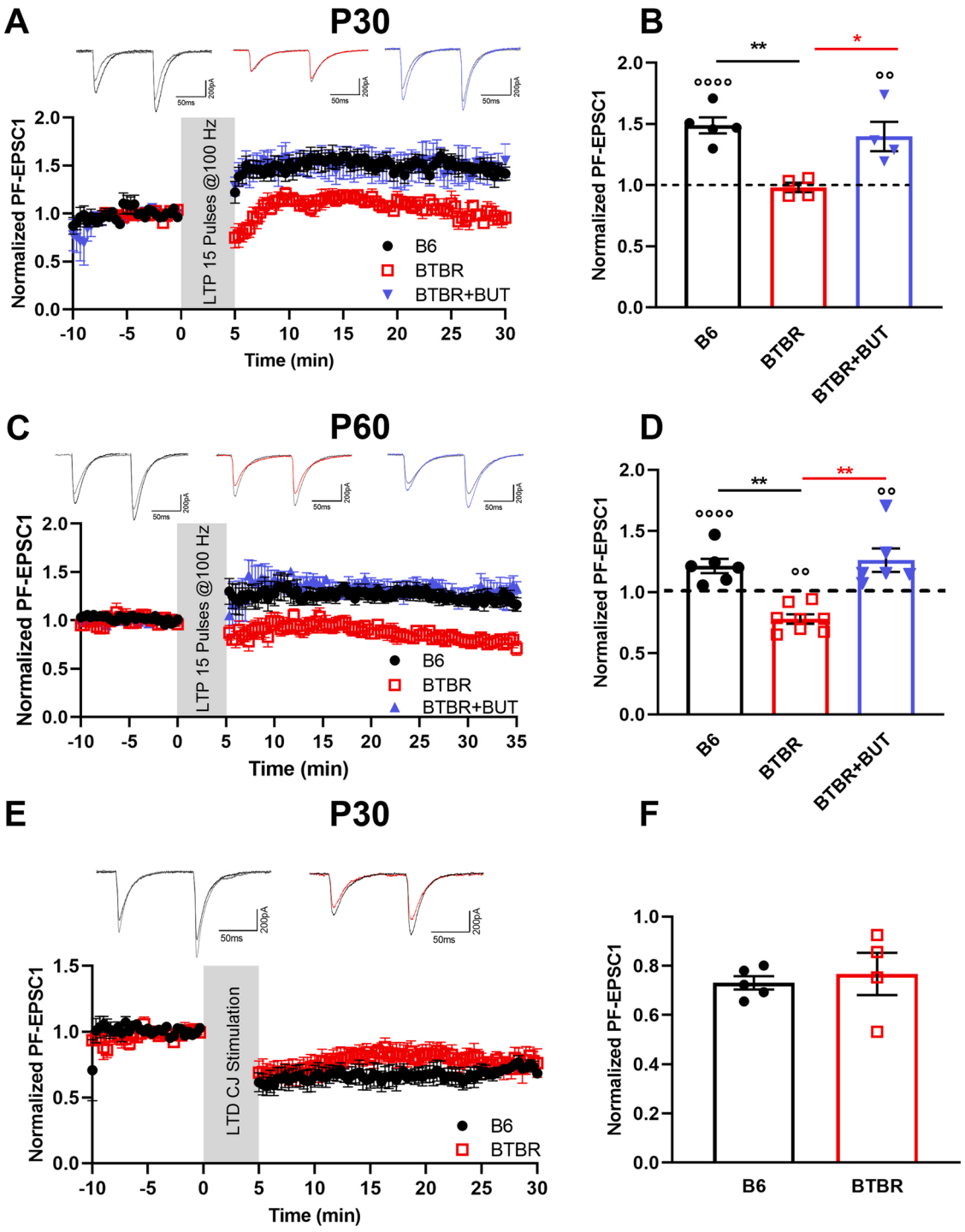

Fig. 8. The impairment of LTP at PF-PC synapse in BTBR mice is restored by maternal BUT treatment. (A) At P30 and (C) at P60, time course of normalized first PF-EPSC (EPSC1) before and after LTP induction recorded from cerebellar slices of B6 and BTBR mice born from BUT-treated or untreated mothers. The inserts on the top show superimposed traces of PF-EPSCs evoked by paired-pulse stimulation before and 30 min after PF tetanic stimulation. (B) At P30 and (D) at P60, the change in normalized PF-EPSC1 relative to control at 30 min after LTP induction in B6 and BTBR mice born from BUT-treated or untreated mothers. (E) The time course of normalized PF-EPSC1 before and after conjunction stimulation of PF and PC depolarization (CJ stimulation) from B6 and BTBR mice; the inserts on the top show the PF-EPSCs evoked by paired-pulse stimulation before and 30 min after LTD induction. (F) The change in normalized PF-EPSC1 relative to control at 30 min after CJ stimulation in juvenile B6 and BTBR mice. Statistical analysis was done using the one-way ANOVA followed by Tukey's post-test except for the LTP data shown in panel D where Kruskal-Wallis with Dunn's post-test was used. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ vs control B6 (black), vs control BTBR (red). °° $p < 0.01$ and °°°° $p < 0.0001$ vs baseline. N = 5–8 slices (4–6 animals)/treatment.

Whole-cell patch-clamp recordings in BTBR cerebellar slices revealed an impairment of intrinsic firing properties of PC in response to high-intensity current injections, particularly at P60. Interestingly, such deficit was rescued in BTBR mice from BUT-treated dams. PCs are responsible for the integration of cerebellar incoming signals and provide the only output of the cerebellar cortex to deep cerebellar nuclei to control motor and cognitive behavior [82]. Therefore, disruptions in PC excitability during development can induce an alteration of the

cerebellar circuits that may lead to behavioral impairment including social deficits. Treatments that rescue PC firing deficits during a specific developmental time window have the potential to improve behavior in mice models of ASD. Indeed, previous studies in mutant mice with a selective deletion of tuberous sclerosis complex in PCs have demonstrated that early rapamycin treatment was able to prevent the PC spontaneous firing rate deficits in correlation with the rescue of motor and social deficits [70,71,83,84].

In BTBR mice, PC excitability deficit was associated with the specific impairment of LTP at PF-PC synapse but not LTD. Here, LTP is induced by high-frequency stimulation of the PFs while LTD is evoked by low-frequency stimulation of PF in conjunction with PC depolarization. Therefore, it is likely that the LTP impairment is due to a reduced ability of PF-PC to respond to the conditioning high-frequency PF burst stimulation. A selective deficit of LTP but not LTD has been also reported in Shank2-deficient mice and has been proposed as a cellular mechanism underlying the pathophysiology of ASD [85]. Overall, the rescue of BTBR PC excitability and cerebellar synaptic plasticity alterations provides a cellular mechanism by which BUT treatment during early developmental periods may rescue ASD-behaviors including social deficits.

Our results are coherent with previous studies showing that BUT and its prodrug tributyrin induce hippocampal LTP and rescue the scopolamine-induced impairment of LTP and memory [86]. In addition, it has been demonstrated that BUT supplementation promotes hippocampal structural plasticity and neurogenesis in association with an increase in brain glucose metabolism [87].

The fact that BUT-treated mice show a recovery of behavior and electrophysiological parameters at P30 and P60 in parallel with the lack of morphological rescue at P60 indicates that the brain plasticity in adult mice is mainly functional [83]. In biological systems, morphological plasticity follows different time windows and mechanisms compared to functional plasticity. For example, in animal models of ataxia, functional alterations proceed in parallel with symptoms, while morphological changes, including cell death, occur weeks or months later [88]. The morphological recovery at P30 suggests that the BUT treatment was not only effective but also that at this age the cerebellar structure of this animal species is plastic enough to reorganize and repair itself.

There are several hypotheses regarding the mechanisms by which BUT may influence behavioral and neuronal activity. SCFAs including BUT are considered key mediators of gut-brain communication directly or via the immune, endocrine, and vagal pathways [89]. BUT can be absorbed from the gut and reach the brain through the monocarboxylate transporters, which are present in the blood-brain barrier, glial cells, and neurons. At a cellular level, SCFAs might affect the CNS by binding the G protein-coupled free fatty acid receptors (FFARs) and/or inhibition of HDACs [90]. Butyrate is a ligand of the FFAR2 and FFAR3, which are expressed in the brain and are linked to signaling cascades that include phospholipase C, mitogen-activated protein kinases (MAPKs), and the transcription factor nuclear factor κB [91]. Many studies indicate all class I histone deacetylase (HDAC) inhibitors and most class II HDACs as the main targets of BUT [92]. Treatment with BUT has been shown to inhibit via acetylation the histone H3 in the hippocampus [28,93] and PFC [33,94]. In PFC, BUT treatment downregulates the neuronal activation marker cFos and genes encoding excitatory neurotransmitter receptors while upregulating inhibitory receptor genes. In such a way, BUT may modulate the excitatory/inhibitory balance, which appears to be altered in ASD.

Although HDAC inhibition has positive effects in different mouse models of ASD, these beneficial effects do not involve all HDAC inhibitors; for example, the administration of valproic acid during pregnancy increases the risk of ASD in the offspring [95].

In addition, it has been demonstrated that BUT attenuates the mitochondrial dysfunction commonly observed in ASD by enhancing oxidative phosphorylation and beta-oxidation [96,97].

BUT is considered one of the major mediators of gut-brain communication [98]. Whether BUT has a direct effect on brain development or acts through the gut-brain axis requires further investigation. Several studies indicate that BUT supplementation modulates the microbiota composition in both healthy and pathological conditions, restoring the Bacteroidetes/Firmicutes ratio [99,100]. The analysis of intestinal microbiota composition in BTBR mouse carried out in adulthood and aging have shown an imbalance of the ratio Firmicutes to Bacteroidetes [37,101,102]. Interestingly, a similar microbiota alteration has been found in autistic children [103,104]. Although no study has reported the microbiota composition in BTBR mice after birth, the microbiota alteration likely occurs at the early stage of development, as demonstrated in other ASD mouse models [105].

5. Conclusion

Here, we demonstrate that treatment of dams with the gut microbial metabolite BUT can prevent the development of aspects related to ASD symptomatology in BTBR mice offspring. This study can be considered the first of its kind to investigate the effect of maternal treatment with microbiota metabolites on ASD offspring and the impact of microbiota metabolites from the very early stage of development.

Based on the results of this study, further investigations will be necessary to understand the molecular mechanism underlying the BUT-induced improvements in autism-like behaviors and the role of microbiota on the beneficial effect of BUT. Despite the intense interest and the recent phase I clinical trials on fecal microbiota transplantation studies in ASD [106,107], the contribution of the microbiome remains an open question [108].

Although BTBR mice recapitulate the autism-relevant behavioral phenotype, such as social deficits and high levels of repetitive behavior, other mouse models of autism would be also useful to confirm the beneficial effect of BUT in ASD. Since we limited our analysis to the cerebellum, we cannot exclude that other brain regions would contribute to the observed behavioral rescue following BUT treatment. Finally, a long-term endpoint evaluation should also be considered to understand whether the benefits of BUT treatment of dams are maintained over time in the offspring.

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CRediT authorship contribution statement

Claudia Cristiano: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Eriola Hoxha:** Methodology, Formal analysis, Writing – review & editing. **Pellegrino Lippiello:** Methodology, Investigation, Formal analysis. **Ilaria Balbo:** Methodology. **Roberto Russo:** Writing – review & editing. **Filippo Tempia:** Supervision. **Maria Concetta Miniaci:** Conceptualization, Formal analysis, Writing – original draft, Supervision.

Conflict of interest statement

The authors declare no competing interests.

Data Availability

Data will be made available on request.

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