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Amygdalar neurotransmission alterations in the BTBR mice model of idiopathic autism

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Autism Spectrum Disorders (ASD) are principally diagnosed by three core behavioural symptoms, such as stereotyped repertoire, communication impairments and social dysfunctions. This complex pathology has been linked to abnormalities of corticostriatal and limbic circuits. Despite experimental efforts in elucidating the molecular mechanisms behind these abnormalities, a clear etiopathogenic hypothesis is still lacking. To this aim, preclinical studies can be really helpful to longitudinally study behavioural alterations resembling human symptoms and to investigate the underlying neurobiological correlates. In this regard, the BTBR T^{+} $Itpr3^{tf}$/J (BTBR) mice are an inbred mouse strain that exhibits a pattern of behaviours well resembling human ASD-like behavioural features. In this study, the BTBR mice model was used to investigate neurochemical and biomolecular alterations, regarding Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF), together with GABAergic, glutamatergic, cholinergic, dopaminergic and noradrenergic neurotransmissions and their metabolites in four different brain areas, i.e. prefrontal cortex, hippocampus, amygdala and hypothalamus. In our results, BTBR strain reported decreased noradrenaline, acetylcholine and GABA levels in prefrontal cortex, while hippocampal measurements showed reduced NGF and BDNF expression levels, together with GABA levels. Concerning hypothalamus, no differences were retrieved. As regarding amygdala, we found reduced dopamine levels, accompanied by increased dopamine metabolites in BTBR mice, together with decreased acetylcholine, NGF and GABA levels and enhanced glutamate content. Taken together, our data showed that the BTBR ASD model, beyond its face validity, is a useful tool to untangle neurotransmission alterations that could be underpinned to the heterogeneous ASD-like behaviours, highlighting the crucial role played by amygdala.

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INTRODUCTION

Autism Spectrum Disorders (ASD) are a group of neurodevelopmental disorders, generally diagnosed by three core behavioural symptoms, such as stereotyped repertoire, communication impairments and social withdrawal [1]. In addition, ASD are characterized by a wide range of additional symptoms, including cognitive dysfunctions, restricted interests, hyperactivity and impulsivity, thus showing comorbidities with different neuropsychiatric disorders, such as schizophrenia, attention-deficit/hyperactivity disorder, anxiety and obsessive-compulsive disorder [2, 3]. ASD prevalence has increased dramatically during the last decade, reaching the 1% estimation from the World Health Organization, that only accounts for approximately 16% of the global paediatric population; while European and US reports estimated around 1.4-2.5% in children of 8 years old on average based on population studies [4–7]. The complex etiopathogenesis underlying ASD onset and development is still unknown, since beyond the mostly hypothesized genetic and environmental involvement, multiple factors, regarding immune, dietary, metabolic and gastrointestinal systems, have recently been implicated [8, 9]. Considering that studies involving humans are often polluted by uncontrollable variables and biases and some contrasting results and inconsistencies in clinical evaluations have also been denounced, animal models can be really helpful to longitudinally study behavioural alterations resembling human symptoms in a translational way and to investigate the underlying neurobiological mechanisms [10, 11]. To this aim, the BTBR T^{+} $Itpr3^{tf}$/J (BTBR) mice are an inbred mouse strain that well resembles the principal behavioural deficits of ASD [2, 12]. Among the different ASD mice models, the BTBR mouse strain shows robust and pronounced deficits in reciprocal social interactions, altered ultrasonic vocalization and repetitive stereotyped repertoire [13, 14]. In addition, BTBR mice display cognitive and emotional abnormalities to the psychiatric comorbidity of ASD [15, 16]. Considering their unique behavioural profile, BTBR mice might be a helpful tool to disentangle the neurobiological alterations that give rise to the heterogeneity of ASD symptoms, to identify putative biomarkers and, ultimately, to develop efficacious pharmacological treatments [17]. Regarding repetitive and restricted behaviours, BTBR mice were able to display both "lower-order" motor stereotypies and "higher-order" cognitive stereotypies [18], making this animal model suitable to study the neurobiological pathways related to these behavioural dysfunctions. Pharmacological treatments to ameliorate repetitive behaviours are mainly focused towards

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impulsivity and irritability, by using typical and atypical antipsychotics, thus they are employed in limited cases. Currently, there are no drugs approved to successfully treat ASD repetitive symptoms [19–22]. Hence, unravelling the burden of underlying mechanisms related to stereotypies would provide a valuable step forward to the development of novel pharmacological interventions to target ASD repetitive repertoire. Although the pathophysiology of ASD is still cryptic, several imaging and *post-mortem* studies reported the crucial involvement of corticostriatal and limbic circuits, by showing cortical, hippocampal and amygdalar anatomical abnormalities and functional alterations [23–27].

Indeed, a number of genes related to ASD are known to alter the neural structure, function and connectivity, as well as neurotransmission and neurotropism [28, 29]. In this regard, the neurotrophin family, notably Brain-Derived Neurotrophic Factor (BDNF) and Nerve Growth Factor (NGF), plays a pivotal role in neurodevelopmental processes which are atypical in ASD pathology [30]. In particular, BDNF, being an essential actor for the proper cerebral development and importantly associated to synaptic plasticity, has been recently proposed as a possible diagnostic marker in children suffering from ASD and different line of evidence have reported its involvement in ASD onset and development [31–33]. As regarding Nerve Growth Factor (NGF), such neurotrophin plays a key role in the regulation of nerve-cell growth, survival and differentiation, being markedly expressed in the central nervous system, particularly in the cerebral cortex, hippocampus (HIPP) and amygdala (AMY) [34]. Research linking NGF and ASD is limited. Few studies report an involvement of the NGF signalling pathway in the pathogenesis of ASD [30, 35], also considering the critical role of this neurotrophin in the immune modulation and in the induction of the release of different neuropeptides and neurotransmitters [36], such as glutamate and dopamine (DA) [37]. On the other hand, cathecolamines are known to increase NGF content [38], thus resulting in a continuous crosstalk between these two pathways. In this regard, cathecolamines, such as DA and noradrenaline (NA) are known to play a pivotal role in the modulation of executive functions, attention, impulsivity and emotional state, which are all processes disrupted in ASD [39, 40]. Indeed, recent studies report a possible implication of DA neurotransmission and metabolism in ASD development [41–43] while a number of evidence demonstrated that neuropathological changes related to the noradrenergic system often occur in ASD [44–46]. In addition, the imbalance of the excitatory-inhibitory synaptic transmission has also been linked to ASD pathology [47]. In this regard, alterations of glutamate and gamma-aminobutyric acid (GABA) receptors expression have been reported in *post-mortem* brains of ASD patients [48] and a decrease in GABA levels have been observed in different brain regions of ASD children [49]. Furthermore, brain regions with dense cholinergic innervation, such as prefrontal cortex (PFC), hippocampus (HIPP) and amygdala (AMY), are linked to social cognition, thus resulting crucially involved in ASD pathophysiology [50]. In this regard, it has been reported that low acetylcoline (ACh) levels in mice displayed reduced social interactions and that, by administrating acetylcholinesterase inhibitors, such impairment might be relieved [51]. Converging evidence have revealed that ASD patients show an atypical social brain circuitry and that, in this important network, AMY is one of the regions with major interest [52]. In this regard, it has been reported that ASD patients showed reduced connectivity between AMY and PFC that was correlated with the severity of social dysfunctions [53]. In addition, a weaker functional connectivity was also found between AMY and occipital cortex [54], suggesting that such region, being the core of social brain network, deserves deeper investigations.

To summarize, a number of molecular alterations, in terms of neurotrophin, cathecolamines and aminoacids have been reported in both animal and human ASD studies, and, from an anatomical point of view, tendency towards overgrowth of PFC and AMY have been detected in individuals with ASD compared to neurotypical controls [55–57]. Moreover, data from structural magnetic resonance imaging highlighted widespread cerebral abnormalities in ASD patients, that involve total brain volume, fronto-parieto-temporal and cerebellar regions [58]. Nonetheless the above mentioned alterations, ASD continue to be purely defined by behavioural dysfunctions, thus to unravel the underpinning mechanisms beyond behavioural traits still represents a demanding task.

To this aim, we employed the BTBR mice model to investigate neurochemical and biomolecular alterations underpinning ASD behavioural dysfunctions. In particular, we quantified NGF and BDNF expression levels, together with GABAergic, glutamatergic, dopaminergic, noradrenergic and cholinergic neurotransmissions in PFC, HIPP, AMY and hypothalamus (HYP).

MATERIALS AND METHODS

Animals

In this study, we focused on male sex since ASD shows a male prevalence, being males three times more diagnosed than females [59]. A total of 20 (2–3 per litter, randomly distributed) 10-weeks-old male mice, C57/BL6J (BL6) (Envigo, San Pietro al Natisone, Italy) and BTBR (Charles-River Italia, Milan, Italy), were used in this experimental study. They were raised at constant room temperature (22 ± 1 °C) with relative humidity (55 ± 5%), under a 12 h light/dark cycle (lights on from 7:00 AM to 7:00 PM) and free access to water and food. All experiments on animals and their care were carried out in accordance with the institutional guidelines of the Italian Ministry of Health (D.Lgs. n. 26/2014), the Guide for the Care and Use of Laboratory Animals: Eight Edition, the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2004), the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific intents, as defined by Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0. The experimental protocol was approved by the Italian Ministry of Health (protocol nr. B2EF8.24). Animals' health state was checked daily during experimental period. Moreover, to accomplish the 3 R's principles, we performed the behavioural test battery on the same group of animals; in order to reduce as much as possible the number of animals used and every procedure was performed with the aim to minimize their suffering.

Battery of behavioural tests

Hole board test. During day 1, BTBR and BL6 animals performed the Hole Board task. This test was carried out in a wooden box (40 × 40 x 35 cm) having 16 holes with a diameter of 3 cm on the ground and placed 5 cm from the floor, as previously described [60]. The test lasted 10 min and an automatic counter registered the number of times the animals poking the hole for a duration of at least 1 sec, reported as number of poking holes.

Open Field test. During day 2, the Open Field test was carried out. The test was performed according [61]. Briefly, the animals were left to explore an open field arena (40 × 40 x 35 cm) for 5 min. ANY-maze tracking software version 7 (Ugo Basile-Varese, Gemonio, Italy) recorded and analyzed the locomotory activity of each mouse by measuring the distance travelled, the duration and the frequency of freezing behaviour and the time spent in the center and in the wall of the arena. Between one test and another, a solution at 70% of ethanol was used to clean arena floor and avoid inter-assay bias.

Social Interaction test. During day 3, the Social Interaction test was performed, as previously described [62]. The animals, after 2 days of individual housing, were left in the same large box used for the Open Field arena and let free to explore one plastic object, one paper cylinder, one ball and an unfamiliar stimulus mouse (same strain, sex and age of subject mouse) for 5 min. A camera recorded the test and a blind observer scored the frequency and duration of social behaviours from an investigative and affiliative point of view and also frequency and duration of non-social behaviour, considering the attitude towards objects, like sniffing, exploring and playing with the objects. [63, 64].

Elevated Zero Maze test. During day 4, the Elevated Zero Maze test was carried out, as previously described [65]. Precisely, a maze built in black acrylic in a circular track 10 cm wide, 105 cm in diameter and 72 cm high was used. Two opposed closed quadrants and two opposed open quadrants with black acrylic walls 28 cm high composed the maze. On the test day the animal was placed at a casually chosen boundary between an open and a closed zone, facing the closed area. After each trial, the maze was cleaned with a 70% ethanol solution. The test lasted 5 min and a blind observer evaluated the time spent in the open and in the closed corridors, expressed in seconds.

Post-mortem tissues analyses

After behavioural tests, the mice were sacrificed by cervical dislocation and brains were randomly divided for ex vivo analysis. PFC, HIPP, HYP and AMY were removed from brains, in accordance with the mouse brain atlas of Paxinos and Franklin, and frozen, stored at −80 °C for subsequent analyses. To perform biomolecular studies, the tissues were homogenated and diluted 1:10 w/v in PBS buffer with 1:100 protease and phosphatase inhibitor (HALT inhibitors, Thermo Fisher Scientific, Cleveland, OH, USA) at 4 °C; while for neurochemical analyses, the samples were homogenated and diluted 1:10 w/v in perchloric acid 0.1 M at 4 °C. In both cases, after dilution, a centrifuge at 10.000 x *g* at 4 °C for 10 min was carried out and the supernatants were analysed.

Western blotting quantification

In this procedure, protein extracts were generated from fresh frozen PFC, HIPP and AMY tissues following samples homogenization with a Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail, EDTA-Free (Thermo Fisher Scientific, Cleveland, OH, USA). Subsequently, lysates were measured for total protein concentration using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Cleveland, OH, USA) and the Multiskan™ FC Microplate spectrophotometer (Thermo Fisher Scientific, USA) at 570 nm. For SDS-PAGE the total amount of samples protein (forty µg) loaded in to the 4–15% Mini-PROTEAN™ TGX Stain-Free™ Protein Gels (Bio-Rad Laboratories Inc, Segrate (MI), Italy) for electrophoresis and then transferred on the Nitrocellulose membrane (Bio-Rad Laboratories Inc, Segrate (MI), Italy) by Pierce™ Power Blotter (Thermo Fisher Scientific, Cleveland, OH, USA). Then, the membranes were blocked in the 5% skimmed milk for 1 h at room temperature followed the incubation with rabbit monoclonal antibodies against NGF (ab52918; 1:1000, Abcam, Cambridge, UK), BDNF (ab226843; 1:1000, Abcam, Cambridge, UK) and mouse monoclonal antibodies against β-actin (ab8226; 1:1000, Abcam, Cambridge, UK) overnight at 4 °C. In add to this, the incubation (1 h at room temperature) with horseradish peroxidase-conjugated specific (Goat anti-rabbit (ab6721; 1:5000, Abcam, UK) and goat anti-mouse (ab205719; 1:5000, Abcam, UK)) secondary antibodies were used. Clarity™ Western ECL Substrate (Bio-Rad Laboratories Inc, Segrate (MI), Italy) was used for protein bands visualization. ChemiDoc™ XRS+ system (Bio-Rad Laboratories Inc, Segrate (MI), Italy) was used to detect chemiluminescence and ImageJ software (version 1.52a; National Institutes of Health, USA) was utilized to quantify the optical densities of the bands that were then normalized versus bands of β-actin.

Neurochemical quantifications

The neurochemical quantifications were carried out in PFC, HIPP and AMY of BTBR and BL6 animals. In particular DA, NA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) were measured by using high-performance liquid chromatography coupled with an electrochemical detector (Ultimate ECD, Dionex Scientific, Milan, Italy). LC18 reverse phase column (Kinetex, 150 mm × 3.0 mm, ODS 5 µm; Phenomenex, Castel Maggiore-Bologna, Italy) was utilized to separate the catecholamines that were detected by a thin-layer amperometric cell (Dionex, ThermoScientific, Milan, Italy) with a 5-mm diameter glassy carbon electrode by using 400 mV as working potential vs. Pd. An aqueous buffer (pH 3.0) composed of 75 mM NaH2PO4, 1.7 mM octane sulfonic acid, 0.3 mM EDTA, acetonitrile 10%, was used as mobile phase and an isocratic pump (Shimadzu LC-10 AD, Kyoto, Japan) worked at 0.7 ml·min-1 as flow rate. To perform the data acquisition and integration, Chromeleon software (version 6.80, Dionex, Thermo Scientific, San Donato Milanese, Italy) was utilized. Moreover, GABA and glutamate amounts were quantified by high-performance liquid chromatography with fluorescence detection, after derivatization with ophthalaldehyde/mercaptopropionic acid (emission length, 460 nm; excitation length, 340 nm) by using an OD column (Kinetex, 150 mm×3.0 mm, ODS 5 µm; Phenomenex, Castel Maggiore-Bologna, Italy). The mobile phase used was a gradient phase of 50 mM sodium acetate buffer, pH 6.95, with methanol increasing linearly from 2 to 30% (v/v) over 40 min. A pump (JASCO, Tokyo, Japan) maintained the flow rate at 0.5 ml/min and the Borwin software (version 1.50; Jasco) was used to analyze the results. Results, after being normalized for total area weight, were expressed as concentration/mg of tissue.

Acetylcholine (ACh) assay

The levels of ACh were quantified in tissue homozenaized by using the commercially available kit (Catalog Number MAK435, Sigma-Aldrich, Milano, Italy), following the manufacturer's instructions. Tissue has been prepared by homogenization in cold 1× PBS and centrifugation (5 min at 14,000 × *g*). In the assay utilized, acetylcholine is hydrolysed by acetylcholinesterase to choline which is oxidized by choline oxidase to betaine and H2O2. H2O2 reacts with a specific dye to form a colored product. The color intensity was analysed by the Multiskan™ FC Microplate spectrophotometer (Thermo Fisher Scientific, USA) at 570 nm and it is directly proportional to the acetylcholine concentration in the sample. Results were expressed in accordance to the linear detection range for the acetylcholine assay method (10-200 µM). Each sample analysis was performed in duplicate to avoid intra-assay variations.

Blindness of the study

For each test, scoring process and analysis, the experimenters were blind with respect to the experimental groups.

Statistical analyses

Sample size calculation has been performed a priori by using G power software. Statistical analyses were carried out by GraphPad Prism software (version 9.5.0; San Diego, CA, USA). In particular, Shapiro-Wilk test for normality and ROUT method to identify statistical outliers were performed for each group. Subsequently, Unpaired Student's *t*-test two-sided, with Welch's correction when needed, was used to analyze data that were expressed as mean ± standard error of the mean (SEM). Correlation between behavioural outcomes and ex vivo results were analyzed by using Pearson correlation. Differences between groups were considered significant with a P value less than 0.05.

RESULTS

BTBR mice showed increased repetitive behaviour and novelty-induced hyperlocomotion

In order to evaluate repetitive behaviours, we performed the Hole Board task. Our results showed that BTBR mice reported a significant increase in the number of poking holes compared to control mice (Supplementary Fig. 1A, Unpaired Student's *t*-test, $P < 0.001$ BTBR vs. BL6). Furthermore, the locomotor activity in a novel environment was investigated in the Open Field paradigm. We observed an increase of the distance travelled in meters in the arena, index of novelty-induced hyperlocomotion, in BTBR mice compared with control animals (Supplementary Fig. 1B, Unpaired Student's *t*-test, $P < 0.0001$ BTBR vs. BL6). Furthermore, BTBR also showed a decrease in freezing duration (Supplementary Fig. 1C, Unpaired Student's *t*-test, $P < 0.05$ BL6 vs. BTBR) and freezing frequency (Supplementary Fig. 1D, Unpaired Student's *t*-test, $P < 0.05$ BL6 vs. BTBR) compared with BL6 animals.

BTBR mice showed reduced social behaviours and increased non-social behaviours

To investigate social dysfunctions, the Social Interaction test was performed. During Social Interaction test, BTBR mice performed significantly less social duration time (sec) compared to BL6 mice (Supplementary Fig. 2A, Unpaired Student's *t*-test, $P < 0.05$ BTBR vs. BL6). Moreover, the time (sec) spent performing non-social interactions in BTBR animals was significantly increased compared to controls (Supplementary Fig. 2B, Unpaired Student's *t*-test, $P < 0.0001$ BTBR vs. BL6). As regarding the frequency of such behaviours, BTBR social frequency measurements were

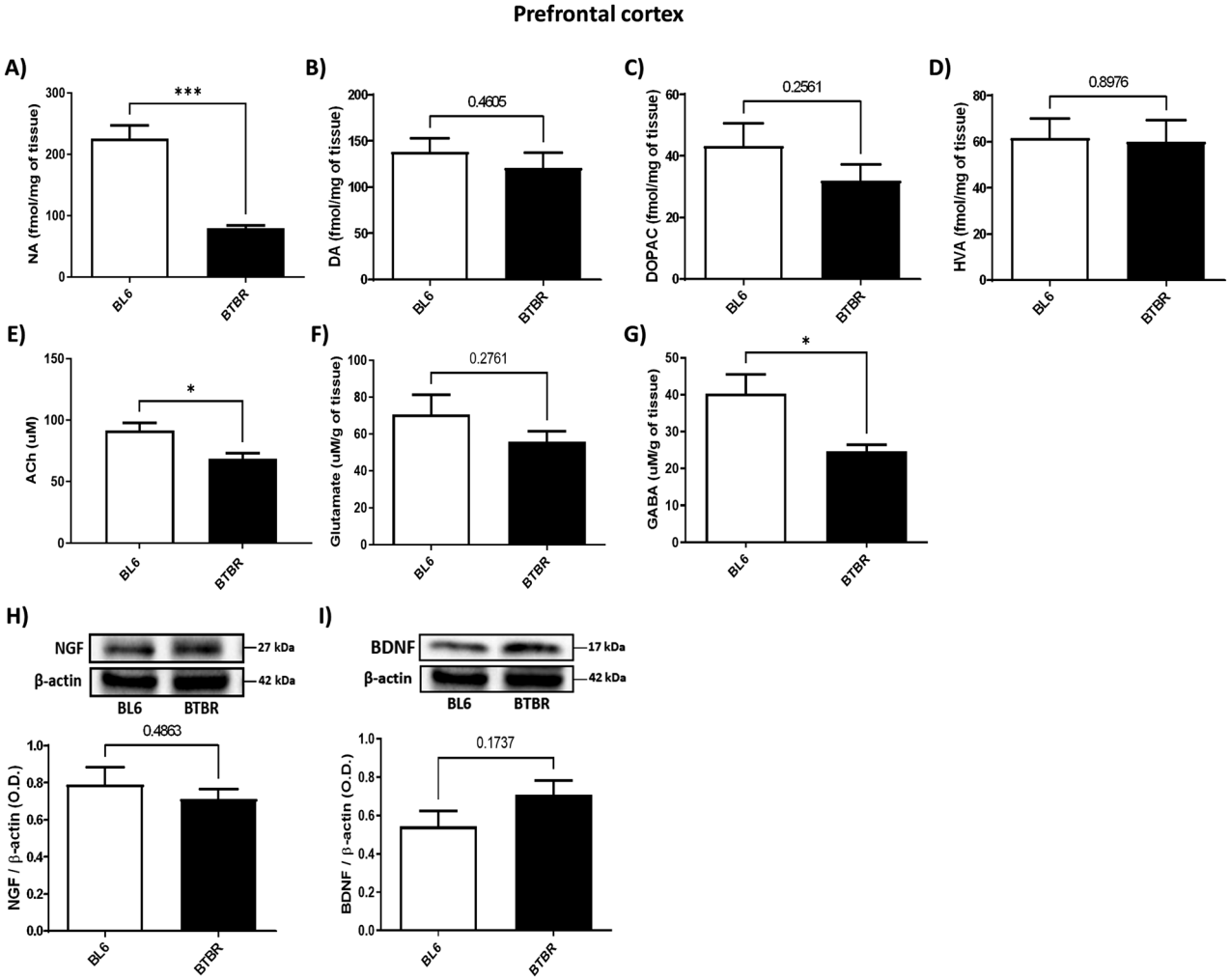

Fig. 1 NA, DA, DOPAC, HV, Ach, Glutamate, GABA, NGF and BDNF levels in PFC of BTBR (black bar) and BL6 (white bar) mice. A NA levels (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, ***P = 0.0008 BTBR vs. BL6; **B** DA amount (fmol/mg) in the PFC of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, n.s.; **C** DOPAC levels (fmol/mg) in the PFC of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, n.s.; **D** HVA levels (fmol/mg) in the PFC of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, n.s.; **E** Ach levels (uM) in the PFC of BL6 ($n = 5$) and BTBR ($n = 4$) mice. Unpaired Student's *t*-test, *$P = 0.0176$ BTBR vs. BL6; **F** Glutamate levels (uM/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, n.s.; **G** GABA levels (uM/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, *$P = 0.0290$ BTBR vs. BL6; **H** NGF expression levels of BTBR ($n = 5$) and BL6 ($n = 4$). Unpaired Student's *t*-test, n.s. Quantification of the optical band density of NGF normalized for optical band density of β-actin housekeeping gene; **I** BDNF expression levels of BTBR ($n = 5$) and BL6 ($n = 4$). Unpaired Student's *t*-test, n.s. Quantification of the optical band density of BDNF normalized for optical band density of β-actin housekeeping gene.

significantly reduced (Supplementary Fig. 2C, Unpaired Student's *t*-test, $P < 0.05$ BL6 vs. BTBR), while there was a significant enhancement in non-social frequency compared to BL6 animals (Supplementary Fig. 2D, Unpaired Student's *t*-test, $P < 0.0001$ BTBR vs. BL6).

BTBR mice showed reduced anxiety-like behaviours

To evaluate the anxiety-like behaviours, we used the Elevated Zero Maze and the Open Field tests. We found that BTBR animals showed an increase in the time spent exploring the open corridors compared to controls (Supplementary Fig. 3A, Unpaired Student's *t*-test, $P < 0.001$ BTBR vs. BL6). Accordingly, the time spent in closed quadrants of BTBR mice was significantly decreased compared to BL6 (Supplementary Fig. 3B, Unpaired Student's *t*-test, $P < 0.001$ BTBR vs. BL6). Moreover, results from Open field test showed that the time spent in the center in BTBR animals was significantly increased compared to control animals (Supplementary Fig. 3C, Unpaired Student's *t*-test, $P < 0.0001$ BTBR vs. BL6), while the time spent in the wall was a significantly reduced, respectively (Supplementary Fig. 3D, Unpaired Student's *t*-test, $P < 0.0001$ BTBR vs. BL6).

Subsequently, we performed ex vivo analyses in PFC, HIPP, HYP and AMY tissues to quantify NA, DA and DA metabolites (DOPAC and HVA) levels, together with Acetylcholine (ACh), Glutamate and GABA, and NGF and BDNF expression levels in both experimental groups.

BTBR mice showed reduced NA, ACh and GABA levels in PFC

Our results reported a significantly decrease of NA levels in the PFC of BTBR animals compared to BL6 (Fig. 1A, Unpaired Student's *t*-test, $P < 0.0001$ BTBR vs. BL6), together with ACh and GABA levels, respectively (Figs. 1E, G, Unpaired Student's *t*-test, $P < 0.05$ BTBR vs. BL6). As regarding DA, DOPAC, HVA, NGF, BDNF and glutamate content, our results did not show any difference between the two groups (Fig. 1, Unpaired Student's *t*-test, n.s.).

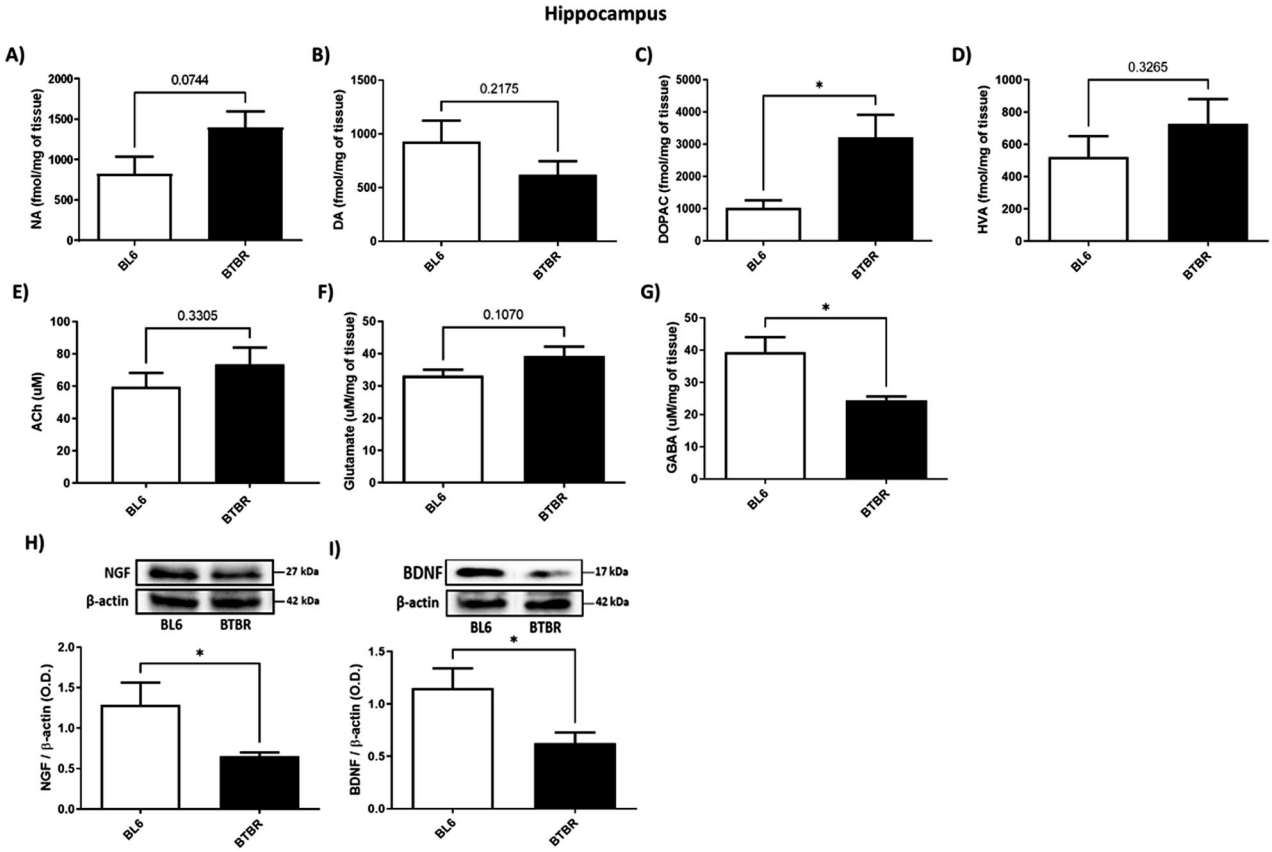

Fig. 2 NA, DA, DOPAC, HV, Ach, Glutamate, GABA, NGF and BDNF levels in HIPP of BTBR (black bar) and BL6 (white bar) mice. A NA amount (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, n.s.; **B** DA levels (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, n.s.; **C** DOPAC amount (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, $^*P = 0.0323$ BTBR vs. BL6; **D** HVA amount (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, n.s.; **E** Ach levels (uM) of BL6 ($n = 5$) and BTBR ($n = 4$) mice. Unpaired Student's *t*-test, n.s.; **F** Glutamate levels (uM/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, n.s.; **G** GABA levels (uM/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, $^*P = 0.0221$ BTBR vs. BL6; **H** NGF expression levels of BTBR ($n = 5$) and BL6 ($n = 4$). Unpaired Student's *t*-test, $^*P = 0.0361$ BTBR vs. BL6. Quantification of the optical band density of NGF normalized for optical band density of β-actin housekeeping gene; **I** BDNF expression levels of BTBR ($n = 5$) and BL6 ($n = 4$). Unpaired Student's t-test, $^*P = 0.0352$ BTBR vs. BL6. Quantification of the optical band density of BDNF normalized for optical band density of β-actin housekeeping gene.

BTBR mice showed enhanced DOPAC and decreased NGF, BDNF and GABA levels in HIPP

As regards HIPP, we did not find any difference in NA, DA, HVA, ACh and glutamate levels between BTBR and BL6 mice (Fig. 2, Unpaired Student's *t*-test, n.s.). Moreover, analysis of DOPAC content reported a significant increase in BTBR animals compared to controls (Fig. 2C, Unpaired Student's *t*-test, $P < 0.05$ BTBR vs. BL6), while NGF and BDNF expression levels, together with GABA content were decreased (Figs. 2G–I, Unpaired Student's *t*-test, $P < 0.05$ BTBR vs. BL6).

BTBR mice did not show any difference in HYP

Concerning HYP analysis, no differences in NA, DA, DOPAC, HVA, ACh, NGF, BDNF, glutamate and GABA levels were found between groups (Fig. 3, Unpaired Student's *t*-test, n.s.).

BTBR mice showed reduced DA, NA, ACh, NGF and GABA and increased DA metabolism and glutamate content in AMY

Our results showed that NA, DA, ACh, NGF and GABA levels in AMY were substantially reduced in BTBR compared to BL6 animals (Fig. 4, Unpaired Student's *t*-test, $P < 0.05$ BTBR vs. BL6). In addition, DOPAC, HVA and glutamate content in AMY of BTBR animals showed a significant enhancement compared to controls (Figs. 4C, D and F, Unpaired Student's *t*-test, $P < 0.001$, $P < 0.05$ BTBR vs. BL6), while BDNF did not exhibit any differences (Fig. 4I, Unpaired Student's *t*-test, n.s.).

BTBR mice showed negative correlation between repetitive behaviours and hyperlocomotion with amygdalar NA levels and between non social behaviours and amygdalar GABA amount

In order to investigate the presence of correlations between ASD behaviours and ex vivo parameters in amygdala, we performed Pearson correlation in mice from both groups. We did not find any significant correlation (data not shown), except for the following reported results; we found a significant negative correlation between the number of poking holes in the Hole Board task and the levels of amygdalar NA (Fig. 5A Pearson correlation, $r = -0.8857$; $P < 0.01$), together with a significant negative correlation between the distance travelled in the Open Field test and the levels of amygdalar NA (Fig. 5B, Pearson correlation, $r = -0.8608$; $P < 0.01$). Moreover, frequency of non-social behaviours in the Social Interaction test and GABA levels in AMY were also negatively correlated (Fig. 5E, Pearson correlation, $r = -0.7203$; $P < 0.05$).

DISCUSSION

In the present study, the BTBR mice model reported ASD-like behavioural features, accompanied by several neurochemical alterations, in particular concerning the amygdalar area.

In this regard, we characterized the BTBR strain from a neurochemical point of view, by analysing dopaminergic,

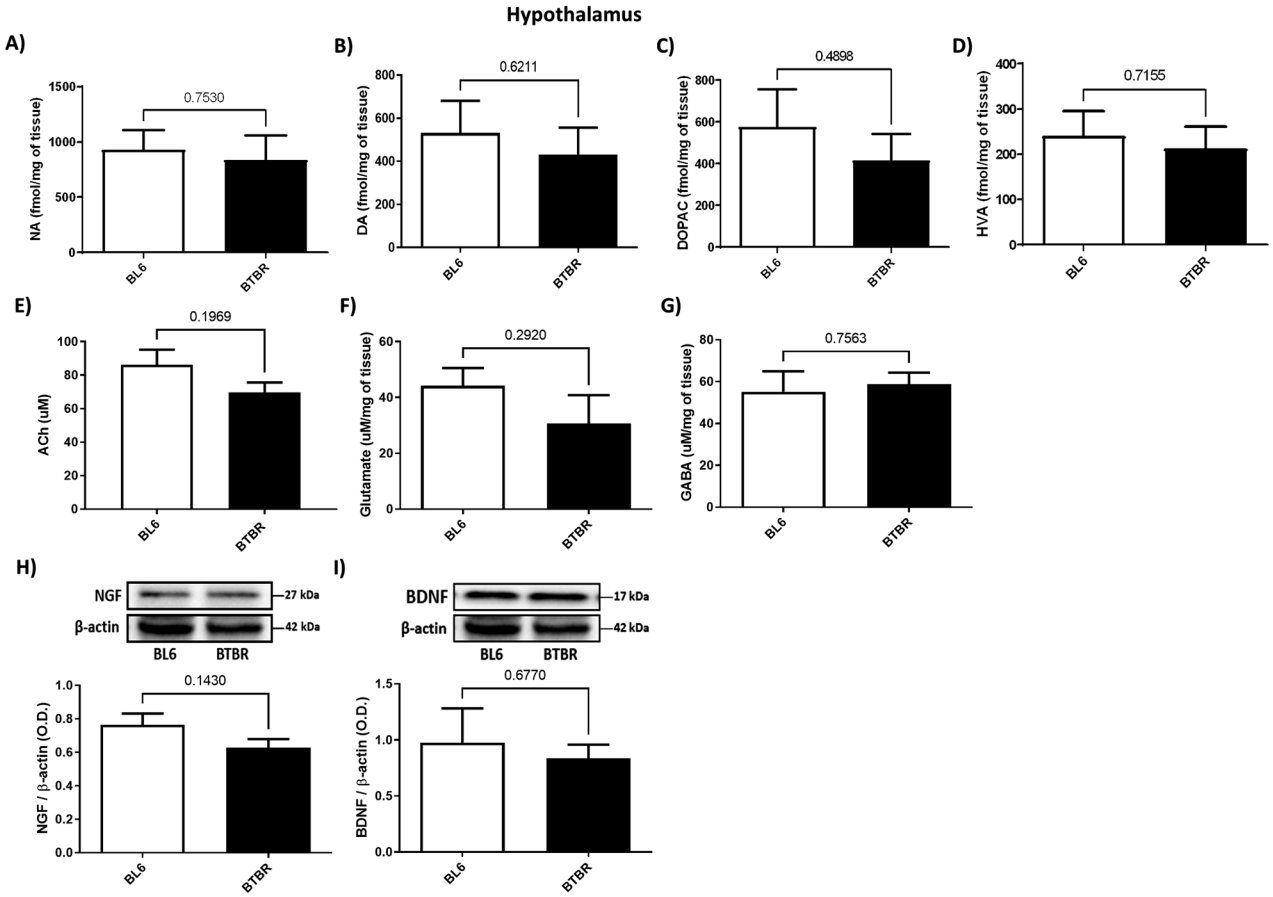

Fig. 3 NA, DA, DOPAC, HV, Ach, Glutamate, GABA, NGF and BDNF levels in HYP of BTBR (black bar) and BL6 (white bar) mice. A NA amount (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, n.s.; **B** DA levels (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, n.s.; **C** DOPAC amount (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, n.s.; **D** HVA amount (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, n.s.; **E** Ach levels (uM) of BL6 ($n = 5$) and BTBR ($n = 4$) mice. Unpaired Student's *t*-test, n.s.; **F** Glutamate levels (uM/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, n.s.; **G** GABA levels (uM/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's t-test, n.s.; **H** NGF expression levels of BTBR ($n = 5$) and BL6 ($n = 4$). Unpaired Student's *t*-test, n.s. Quantification of the optical band density of NGF normalized for optical band density of β-actin housekeeping gene; **I** BDNF expression levels of BTBR ($n = 5$) and BL6 ($n = 4$). Unpaired Student's *t*-test, n.s. Quantification of the optical band density of BDNF normalized for optical band density of β-actin housekeeping gene.

noradrenergic and cholinergic, together with excitatory-inhibitory neurotransmissions and NGF and BDNF expression levels, in four different brain areas that are involved in ASD and that are strictly interplayed among each other. As regards PFC, our results showed a decrease in NA, ACh and GABA levels, while the HIPP was characterized by enhanced DA metabolism and reduced NGF, BDNF and GABA content. Concerning HYP, no differences were retrieved among the different neurotransmitters analysed. Interestingly, we found significant alterations in the AMY, in which there was a decrease in NA, DA, ACh, GABA and NGF levels, together with an increase in DA metabolites and glutamate content, thus denoting massive dysfunctions in this brain region. According to our results, it has been reported that a significant activation of the DA turnover in AMY exacerbated the ASD-like behaviours in BTBR mice [66] and that a reduction in the DA release in AMY was associated with an enhancement in grooming episodes [67]. Moreover, it has been reported that impairment during AMY development is a crucial component of the neurophenotype of ASD, occurring certain time before behavioural diagnosis of ASD can reliably be made [68]. In addition, the increased AMY growth in ASD children has been associated with an enhancement of social dysfunctions [68]. Furthermore, it has been demonstrated that lesions in the AMY altered fear response and reduced anxiety [69]. However, it has to be taken into account that the basolateral nucleus of the AMY sends projections to the PFC and HIPP [70] and that these three areas are in continuous crosstalk. Indeed, several studies reported a dopaminergic modulation in widespread forebrain areas of ASD patients [67, 71–76]. In regards to ACh content, we found a decrease in PFC and AMY according to neurochemical alterations in the cholinergic pathway observed in a *postmortem* study involving ASD patients [77]. Moreover, it has been reported that ACh increase could lead to an improvement of cognitive deficits in ASD and other neuropsychiatric disorders [78]. Interestingly, reduced ACh in the basal forebrain has been linked to decreased social interactions and social memory dysfunctions in mice [51]. Furthermore, we also found a decrease in cortical and amygdalar NA levels in BTBR mice. In this regard, it has been reported that stereotyped behaviours, considered as derivatives of poor adaptive behaviours, are principally mediated by the noradrenergic system and that the administration of an alpha-2-receptor agonist might be helpful to improve ASD behavioural dysfunctions [79]. In line with our results, another model of ASD, the Engrailed-2 knock-out (EN2KO) mice, also reported NA neurotransmission alterations in the ventral hindbrain [80]. In addition, it has been shown that the administration of atomoxetine, a NA reuptake inhibitor, could be effective in treating hyperactivity in children with ASD [81]. In addition, we found a significant negative

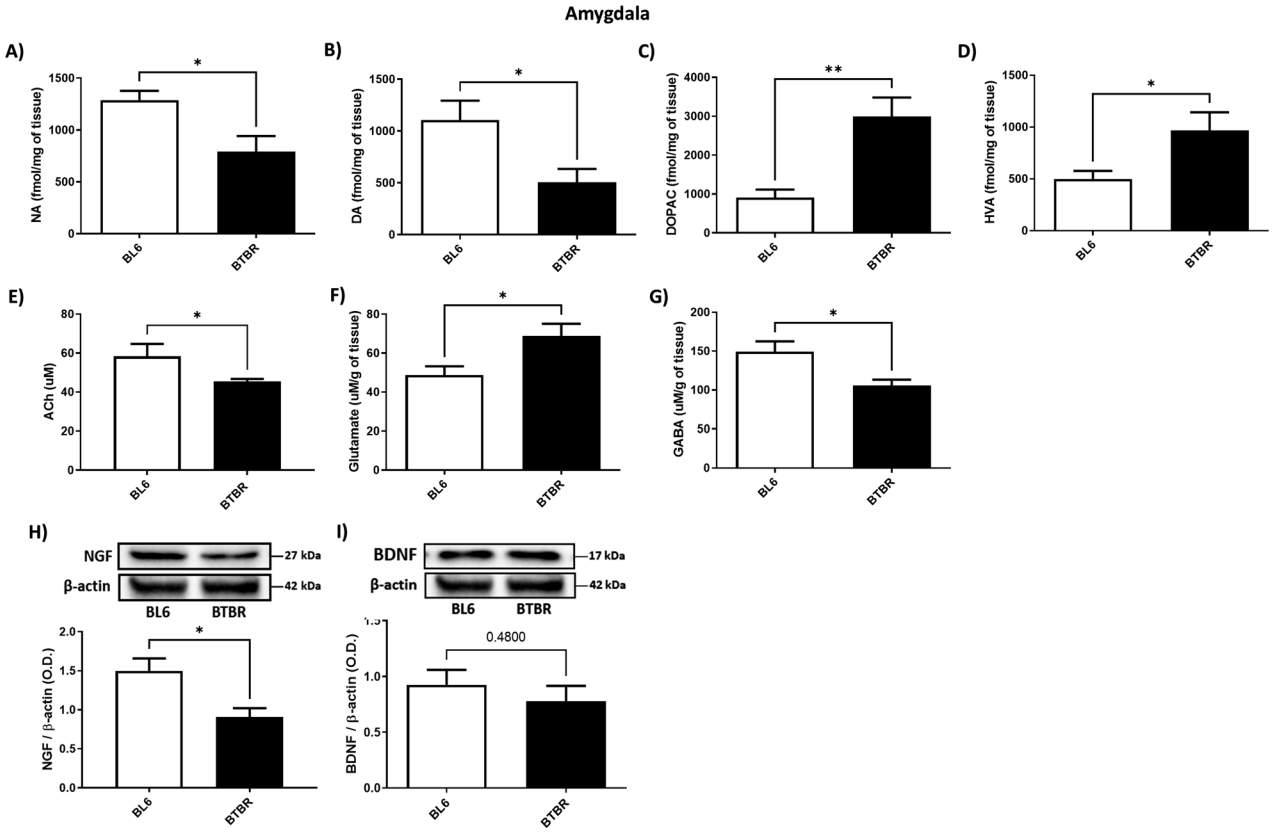

Fig. 4 NA, DA, DOPAC, HV, Ach, Glutamate, GABA, NGF and BDNF levels in AMY of BTBR (black bar) and BL6 (white bar) mice. A NA levels (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, $^{*}P = 0.0221$ BTBR vs. BL6; **B** DA levels (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, $^{*}P = 0.0266$ BTBR vs. BL6; **C** DOPAC levels (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, $^{**}P = 0.0100$ BTBR vs. BL6; **D** HVA levels (fmol/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, $^{*}P = 0.0469$ BTBR vs. BL6; **E** Ach levels (uM) of BL6 ($n = 5$) and BTBR ($n = 4$) mice. Unpaired Student's *t*-test, $^{*}P = 0.0286$ BTBR vs. BL6; **F** Glutamate levels (uM/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, $^{*}P = 0.0366$ BTBR vs. BL6; **G** GABA levels (uM/mg) of BL6 ($n = 5$) and BTBR ($n = 5$) mice. Unpaired Student's *t*-test, $^{*}P = 0.0244$ BTBR vs. BL6; **H** NGF expression levels of BTBR ($n = 5$) and BL6 ($n = 4$). Unpaired Student's *t*-test, $^{*}P = 0.0167$ BTBR vs. BL6. Quantification of the optical band density of NGF normalized for optical band density of β-actin housekeeping gene; **I**) BDNF expression levels of BTBR ($n = 5$) and BL6 ($n = 4$). Unpaired Student's *t*-test, n.s. Quantification of the optical band density of BDNF normalized for optical band density of β-actin housekeeping gene.

correlation between the number of poking holes (in the Hole Board test) with the amount of NA in AMY. Moreover, the levels of NA in AMY were also negatively correlated with the distance travelled, thus denoting a significant inverse correlation between repetitive behaviours and hyperactivity with NA levels. Accordingly, an important role of NA in attention-deficit/hyperactivity disorders has been reported, therefore different treatments for hyperactivity aim to increase NA brain levels [82]. On the other hand, the neurobiology of stereotyped behaviours is still controversial [83]. However, it has been demonstrated that mice lacking NA expressed excessive grooming behaviour, a well-known repetitive behaviour [84], and that NA upregulation reduced both hyperactivity and stereotypic behaviours [44], hence suggesting that NA alterations might be crucially involved in the development of such behavioural features and that the targeted modulation of noradrenergic neurotransmission could result in improving stereotyped repertoire and hyperactivity.

Furthermore, different studies reported an interplay between NA neurotransmission and NGF expression [85–87]. In particular, it has been shown that, in cellular models, monoamine oxidase inhibitors increased NGF expression [87] and that NA can exert neuroprotective properties by inducing NGF expression [85]. In this regard, our results showed that BTBR animals had decreased NGF and BDNF expression levels in HIPP, a brain region in which such neurotrophins are mainly present [88]. Accordingly, hippocampal BDNF and NGF deficiency has been associated with ASD development [89, 90], however results are contrasting, since BDNF brain levels fluctuations have been reported in response to several known, such as brain areas and age, and unknown factors [91]. In addition, NGF levels were reduced also in AMY, a brain region in which NGF signalling has been linked to stress, reward and neuroprotection [92]. Moreover, NGF expression might be modulated also by the GABAergic system. Indeed, it has been demonstrated that NGF production is predominantly localized in GABAergic inhibitory neurons [93]. In our results, we also found impairments of excitatory-inhibitory neurotransmissions. In particular, we showed an increase in glutamate and a reduction in GABA levels in the AMY, while in PFC and HIPP we only reported a decrease in GABA content. Thus, BTBR animals demonstrated a perturbation of the excitatory-inhibitory balance, by showing disruptions in the PFC and HIPP and disequilibrium in favour of glutamate in AMY. These data corroborate with our previous studies in which BTBR housed in a semi-natural environment showed a decrease in GABA levels in AMY [94]. Accordingly, great interest is moving toward GABA involvement in sociability pathways. Indeed, it has been shown that a reduction in GABA functions in the basolateral AMY triggered social interaction dysfunctions [95]. Regarding glutamate, already in 2008 Fatemi [96] proposed the hyperglutamatergic theory of autism.

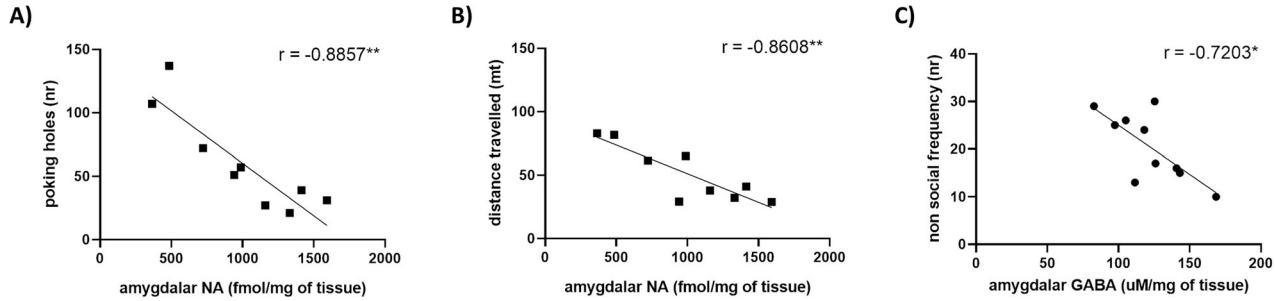

Fig. 5 Correlation between repetitive behaviours and hyperlocomotion with amygdalar NA levels and between non social behaviours and amygdalar GABA amount in BTBR and BL6 mice. A Correlation between poking holes (nr) and amygdalar NA (fmol/mg of tissue). Pearson correlation, $r = -0.8857$; **P = 0.0015; **B** Correlation between distance travelled (mt) and amygdalar NA (fmol/mg of tissue). Pearson correlation, $r = -0.8608$; $^{**}P = 0.0029$; **C** Correlation between non social behaviours frequency (nr) and amygdalar GABA (uM/mg of tissue). Pearson correlation, $r = -0.7203$; $^{*}P = 0.0188$.

Furthermore, the reduction in GABA levels in AMY has been associated with decreased sociability, especially considering that AMY is a crucial component of the social brain [95]. Concerning behavioural alterations, our results confirmed stereotyped repertoire, social interaction deficits and novelty-induced hyperlocomotion of BTBR mice [97–100], but also showed an increase of the time spent performing non-social exploration, together with the frequency of such behaviour. In this regard, the exploration of three objects non socially-related might resemble the absence of interests in people and in developing relationships, an important behavioural feature of ASD patients [101]. Moreover, we found a significant negative correlation between GABA amount in AMY and frequency of non-social behaviours, which could mimic the indifference and the lack of interest for the surrounding social stimuli often described in ASD patients [102]. Lastly, we also showed that BTBR animals display lower anxiety levels, which could also be interpreted as enhanced hyperactivity and impulsiveness, important traits typical of ASD patients [103].

In conclusion, our neurochemical characterization of the BTBR strain suggested that such idiopathic animal model might be useful to unravel neurobiological and neurochemical correlates of ASD behavioural dysfunctions, highlighting the important role of neurotransmitter alterations in a specific brain region, such as AMY, and strengthening that, beyond the well-known face validity, BTBR mice also exhibit a high-grade of construct validity.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Substantial contributions to the conception or design of the work: MB and LT. Acquisition, analysis and interpretation of data for the work: MAP, MS, LPA, SD, GC, VS, PT, SS, MGM. Drafting the work: MB, VS, PT, SS, MGM, LT. Revising the work critically: MB, MAP, MS, LPA, SG, AR, SD, GC, VS, PT, SS, MGM, LT. Final approval: MB, MAP, MS, LPA, SG, AR, SD, GC, VS, PT, SS, MGM, LT. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work: MB, MAP, MS, LPA, SG, AR, SD, GC, VS, PT, SS, MGM, LT.

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COMPETING INTERESTS

The authors declare no competing interests.

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