Biomedicine & Pharmacotherapy 138 (2021) 111517

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

The histamine H3R and dopamine D2R/D3R antagonist ST-713 ameliorates autism-like behavioral features in BTBR T+tf/J mice by multiple actions

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ARTICLE INFO

Keywords:
Autistic spectrum disorder
BTBR mice
Histamine H3 receptor antagonist
Dopamine D2/D3R antagonist
Stereotyped repetitive behavior
Neuroinflammation

ABSTRACT

Several brain neurotransmitters, including histamine (HA), acetylcholine (ACh), and dopamine (DA) are suggested to be involved in several brain disorders including cognitive deficits, depression, schizophrenia, anxiety, and narcolepsy, all of which are comorbid with Autism spectrum disorder (ASD). Therefore, the ameliorative effects of the novel multiple-active compound ST-713 with high binding affinities at histamine H3 receptor (H3R), dopamine D2sR and D3R on ASD-like behaviors in male BTBR T+tf/J mice model were assessed. ST-713 (3-(2-chloro-10*H*-phenothiazin-10-yl)-*N*-methyl-*N*-(4-(3-(piperidin-1-yl)propoxy)benzyl)propan-1-amine; 2.5, 5, and 10 mg/kg, i.p.) ameliorated dose-dependently social deficits, and significantly alleviated the repetitive/compulsive behaviors of BTBR mice (all $P < 0.05$). Moreover, ST-713 modulated disturbed anxiety levels, but failed to obliterate increased hyperactivity of tested mice. Furthermore, ST-713 (5 mg/kg) attenuated the increased levels of hippocampal and cerebellar protein expressions of NF-κB p65, COX-2, and iNOS in BTBR mice (all $P < 0.05$). The ameliorative effects of ST-713 on social parameters were entirely reversed by co-administration of the H3R agonist (*R*)-α-methylhistamine or the anticholinergic drug scopolamine. The obtained results demonstrate the potential of multiple-active compounds for the therapeutic management of neuropsychiatric disorders, e.g. ASD.

1. Introduction

Autism spectrum disorder (ASD) is commonly diagnosed neurodevelopment disorder, defined by deficits in social communication, stereotypical patterns of restricted and repetitive behaviors [1–6]. Epidemiological studies reported that approximately half of the children diagnosed with an ASD disorder also have intellectual impairments, of which the general cognitive deficits are fundamental to many aspects of social cognitive dysfunctions [7]. At present, behavioral intervention is the only effective form of treatment, with many positive outcomes reported [7–9]. The only approved pharmacological treatments for ASD are risperidone (Risperdal®) and aripiprazole (Abilify®), which only target the associated symptoms of irritability that include self-injury, tantrums and aggression [2,4,5,10].

Recent progresses in drug developments focus on novel agents with multiple pharmacological effects for multifactorial diseases, such as ASD [2,4,11,12]. In search of specific markers for ASD, several research efforts have concentrated on the study of various brain neurotransmitters [2]. Hence, evaluation of the function of various brain neurotransmitters, e.g., histamine (HA), dopamine (DA), acetylcholine (ACh), serotonin (5-HT), γ-aminobutyric acid (GABA), and glutamate (Glu) in early stage of brain development became an interesting field of research to develop innovative therapeutics [2,13–19]. Preclinical as well as clinical evidences revealed the involvement of dopamine (DA) and dopaminergic system (DS) dysfunction in the phenotypic outcomes of ASD-related behavioral deficits, in both humans and animal models [2,6]. Also, there is a wide clinical use of antipsychotics that mainly target the D2 receptors (D2Rs) in schizophrenia which shares similarities in several features with ASD [20].

Accordingly, the brain DS with DA has a crucial role in controlling

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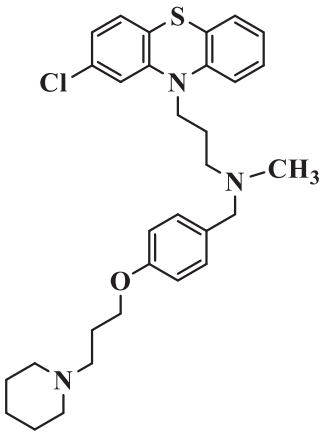
https://doi.org/10.1016/j.biopha.2021.111517
Received 10 January 2021; Received in revised form 7 March 2021; Accepted 14 March 2021
Available online 24 March 2021

ASD-related behavioral features including attention, cognitive flexibility, social interaction, and stereotypical behaviors [2,21]. Previous report suggested that mice with increased dopaminergic neurotransmission exhibited significant deficits in sociability and repetitive behaviors, while these behavioral alterations were reversed by administration of several antagonists targeting D1Rs [22]. Also, D1R agonists were found to induce typical ASD-like features in normal mice, and these features were comparable to those witnessed in the D2R knockout (KO) mice, indicating that modulation of the brain DS is strongly contributing to the ASD-like behaviors in mice [15,16,21]. On the other side, brain HA provides its effects through binding to four known histamine receptor (HR) subtypes belonging to the family of G-protein-coupled receptors, and designated H1 to H4 receptors (H1R-H4R). The histamine H3 receptor (H3R) initially described in 1983 was found to negatively regulate HA synthesis and release, acting as presynaptic auto-receptors [23,24] or hetero-receptors modulating the release of several other brain neurotransmitters like ACh, 5-HT, DA, GABA, and Glu in various brain regions [19,25]. Evidence from previous studies suggested indirectly that histaminergic neurotransmission system may play an important role in schizophrenia (SCH) so that potent and selective H3R antagonists or inverse agonists potentially lead to therapeutic improvements of cognitive symptoms associated with SCH and ASD [2,18,19,26,27]. However, there are up to-date limited literature studies regarding the relationship between H3R antagonists and treatment of ASD behavioral impairments, and only few H3R antagonists were found able to mitigate impaired ASD-like features in preclinical experiments in rodents [3–5,28,29].

Moreover, the imbalance at the level of oxidant-antioxidant in ASD patients was found to contribute significantly to the pathogenesis and severity of ASD along with numerous proinflammatory cytokines, e.g. nuclear factor κ-B (NF-κB) and inducible nitric oxide synthase (iNOS), which were associated with the neurobehavioral dysregulations [30,31]. Accordingly, previous clinical observations revealed the increase in protein and lipid peroxidation products such as nitrotyrosine both in the periphery and CNS of ASD patients [30,32–36]. In addition, several other studies showed dysregulations in enzymatic and non-enzymatic antioxidants, e.g. consistent decreased levels of glutathione in brain and blood of patients diagnosed with ASD [30,37–39]. Notably, a recent preclinical study revealed that oxidant-antioxidant balance plays an essential role in the severity of ASD-like repetitive behaviors in BTBR mice, demonstrating BTBR mice as a useful model in exploration of antioxidant intervention strategies that have translational value [30].

Pursuing the discovery of effective pharmacological interventions with consideration that brain HA and DA are involved in the (patho) physiology of ASD, the novel multiple-targeting H3R and D2R/D3R ligand ST-713 [3-(2-chloro-10*H*-phenothiazin-10-yl)-*N*-methyl-*N*-(4-(3-(piperidin-1-yl)propoxy)benzyl)-propan-1-amine] has been studied, which shows potent and selective *in-vitro* binding affinities at *h*H3R (*h*H3R K_i = 1.21 nM), and balanced affinities at *h*D2/*h*D3R (*h*D2R K_i = 41 nM, *h*D3R K_i = 50 nM), (Fig. 1) [27]. Moreover, ST-713 displayed high safety profile as it exhibited *in-vitro* at high concentration (1 μM) moderate cytotoxicity (22.2 ± 26.5% of cell death) and in a concentration of 10 μM cytotoxicity (98.5 ± 1.0% of cell death) on SH-SY5Y neuroblastoma cell [40]. In addition, ST-713 demonstrated in oxygen radical absorbance capacity study no notable antioxidative property [41]. In the present series of behavioral and biochemical experiments, the *in-vivo* effects of subchronic systemic administration of ST-713 on deficits in social features and the presence of repetitive and restricted behaviors in BTBR mice as an idiopathic mouse model of ASD were assessed. Also, the effects of ST-713 on locomotor activity and anxiety-like behaviors on treated animals were recorded, since anxiety and motor activity could confound the performance of ST-713-treated animals [4,42]. Additionally, the abrogative effects of CNS-penetrant H1R antagonist pyrilamine (PYR), H2R antagonist zolantidine (ZOL), H3R agonist (*R*)-α methyl histamine (RAM), and cholinergic muscarinic antagonist scopolamine (SCO) on the ST-713 provided effects were assessed in a battery of standard behavioral tests to further explain whether brain histaminergic as well as dopaminergic neurotransmission are involved in the effects delivered by the multiple-active novel compound ST-713.



ST-713

K_i (*h*H3R)[a] = 1.21 ±0.04 nM
K_i (*h*H1R)[b] = 205 ±4 nM
K_i (*h*H4R)[c] = 210 ±6 nM
K_i (*h*D2R)[d] = 41 ±17 nM
K_i (*h*D3R)[e] = 50 ±19 nM
K_i (*h*D1R)[e] = 232 ±74 nM
K_i (*h*D5R)[e] = 153 ±36 nM

Fig. 1. *In-vitro* pharmacological binding profile of ST-713 at selected human histamine and dopamine receptor subtypes. [a][^{3}H]N^α-Methylhistamine binding assay performed with cell membrane preparation of HEK cells stably expressing the human H3R (n = 4). [b][^{3}H]Pyrilamine binding assay performed with cell membrane preparation of CHO cells stably expressing the human H1R (n = 2). [c][^{3}H]Histamine binding assay performed with cell membrane preparation of Sf9 cells transiently expressing the human histamine H4R and co-expressed with $G_{\alpha i2}$ and $\beta_{1\gamma 2}$ subunits (n = 2). [d,e]Displacement assay was carried out as described previously using membrane suspension of cell lines stably expressing the human dopamine *h*D1Rs and *h*D5Rs (HEK) against [^{3}H]SCH23390 and *h*D2SRs, *h*D3Rs (CHO) using [^{3}H]spiperone (n = 3) [27].

2. Material and methods

2.1. Animals

BTBR T+Itpr3tf/J (BTBR) mice (aged 10–12 weeks, weighing 30–35 g) were procured from Jackson Laboratory (Boulevard, Bethesda, MD 20892-4874, USA), and were bred in the local central animal facility of the College of Medicine and Health Sciences, United Arab Emirates University. The inbred C57BL/6J (C57) mice (aged 10–12 weeks, weighing 20–25 g) were also obtained from animal facility, College of Medicine and Health Sciences, United Arab Emirates University. BTBR mice were used in the current study as numerous previous reports have shown that BTBR mice exhibit peripheral and CNS abnormalities, e.g. abnormal inflammatory profile, which associated with ASD features and are similar to the patients diagnosed with ASD [2,6,43–51]. Accordingly, behavioral patterns are dysregulated in BTBR mice which are similar to human ASD subjects such as deficits in social communication and repetitive/compulsive behaviors, and repetitive self-grooming behavioral pattern. This contrasts with the background strain, i.e. C57BL/6J mice (C57), that exhibit high sociability and normal grooming behavioral pattern [52,53]. The animals were maintained in a separate air-conditioned room with controlled temperature and humidity (24 ± 2 °C and 55% ± 15%, respectively), 12 h light/dark cycle, and ad libitum to food and water. All the experiments were performed between 9.00 am and 3.00 pm. The procedures were approved by the Institutional Animal Ethics Committee of College of Medicine and Health Sciences/United Arab Emirates University (Approval No. ERA-2019-6013). To reduce the suffering of animals, minimum number of animals were used in this study, whereas the objectives were not compromised.

2.2. Drugs

Synthesis and *in-vitro* profiling of ST-713 was carried out according to previously described methodologies in the Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Germany [27]. The reference drugs donepezil (DOZ, 1 mg/kg, i.p.) hydrochloride, Chlorpromazine (CPZ, 1.5 mg/kg, i.p.), the brain-penetrant H1R antagonist pyrilamine (PYR, 10 mg/kg, i.p.), the brain-penetrant H2R antagonist zolantidine (ZOL, 10 mg/kg, i.p.), the muscarinic anticholinergic scopolamine (SCO, 0.3 mg/kg, i.p.), the CNS-penetrant H3R agonist (*R*)-α-methylhistamine (RAM, 10 mg/kg, i.p.), Bovine serum albumin (BSA), primary antibodies (mouse anti-Cyclooxygenase-2 (COX-2)), mouse anti-Nuclear factor κ-B (NF-κBp65), mouse anti-inducible nitric oxide synthase (iNOS), mouse anti-actin, and secondary antibody (anti-mouse IgG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene fluoride (PVDF) membrane was procured from Bio-Rad Laboratories (CA, USA). Chemiluminescence pico kit and Pierce™ BCA protein assay kit was obtained from Thermo Fisher Scientific (Rockford, IL, USA). All the reagents used were of analytical grade. All drugs were dissolved in saline and all doses were expressed in terms of the free base.

2.3. Experimental design and treatments

All the mice were acclimatized for 1 week before start of the experiment, and subsequently randomly divided in to thirteen groups of seven mice each. Total duration of the experiment was 21 days, the daily intraperitoneal (i.p.) chronic treatment started 1 week before the

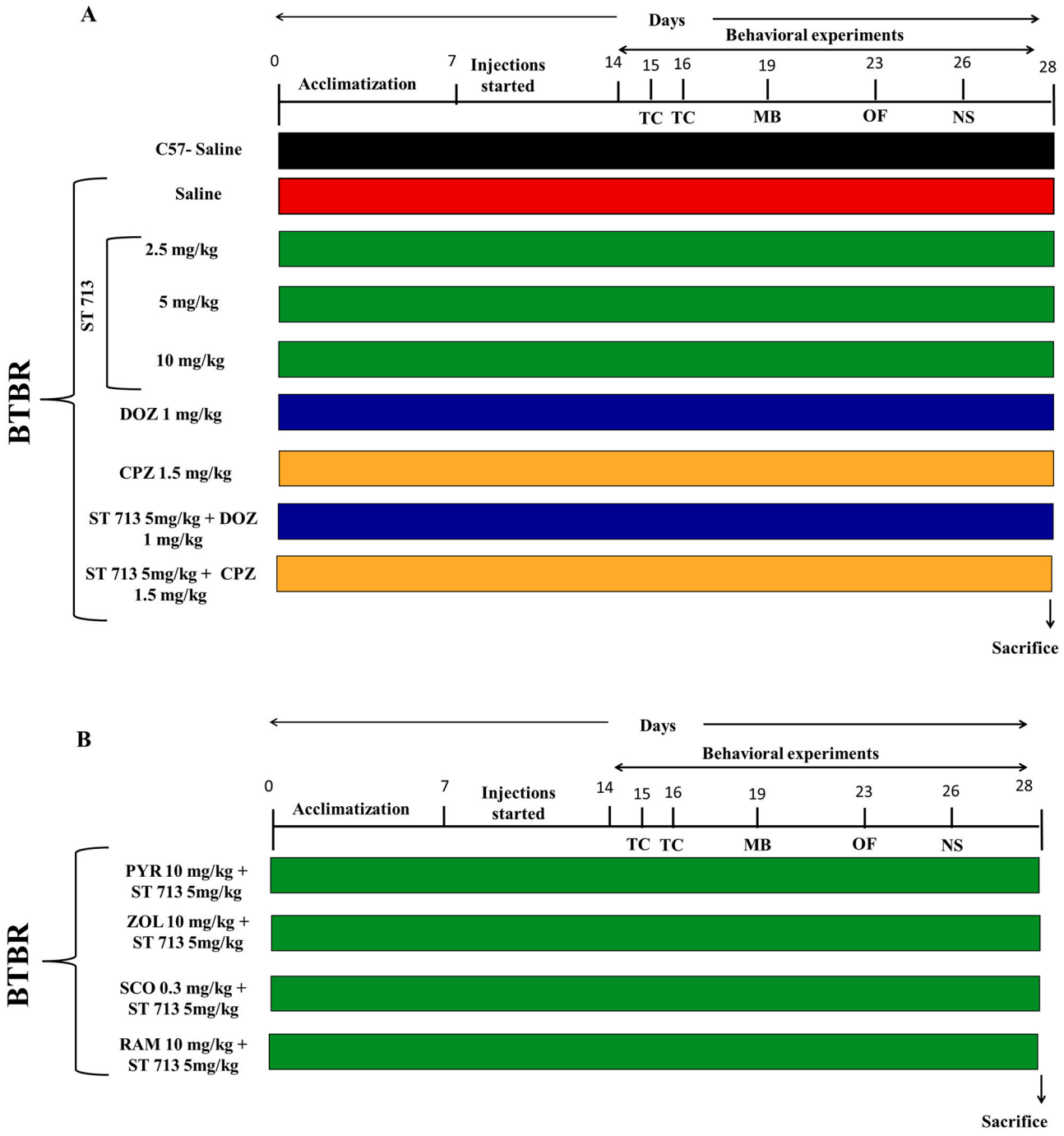


Fig. 2. Schematic experimental design.

behavioral experiments, and continued until the sacrifice. The doses as well as saline were injected i.p. 30–45 min before commencement of the behavioral experiments each day. The compounds were dissolved in physiological saline before administration and the volume was normalized according to body weight (10 ml/kg). Group 1, C57 mice injected with saline served as control. Group 2, BTBR mice treated with saline served as autistic control. Groups 3–5, BTBR mice received i.p. injections of different doses of ST-713 (2.5, 5, and 10 mg/kg), respectively. Group 6 and 8, BTBR mice were injected with DOZ (1 mg/kg, i.p.) and the latter were co-injected with ST-713 (5 mg, i.p.). Groups 7 and 9, BTBR mice received i.p. injections of CPZ (1.5 mg/kg), in addition, Group 9 were co-injected with ST-713 (5 mg/kg i.p.). For abrogation studies, Groups 10–13, BTBR mice were treated with ST-713 (5 mg/kg, i.p.) along with PYR (10 mg/kg, i.p., Group 10), ZOL (10 mg/kg, i.p., Group 11), SCO (0.3 mg/kg, i.p., group 12), and RAM (10 mg/kg, i.p., Group 13), respectively (Fig. 2). Also, C57 mice were administered with ST-713 (2.5, 5, 10 mg/kg), DOZ (1 mg/kg), CPZ (1.5 mg/kg), ST-713 (5 mg)+DOZ(1 mg), and ST-713(5 mg)+CPZ(1.5 mg) to assess the effects of used treatments on the behavioral parameters of normo-social comparator C57 mice (Table 3). Following behavioral tests, i.e. on day 21 of systemic treatment, all animals were sacrificed. The skull was removed carefully, brains were removed, and both hemispheres were separated on ice plate. The hippocampus and cerebellum were isolated from brain and were frozen immediately with liquid nitrogen for further biochemical assays.

2.4. Behavioral tests

2.4.1. Three chamber (TC) test

TC test was performed according to previously described protocols [4,5,10,47,54]. Briefly, the cage consisted of three chambers, each chamber has (40 × 20 × 22 cm) equal volume of space. The center chamber had two square shaped openings with doors, which provided access to left and right chambers. Two plastic round wired cages were used to separate the stranger mice from experimental mouse. Two stranger mice (10-12 weeks old) were habituated in that plastic wire cage for 30 min, 1 day prior to the commencement of experiments. In the TC test, the total duration of one trial was 30 min, consisting of two five min sessions and two ten min sessions. During the first five min of habituation session, the test mouse was placed in the center chamber with no access to other two chambers. For the second 5 min session, it was allowed to access all three chambers by opening the doors. Following habituation and before starting the first 10 min of test session, a stranger mouse was placed in a wire cage in one side of the chamber (same gender and age as test mouse but with no previous contact) referred to as novel mouse (NM), meanwhile in the opposite chamber an empty wire cage was placed, representing novel object (NO). The position of the stranger mice was altered regularly to avoid side preferences. In this session, test mouse was allowed to access all three chambers for 10 min. This was followed by the second 10 min test session immediately by placing a second stranger mouse in the empty wire cage. In this context, the first stranger mouse was referred to as familiar mice (FM) and second stranger mouse was referred to as novel mouse (NM). Similar pattern of time duration was counted as in first 10 min test session. The whole experiment was recorded and the duration for each session was calculated accordingly using EthoVision® Software (Noldus, Netherlands). To allow the direct comparison of social behavior of the treated groups, the sociability index (SI) and social novelty preference index (SNI) were evaluated. The SI was calculated as [Time exploring NM – Time exploring NO] / [Time exploring NM + Time exploring NO]; while SNI was calculated as [Time exploring NM – Time exploring FM] / [Time exploring NM + Time exploring FM]. The chambers were thoroughly cleaned with a tissue dampened with 70% (volume/volume; vol/vol) alcohol to remove the odor after each mouse completed sociability and social novelty tests.

Table 1
ST-713 treatment attenuates marble burying in BTBR mice.

Treatment group	Marbles buried (mean ± SEM)
SAL (C57)	3.50 ± 0.51
SAL (BTBR)	9.00 ± 1.20**
ST-713 (2.5 mg/kg)/BTBR	8.83 ± 1.14
ST-713 (5 mg/kg)/BTBR	4.83 ± 0.93#
ST-713 (10 mg/kg)/BTBR	5.00 ± 0.70#
DOZ (1 mg/kg)/BTBR	3.83 ± 0.68##
CPZ (1.5 mg/kg)/BTBR	8.33 ± 0.99
ST-713 (5 mg) + DOZ	8.16 ± 0.76
ST-713 (5 mg) + CPZ	7.66 ± 0.65
ST-713 (5 mg) + PYR	4.33 ± 0.38
ST-713 (5 mg) + ZOL	5.17 ± 0.44
ST-713 (5 mg) + SCO	9.17 ± 1.09$
ST-713 (5 mg) + RAM	8.83 ± 1.50$

Mean (± SEM) marbles buried (n = 7). MB test was carried out in BTBR and C57 mice. Each mouse received an i.p. injection ST-713 (2.5, 5, or 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), or CPZ (1.5 mg/kg, i.p.) chronically for 21 days. BTBR mice buried significantly more marbles compared to that of C57 mice. ST-713 (5 and 10 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) attenuated the increased number of buried marbles. Effects of chronic (21 days) systemic co-injection of PYR (10 mg/kg, i.p.), ZOL (10 mg/kg, i.p.), SCO (0.3 mg/kg, i.p.), and RAM (10 mg/kg, i.p.) on the ST-713-(5 mg)-provided attenuation of increased number of marbles buried were evaluated in MBT.

** $P < 0.001$ vs. saline-treated C57 mice.
# $P < 0.05$ vs. saline-treated BTBR mice.
$P < 0.01$ vs. saline-treated BTBR mice.
$ $P < 0.05$ vs. ST-713-(5 mg)-treated BTBR mice.

Table 2
Effects of ST-713, DOZ, and CPZ systemic pretreatment on anxiety levels and locomotor activity in BTBR mice in OF test.

Treatment group	Total distance travelled (cm)	Time spent in center (s)	Time spent in periphery (s)
SAL (C57)	2433.15 ± 264.23	40.40 ± 4.83	571.05 ± 7.33
SAL (BTBR)	4221.00 ± 221.96**	73.75 ± 7.15**	537.25 ± 3.81*
ST-713 (2.5 mg/kg)/BTBR	3698.26 ± 452.80	54.94 ± 1.42#	536.15 ± 7.52
ST-713 (5 mg/kg)/BTBR	3838.63 ± 440.11	53.64 ± 2.90#	535.84 ± 3.13
ST-713 (10 mg/kg)/BTBR	3863.22 ± 521.57	57.20 ± 3.33#	534.69 ± 2.43
DOZ (1 mg/kg)/BTBR	3632.34 ± 298.16	41.11 ± 3.94##	536.02 ± 1.70
CPZ (1.5 mg/kg)/BTBR	3888.22 ± 240.95	71.50 ± 5.40	539.50 ± 2.28

BTBR mice demonstrated elevated impulsive attitude and deficits in cognition as well as locomotor activity behaviors that were significantly increased compared to C57 mice. ST-713 (2.5, 5, or 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), or CPZ (1.5 mg/kg, i.p.) were administered chronically for 21 days. ST-713 (2.5, 5 and 10 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) attenuated the increased time spent in the central arena, but failed to alter the increased total distance travelled as well as the time spent in the periphery in BTBR mice in the OF test. Data are expressed as the means ± SEM (n = 4).

* $P < 0.05$ vs. C57 mice.
** $P < 0.01$ vs. C57 mice.
# $P < 0.05$ vs. saline-treated BTBR mice.
$P < 0.01$ vs. saline-treated BTBR mice.

2.4.2. Marble burying (MB) test

To analyze the repetitive behaviors of the animals, MB test was performed, following previously described experimental protocols [4,5,10,54–56]. In brief, clean and autoclaved rat cages (26 cm × 48 cm × 20 cm) were used for this experiment. All the cages were filled 5 cm depth with wooden waste as bedding material and leveled by using another flat surfaced cage. Each mouse was kept in a separate cage for 10 min for habituation, then they were removed, 20

Table 3
Effects of ST-713, DOZ, and CPZ systemic pretreatment on behavioral parameters of control C57 mice.

Behavioral test		SAL	ST-713 (mg/kg, i.p.)			DOZ (1 mg/kg, i.p.)	CPZ (1.5 mg/kg, i.p.)	ST-713 (5 mg/kg)	
			2.5	5	10			DOZ	CPZ
Sociability	SI	40 ± 6.28	39.00 ± 5.90	38.00 ± 5.34	40 ± 6.28	42 ± 5.69	37 ± 5.26	44 ± 6.41	36 ± 3.85
Social novelty	SNI	33.86 ± 6.58	31.00 ± 5.42	32.43 ± 4.24	33.86 ± 6.58	34.57 ± 6.22	31.86 ± 5.84	35.00 ± 5.97	31.86 ± 5.84
Marble burying	Marble buried (%)	17.50 ± 2.57	16.67 ± 2.26	16.00 ± 2.47	16.17 ± 2.49	15.83 ± 2.27	18.17 ± 2.34	15.67 ± 2.39	17.67 ± 2.65
Nestlet shredding	Nestlet shredded (%)	1.31 ± 0.12	1.29 ± 0.12	1.25 ± 0.14	1.27 ± 0.13	1.25 ± 0.11	1.35 ± 0.12	1.25 ± 0.11	1.35 ± 0.10
Open field	Time in center (s)	30.30 ± 9.70	31.52 ± 7.67	29.50 ± 5.30	28.25 ± 9.41	33.06 ± 6.22	26.75 ± 7.14	–	–
	Time in periphery (s)	571.05 ± 7.33	567.00 ± 10.03	564.07 ± 14.98	574.25 ± 8.92	574.00 ± 10.16	577.75 ± 11.46	–	–
	Total distance (cm)	2433.15 ± 264.23	2488.13 ± 234.97	2222.41 ± 238.61	2369.94 ± 294.73	2220.86 ± 318.27	2481.26 ± 271.26	–	–

Data are expressed as the means ± SEM (n = 7). There was no significant difference between saline, ST713 (2.5, 5 and 10 mg), DOZ and CPZ treated C57 control mice.

black painted glass marbles were kept evenly spaced over the bedding. The mice were then put back into the center of the respective cages and allowed to move freely. After 30 min the mice were removed again, and buried marbles were counted manually. Marbles covered by > 50% with bedding were considered as buried marbles (Table 1). Marbles that were no longer visible were considered completely buried. The marbles used in the tests were thoroughly spared with 70% (volume/volume; vol/vol) alcohol and let overnight to remove the odor of tested mice and to make sure that the marbles are alcohol-oder free as well as after each mouse was tested.

2.4.3. Nestlet shredding (NS) test

To examine compulsive/aggressive behaviors of the animals, NS test was performed according to previous preclinical studies in rodents [3–5,10,55]. A clean autoclaved mice cage (19 cm × 29 cm × 13 cm) was added with wooden waste to make 0.5 cm depth bedding. Each mouse was kept in a separate cage for 10 min for habituation, then one piece of commercially available, pre-weighed cotton nestlet [(5 cm × 5 cm), approximately 2.5 g] was placed in each cage. All the mice were allowed to explore freely for 30 min before returned to their home cages. The remaining unshredded nestlet was carefully collected with forceps and allowed to dry overnight. Dried nestlets were weighed individually and the percentage of cotton shredded by each mouse was calculated accordingly. New cages were used for each test mouse.

2.4.4. Open field (OF) test

To analyze the effect of ST-713 on locomotion and anxiety behaviors in animals, OF test was carried out. In addition to locomotor activity, the OF test is usually used to measure anxiety-like behaviors in experimental rodents [4,5,17,42,54,57–60]. The test provides a unique opportunity to systematically assess novel environment exploration, general locomotor activity, and provide an initial screen for anxiety-related behavior in experimental rodents [60]. The OF box consisted of a square box (45 × 45 × 30 cm). A 23 cm × 23 cm area in the center region was defined as central arena, the remaining was defined as periphery area. The mice with higher anxiety degree prefer to stay closer to the walls of the box and spend less time in the center. Whereas increased time spent in the central area indicates low anxiety level and high exploratory behaviors [61]. First 5 min of the experiment were considered as habituation, followed by 10 min test session. Total distance moved in the whole arena, time spent in the center and in the periphery were recorded for 10 min using a charge-coupled device camera-assisted motion tracking apparatus and software (EthoVision 3.1, Noldus Information Technology, the Netherlands) (Table 2). After each mouse has completed the test, the OF chamber was cleaned thoroughly with 70% (volume/volume; vol/vol) alcohol.

2.5. Brain collection and tissue processing for Western blot analysis of proinflammatory markers

At the end of the behavioral assessments, the animals were sacrificed following previously published protocols [4,5,10]. Briefly, animals were deeply anesthesia with pentobarbital (40 mg/kg, body weight, i.p.) and perfused via intracardial infusion to wash out the blood, using 1× PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) at pH 7.4. The blood removal was confirmed by the observation of whitish color liver, heart, and kidney, indicating that they are blood free. The brains were then quickly removed and placed on an ice plate. The cerebellum and hippocampus were excised from the brain and snap-frozen in liquid nitrogen for further use in biochemical assessments [67,84]. On the day of biochemical tests, all tissues were homogenized in an ice-cold RIPA buffer (containing protease inhibitors and phosphatase inhibitors) and the homogenates were centrifuged at 12,000 rpm/min for 30 min at 4 °C. The protein concentrations of each sample were measured by the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). The protein samples were

separated by 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes, which were activated with 100% methanol in advance. The membranes were then incubated with 5% bovine serum albumin (BSA) for 1 h. After blotting, the membranes were then incubated overnight with the specific primary antibodies, against cyclooxygenase-2 (COX-2) (1:1000), NF-κB p65 (1:1000), inducible nitric oxide synthase (iNOS) (1:1000), and actin (1:1000), at 4 °C with gentle rocking. Prior to the 1-h incubation with horseradish peroxidase-labeled secondary antibody, the membranes were washed three times with tris-buffered saline (TBS). Detection of the protein bands was carried out using an enhanced chemiluminescence pico kit following the manufacturer's instructions. The band intensity for protein levels were quantified using ImageJ software (NIH, USA).

2.6. Statistical analysis

For behavioral assessments, data were expressed as means ± SEM. The data were analyzed for normality by assessing the sample distribution or skewness (− 1.5 to + 1.5 was considered as normally distributed). After the results had passed the tests for normality, the effects of drug treatments were analyzed by two-way analysis of variance (ANOVA) and post hoc comparisons were performed with Tukey's test in case of a significant main effect. Western blot data were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison test. For statistical comparisons, the software package SPSS 25.0 (IBM Middle East, Dubai, UAE) was used. The p values less than 0.05 were considered as statistically significant.

3. Results

3.1. Effects of ST-713 on social deficits in BTBR mice

The effects of chronic systemic administration of ST-713 (2.5, 5, and 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), and CPZ (1.5 mg/kg, i.p.) on autistic-like sociability deficits in TC paradigm are shown in Fig. 3A. The results of two-way ANOVA showed that there was a significant main effect for strain [$F_{(1,96)} = 123.78$, $P < 0.01$], treatment [$F_{(7,96)} = 7.78$, $P < 0.01$] and also for the strain × treatment interaction [$F_{(7,96)} = 6.21$, $P < 0.01$] (Table 4). As observed in the Tukey post hoc analyses, BTBR mice displayed significantly lower percentage of SI when compared to C57 mice ($F_{(1,12)} = 38.31$, $P < 0.001$) (Fig. 3A). ST-713 (5 and 10 mg/kg) and DOZ (1 mg/kg, i.p.) significantly improved sociability of BTBR mice when compared with saline-treated BTBR mice, with ($F_{(1,12)} = 26.71$, $P < 0.001$), ($F_{(1,12)} = 40.85$, $P < 0.001$), and ($F_{(1,12)} = 10.61$, $P < 0.01$), respectively (Fig. 3A). However, the similar improvement could not be observed when BTBR mice were treated with ST-713 (2.5 mg/kg, i.p.) ($F_{(1,12)} = 0.36$, $P = 0.56$). In addition, the reference drug CPZ (1.5 mg/kg, i.p.) deteriorated the sociability deficits in BTBR mice when compared with saline-treated ones ($F_{(1,12)} = 6.91$, $P < 0.05$). Interestingly, when co-injected with CPZ (1.5 mg/kg, i.p.), ST-713 (5 mg/kg, i.p.) ameliorated the negative effects observed by CPZ when administered alone ($F_{(1,12)} = 9.87$, $P < 0.01$), but failed to further improve the enhancement obtained with DOZ ($F_{(1,12)} = 0.04$, $P = 0.84$) (Fig. 3A). Moreover, ST-713 (5 mg)-provided effects on sociability were completely reversed by co-administration with the H3R agonist RAM and the anticholinergic SCO, with ($F_{(1,12)} = 6.40$, $P < 0.05$) and ($F_{(1,12)} = 7.12$, $P < 0.05$), respectively (Fig. 3B). Notably, H1R antagonist PYR or the H2R antagonist ZOL failed to reverse the ST-713 caused effects with ($F_{(1,12)} = 0.39$, $P = 0.54$) and ($F_{(1,12)} = 0.23$, $P = 0.64$), respectively (Fig. 3B). Moreover, systemic pretreatments with ST-713 (2.5, 5, and 10 mg/kg), DOZ (1 mg/kg), CPZ (1.5 mg/kg), ST-713 (5 mg)+DOZ, and ST-713(5 mg)+CPZ did not alter SI observed in control C57 mice in NS test (Table 3).

3.2. Effects of ST-713 on social novelty preference deficits in BTBR mice

Similarly, the effects of chronic systemic administration of ST-713 (2.5, 5, and 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), and CPZ (1.5 mg/kg, i.p.) on autistic-like social novelty preference were assessed (Fig. 4A). The results of two-way ANOVA showed that there was a significant main effect for strain [$F_{(1,96)} = 56.23$, $P < 0.01$], treatment [$F_{(7,96)} = 9.28$, $P < 0.01$] and also for the strain × treatment interaction [$F_{(7,96)} = 8.25$, $P < 0.01$] (Table 4). As observed in the Tukey post hoc analyses, BTBR mice displayed significantly lower percentage of SNI

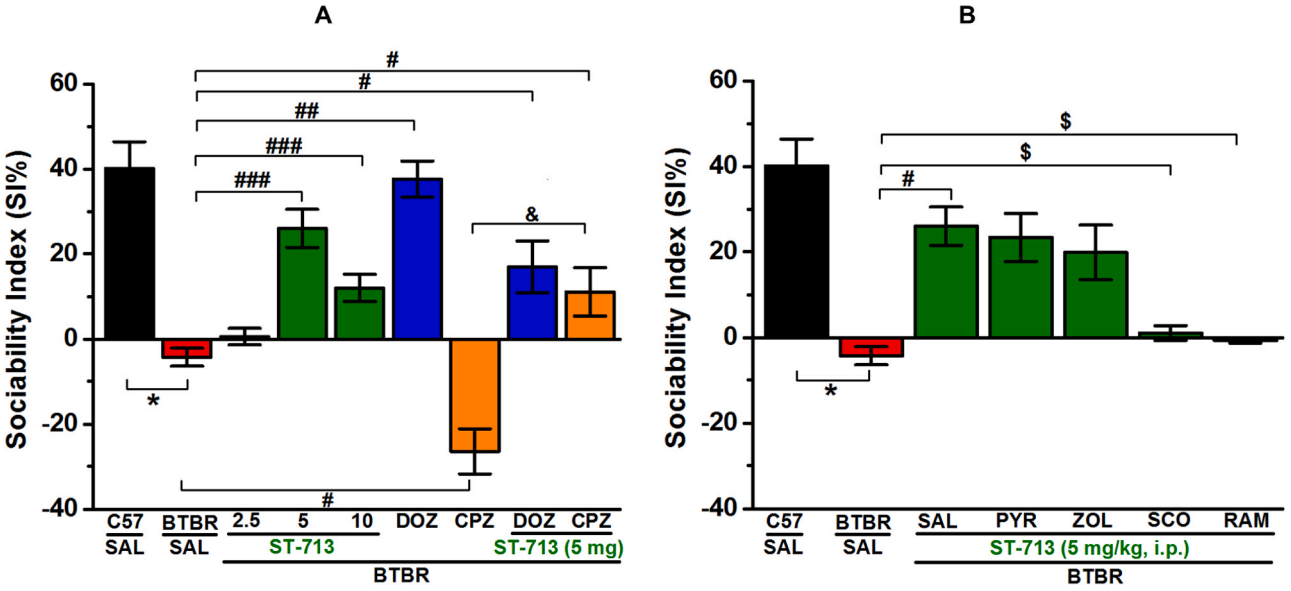


Fig. 3. ST-713 ameliorated sociability deficits of BTBR mice in TC test. After 10 min of habituation, male subjects were allowed to explore all chambers for two 10 min sessions. C57 and BTBR mice were injected with saline, and BTBR mice were administered with ST-713 (2.5, 5 or 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), or CPZ (1.5 mg/kg, i.p.) chronically for 21 days. The results obtained were Sociability Index (SI) (A), and the effects of chronic (21 days) systemic co-injection of PYR (10 mg/kg, i.p.), ZOL (10 mg/kg, i.p.), SCO (0.3 mg/kg, i.p.), and RAM (10 mg/kg, i.p.) on the ST-713-(5 mg)-provided improvement of sociability (B). Figures show mean ± SEM (n = 7). $^{*}P < 0.05$ vs. SI of saline-treated C57 mice. $^{\#}P < 0.05$ vs. SI of saline-treated BTBR mice. $^{\#\#}P < 0.01$ vs. SI of saline-treated BTBR mice. $^{\#\#\#}P < 0.001$ vs. SI of saline-treated BTBR mice. $^{\$}P < 0.05$ vs. ST-713-(5 mg)-treated BTBR mice. $^{\&}P < 0.01$ vs. SI of CPZ-treated BTBR mice.

Table 4
Results observed following two-way ANOVA analyses of ST-713, DOZ, and CPZ systemic pretreatment on behavioral parameters.

Behavioral test		Effect of strain	Effect of treatment	Effect of strain × treatment interaction
Sociability (SI)		$F_{(1,96)} = 123.78$	$F_{(7,96)} = 7.78$	$F_{(7,96)} = 6.21$
Social novelty preference (SNI)		$F_{(1,96)} = 56.23$	$F_{(7,96)} = 9.28$	$F_{(7,96)} = 8.25$
MB test		$F_{(1,80)} = 54.92$	$F_{(7,80)} = 3.98$	$F_{(7,80)} = 2.97$
NS test		$F_{(1,80)} = 23.25$	$F_{(7,80)} = 4.65$	$F_{(7,80)} = 4.54$
OF test	Total distance travelled (cm)	$F_{(1,36)} = 45.58$	$F_{(5,36)} = 0.26^{a}$	$F_{(5,36)} = 0.13^{b}$
	Time spent in the periphery (s)	$F_{(1,36)} = 41.46$	$F_{(5,36)} = 0.21^{c}$	$F_{(5,36)} = 0.12^{d}$
	Time spent in center of arena (s)	$F_{(1,36)} = 37.26$	$F_{(5,36)} = 1.15^{e}$	$F_{(5,36)} = 1.15^{f}$

All P values < 0.01, except $^{a} = 0.93$, $^{b} = 0.98$, $^{c} = 0.96$, $^{d} = 0.99$, and $^{e,f} = 0.35$.

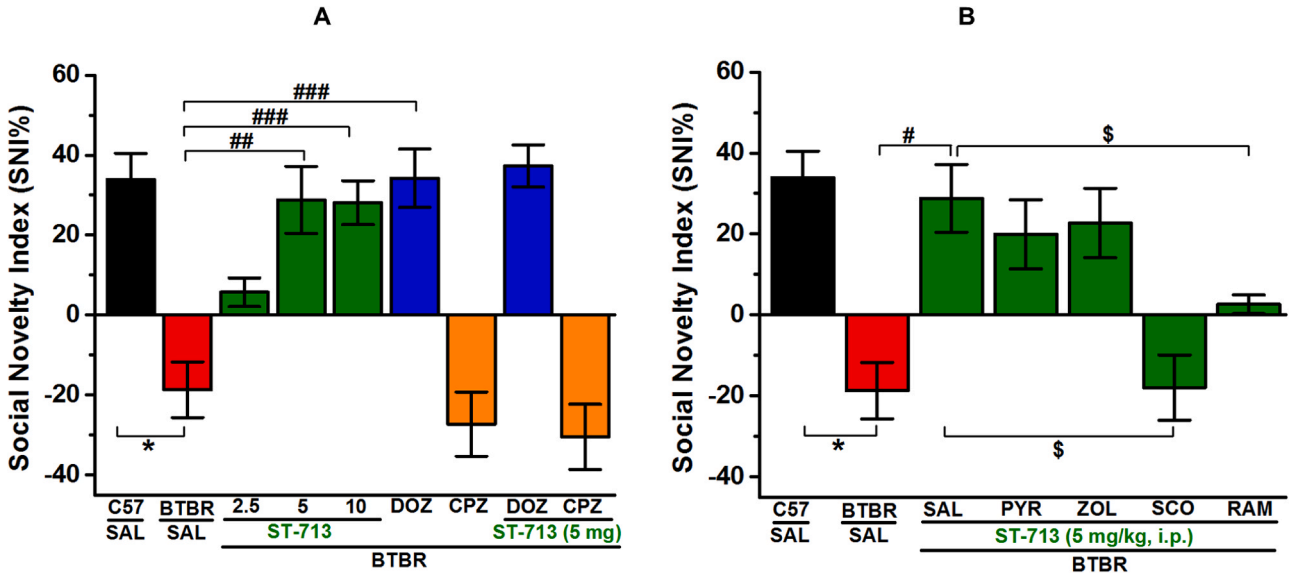


Fig. 4. ST-713 alleviated social novelty deficits of BTBR mice in TC test. After 10 min of habituation, male subjects were allowed to explore all chambers for two 10 min sessions. C57 and BTBR mice were injected with saline, and BTBR mice were administered with ST-713 (2.5, 5 or 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), or CPZ (1.5 mg/kg, i.p.) chronically for 21 days. The results obtained were Social Novelty Index (SNI) (A), and the effects of chronic (21 days) systemic co-injection of PYR (10 mg/kg, i.p.), ZOL (10 mg/kg, i.p.), SCO (0.3 mg/kg, i.p.), and RAM (10 mg/kg, i.p.) on the ST-713-(5 mg)-provided improvement of SNI (B). Figures show mean ± SEM (n = 7). $^{*}P < 0.05$ vs. SI of saline-treated C57 mice. $^{\#}P < 0.05$ vs. SI of saline-treated BTBR mice. $^{\#\#}P < 0.01$ vs. SI of saline-treated BTBR mice. $^{\#\#\#}P < 0.001$ vs. SI of saline-treated BTBR mice. $^{\$}P < 0.05$ vs. ST-713(5 mg)-treated BTBR mice.

when compared to C57 mice ($F_{(1,12)} = 25.74$, $P < 0.001$) (Fig. 4A). As expected, ST-713 (5 and 10 mg/kg) and DOZ (1 mg/kg, i.p.) significantly improved percentage of SNI in BTBR mice when compared with saline-treated BTBR mice, with ($F_{(1,12)} = 16.26$, $P < 0.01$), ($F_{(1,12)} = 23.87$, $P < 0.001$), and ($F_{(1,12)} = 23.51$, $P < 0.001$), respectively (Fig. 4A). Unlike the results observed for SI, the reference drug CPZ (1.5 mg/kg, i.p.) failed to further worsen the social novelty behaviors (SNI) in BTBR mice when compared with saline-treated BTBR mice, with ($F_{(1,12)} = 0.56$, $P = 0.47$). Interestingly, ST-713 (5 mg/kg, i.p.) when co-injected with CPZ (1.5 mg/kg, i.p.), failed to ameliorate the effects observed with CPZ when administered alone ($F_{(1,12)} = 0.06$, $P = 0.80$), or to further increase the enhancing effects obtained with DOZ when administered alone ($F_{(1,12)} = 0.09$, $P = 0.76$) (Fig. 4A). Moreover, ST-713 (5 mg)-provided effects on percentage of SNI were completely abrogated by co-administration with the H3R agonist RAM and the anticholinergic SCO, with ($F_{(1,12)} = 7.08$, $P < 0.05$) and ($F_{(1,12)} = 9.67$, $P < 0.01$), respectively (Fig. 4B). Also similar to the results observed for SI, H1R antagonist PYR or the H2R antagonist ZOL failed to nullify the ST-713-provided effects on percentage of SNI with ($F_{(1,12)} = 0.39$, $P = 0.54$) and ($F_{(1,12)} = 0.17$, $P = 0.68$), respectively (Fig. 4B). Notably, systemic pretreatments with ST-713 (2.5, 5, and 10 mg/kg), DOZ (1 mg/kg), CPZ (1.5 mg/kg), ST-713(5 mg)+DOZ, and ST-713(5 mg)+CPZ did not alter SNI assessed in control C57 mice in NS test (Table 3).

3.3. Effects of ST-713 on stereotyped repetitive and compulsive behavior of BTBR mice in MB test

The ameliorating effect of chronic ST-713 treatment on repetitive behavior in BTBR mice was assessed by MB test (Fig. 5A,B and Table 1) and NS test (Fig. 5C,D). The results of two-way ANOVA showed that there was a significant main effect for strain [$F_{(1,80)} = 54.92$, $P < 0.01$], treatment [$F_{(7,80)} = 3.98$, $P < 0.01$] and also for the strain × treatment interaction [$F_{(7,80)} = 2.97$, $P < 0.01$] (Table 4). The percentage of buried marbles were expectedly as expected increased in saline treated BTBR mice when comparing with saline-treated C57 mice ($F_{(1,12)} = 14.76$, $P < 0.01$) (Fig. 5A, Table 1). Chronic systemic administration of ST-713 (5 and 10 mg/kg, i.p.) and DOZ (1 mg/kg) significantly counteracted the increased percentage of buried marbles in BTBR mice, with ($F_{(1,12)} = 6.29$, $P < 0.05$), ($F_{(1,12)} = 6.86$, $P < 0.05$), and ($F_{(1,12)} = 11.63$, $P < 0.01$), respectively. However, the BTBR mice administered with lower dose of ST-713 (2.5 mg/kg) or CPZ did not display any amelioration in the MB test, with ($F_{(1,12)} = 0.01$, $P = 0.93$) and ($F_{(1,12)} = 0.03$, $P = 0.73$), respectively (Fig. 5A, Table 1). Analyses of data characterizing the number of marbles buried yielded essentially the same results (Table 1). Moreover, ST-713 (5 mg/kg) associated reduction on percentage of marbles buried were completely reversed by co-administration with the H3R agonist RAM and the anticholinergic SCO, with ($F_{(1,12)} = 13.20$, $P < 0.05$) and ($F_{(1,12)} = 6.18$, $P < 0.05$), respectively (Fig. 5B, Table 1). Similar to the results observed for SI, H1R

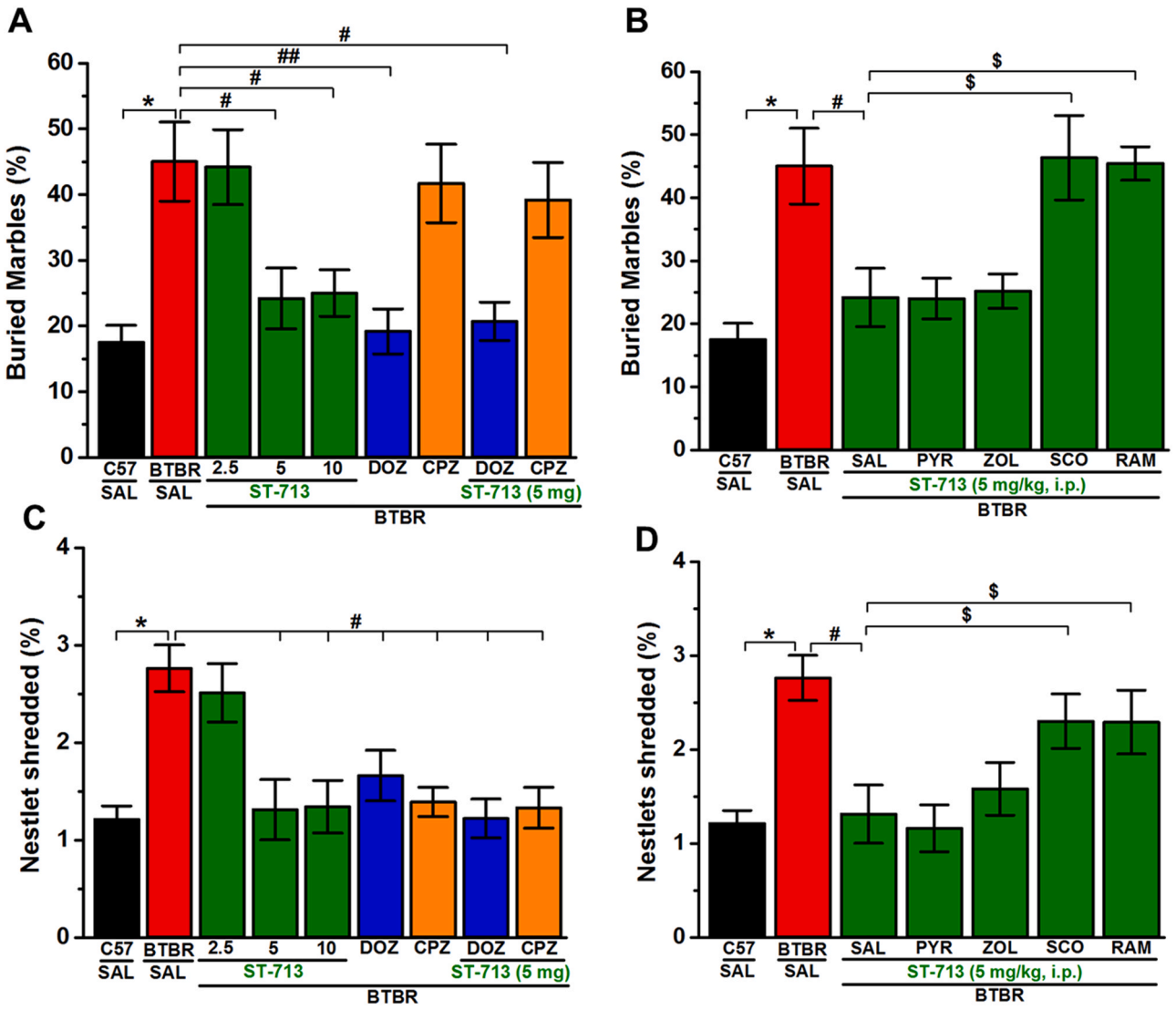


Fig. 5. ST-713 attenuated stereotyped repetitive and compulsive behavior of BTBR mice in MB and NS tests. Stereotyped repetitive paradigms were assessed in MB test (A,B) and NS test (C,D) in a 30-min testing sessions. BTBR mice demonstrated elevated stereotyped, repetitive and compulsive behaviors that were significantly increased compared to C57. ST-713 (2.5, 5, or 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), or CPZ (1.5 mg/kg, i.p.) were administered chronically for 21 days (A,C). Effects of chronic (21 days) systemic co-injection of PYR (10 mg/kg, i.p.), ZOL (10 mg/kg, i.p.), SCO (0.3 mg/kg, i.p.), and RAM (10 mg/kg, i.p.) on the ST-713-(5 mg)-provided attenuation of stereotyped repetitive and compulsive behaviors of BTBR mice were evaluated in MB test (B) and NS test (D). Figures show mean ± SEM (n = 6). $^{*}P < 0.05$ vs. Saline-treated C57 mice. $^{\#}P < 0.05$ vs. Saline-treated BTBR mice. $^{\#\#}P < 0.01$ vs. Saline-treated BTBR mice. $^{\$}P < 0.05$ vs. ST-713-(5 mg)-treated BTBR mice.

antagonist PYR or the H2R antagonist ZOL failed to nullify the ST-713-provided reduction on percentage of buried marbles, with ($F_{(1,12)} = 0.00$, $P = 0.98$) and ($F_{(1,12)} = 0.03$, $P = 0.87$), respectively (Fig. 5B, Table 1). Interestingly, systemic pretreatments with ST-713 (2.5, 5, and 10 mg/kg), DOZ (1 mg/kg), CPZ (1.5 mg/kg), ST-713 (5 mg)+DOZ, and ST-713(5 mg)+CPZ did not alter burying behaviors assessed in control C57 mice in MB test (Table 3).

3.4. Effects of ST-713 on obsessive-compulsive features of BTBR mice in NS test

The counteracting effect of chronic systemic injections of ST-713 on increased percentage of shredded nestlet in BTBR mice is shown in Fig. 5C,D. The results of two-way ANOVA showed that there was a significant main effect for strain [$F_{(1,80)} = 23.25$, $P < 0.01$], treatment [$F_{(7,80)} = 4.65$, $P < 0.01$] and also for the strain × treatment interaction [$F_{(7,80)} = 4.54$, $P < 0.01$] (Table 4). As expected, saline treated BTBR mice has shredded significantly ($F_{(1,12)} = 28.88$, $P < 0.01$) more nestlet when compared with saline-treated C57 mice. Systemic treatment with ST-713 (5 and 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), and CPZ (1.5 mg/kg, i.p.) significantly mitigated the increased compulsive/obsessive behavior of BTBR mice in NS test, with ($F_{(1,12)} = 13.20$, $P < 0.05$; $F_{(1,12)} = 13.20$, $P < 0.05$; $F_{(1,12)} = 13.20$, $P < 0.05$; and $F_{(1,12)} = 13.20$, $P < 0.05$), while low dose of ST-713 (2.5 mg/kg, i.p.) failed to modify the observed percentage of nestlet shredded ($F_{(1,12)} = 0.00$, $P = 0.92$) (Fig. 5C). In addition, systemic co-administration of PYR or ZOL was not able to abrogate the ST-713-provided mitigating effects on repetitive/compulsive behaviors in BTBR mice (all P values > 0.05) (Fig. 5D). As expected, co-injection with RAM and SCO entirely reversed the ST-713 caused benefit in the NS test (all P values < 0.05) (Fig. 5D). On the other hand, systemic pretreatments with ST-713 (2.5, 5, and 10 mg/kg), DOZ (1 mg/kg), CPZ (1.5 mg/kg), ST-713(5 mg)+DOZ, and ST-713(5 mg)+CPZ did not alter shredding behaviors measured in control C57 mice in NS test (Table 3).

3.5. Effects of ST-713 on anxiety and locomotor activity in BTBR mice in OF test

OF test was applied to assess anxiety-associated behavior and locomotion of treated mice. The two-way ANOVA results for total distance travelled and time spent in the center of arena by the C57 and BTBR mice showed a significant main effect for strain [$F_{(1,36)} = 45.58$, $P < 0.01$], [$F_{(1,36)} = 37.26$, $P < 0.01$]whereas, there was no significant difference between the treatment [$F_{(5,36)} = 0.26$, $P = 0.93$], [$F_{(5,36)} = 1.15$, $P = 0.35$] and also for the strain × treatment interaction [$F_{(5,36)} = 0.13$, $P = 0.98$], [$F_{(5,36)} = 1.15$, $P = 0.35$] respectively (Table 4). As observed in Table 2, BTBR mice showed significant increase in the distance travelled ($F_{(1,12)} = 20.13$, $P < 0.01$) and time spent in the center of arena ($F_{(1,12)} = 8.67$, $P < 0.05$) when compared with saline-treated C57 mice. However BTBR mice displayed significant decrease in time spent in the periphery ($F_{(1,12)} = 13.20$, $P < 0.05$) when compared with saline-treated C57 mice. In addition, no significant alteration was found in BTBR mice following the chronic systemic exposure to ST-713 (2.5, 5 and 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), or CPZ (1.5 mg/kg, i.p.) on total distance travelled or time spent in the periphery (all $P > 0.05$). Analysis of variance revealed that the with ST-713 (2.5, 5 and 10 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) treated BTBR mice spent significantly less time in the center of the arena, with ($F_{(1,12)} = 4.89$, $P < 0.05$), ($F_{(1,12)} = 5.89$, $P < 0.05$), ($F_{(1,12)} = 11.20$, $P < 0.05$), and ($F_{(1,12)} = 10.20$, $P < 0.05$), respectively (Table 2). However, CPZ (1.5 mg/kg, i.p.) failed to alter the time spent in the center of the arena ($F_{(1,12)} = 0.05$, $P = 0.84$). Interestingly, systemic pretreatments with ST-713 (2.5, 5, and 10 mg/kg), DOZ (1 mg/kg), CPZ (1.5 mg/kg), ST-713(5 mg)+DOZ, and ST-713(5 mg)+CPZ did not alter the behavioral parameters assessed in control C57 mice in OF test

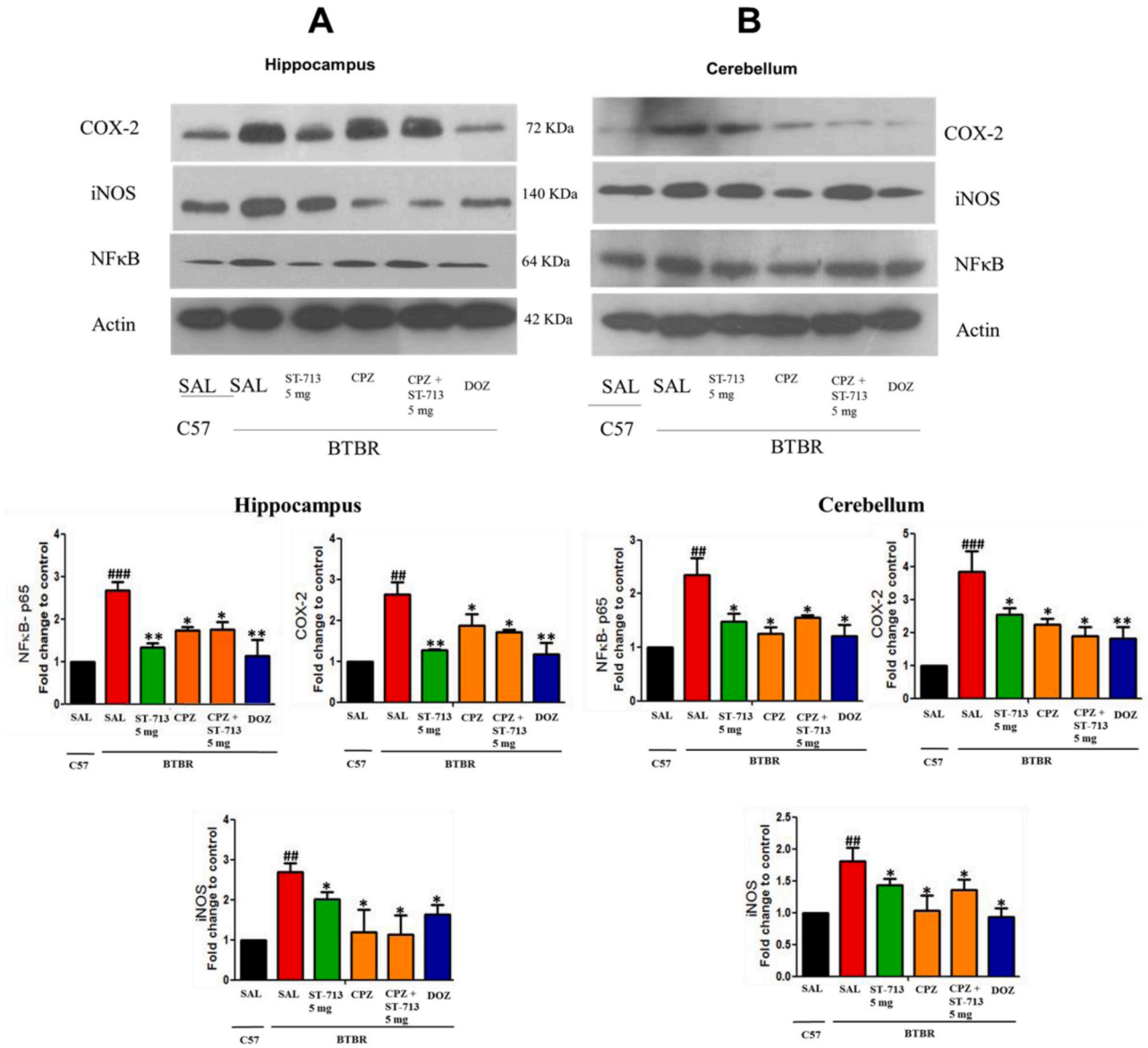


Fig. 6. ST-713 mitigated hippocampal and cerebellar proinflammatory markers. Hippocampal tissues (A) and cerebellar tissues (B) of treated groups were subjected to immunoreactions with anti-COX2, anti-iNOS, and anti-NF-kB p65. Levels of protein expression were determined using western blotting in both the hippocampus and cerebellum. The BTBR mice showed significant increase in NF-kB p65, iNOS and COX-2 compared to saline-treated C57 mice group in both brain regions (A,B). Chronic systemic pretreatment of BTBR mice with ST-713 (5 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), or CPZ (1.5 mg/kg, i.p.) remarkably decreased the expression levels of NF-kBp65 and COX-2 in both brain regions (A,B). Immunoblot were quantified using Image J and corresponding results were represented as fold change of control. The column heights are the means ± SEM. of three determinants (n = 3). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs saline-treated C57 mice; $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. saline-treated BTBR mice.

(Table 3).

3.6. Effects of ST-713 on hippocampal and cerebellar proinflammatory markers

Fig. 6 depicts the effects of ST-713 (5 mg/kg, i.p.) on the protein expression of NF-κB p65, COX-2, and iNOS in the hippocampal and cerebellar tissues of BTBR mice, and Fig. 6A represents the corresponding densitometric analysis. Statistical analysis of saline treated BTBR mice showed significant increase in the expression of the proinflammatory markers in the hippocampus and cerebellum (NF-κB p65, COX-2, and iNOS) (all $P < 0..01$), when compared with saline treated C57 mice. Systemic administration of ST-713 at a dosage of 5 mg/kg significantly decreased both hippocampal and cerebellar protein expression of NF-κB p65, COX-2, and iNOS ($P < 0.05$), as compared with saline-treated BTBR mice (Fig. 6B). In addition, significant reduction of all proinflammatory markers was observed following the treatment with CPZ or DOZ in both brain regions (all $P < 0.05$). Interestingly, systemic administration with a combination of CPZ and ST-713 (1.5 mg and 5 mg/kg, respectively) mitigated the increase of all markers assessed in both brain regions, when compared with saline-treated BTBR mice (all $P < 0.05$) (Fig. 6B).

4. Discussion

Alteration in histaminergic, dopaminergic, and cholinergic neurotransmissions are supposed to play a critical role in the phenotypic features of ASD-related behaviors [1,2,62–65]. Therefore, the aim of the current study was to assess the *in-vivo* effects of the novel multiple-active H3R and D2R/D3R ligand ST-713 on the modulating effects of brain HA, DA, and ACh transmission in BTBR mice. Male BTBR mice as an idiopathic mouse model of ASD were used in the current series of experiments. Male mice were chosen to carry out the experiments, as ASD was found to affect females less frequently than males, and several sex-differential genetic and hormonal factors may contribute [26,66]. ASD as a heterogeneous disorder with several associated symptoms make specific treatment for all patients with ASD very challenging. To include female mice with the hormonal fluctuation in addition to the very well-known heterogeneity of ASD will definitely affect the severity of the symptoms and consequently the level of observed enhancement provided by test compound ST-713. To exclude all these confounding aspects, we decided to test our novel compound on male BTBR mice. Our observations revealed that ST-713 significantly improved social deficits of BTBR mice which was displayed by the significant improvement in sociability as well as social novelty behaviors provided by a dose of 5 mg/kg. However, systemic administration with the lower dose (2.5 mg/kg) of ST-713 failed to obliterate the deficits in all behavioral assessments conducted in the current study. Notably, the optimal enhancing effects on sociability and social novelty were observed with a dose of 5 mg/kg of ST-713, as the results observed with the higher dose (10 mg/kg) were lower than those observed with 5 mg/kg of ST-713. The latter observation may be explained with the possibility of off-target effects for ST-713 at higher doses. The latter findings regarding the dose-dependency are in agreement with previous results of a memory-enhancing effect observed for dual-active compounds targeting H3R and acetylcholine esterase inhibitor (AChEI), where the effect of lower dose (1.25 mg/kg) was significantly higher when compared to the higher doses 2.5 and 5 mg/kg [12,17]. Moreover, the similar dose-effectiveness-relationship was observed in the study of a dual-active H3R antagonist and AChEI in a recent report in BTBR mice [58]. In further abrogative studies, the ST-713-provided improvement in sociability and social novelty (5 mg, being the optimal dose that relieved the assessed behavioral deficits) were completely counteracted when mice were co-administered with the brain-penetrant H_3R agonist RAM or the antimuscarinic compound SCO. The results supported our hypothesis that the benefit of ST-713 on social parameters were due to the release of several neurotransmitters, such as HA and ACh, which might be based on the inhibition of the H_3R-auto- and hetero-receptors, respectively. Importantly, co-administration of ST-713 with the brain-penetrant H1R antagonist (PYR) or H2R antagonist (ZOL) failed to counteract the ameliorative effects of ST-713, which also confirmed our hypothesis that antagonism on H3R, not H1R or H2R, was responsible for the observed effects. These observed results are in harmony with a previous study in which H3R antagonism was found to decrease the impaired social behaviors in animals induced by phencyclidine [28]. Notably, the reference drug DOZ enhanced both sociability as well as social novelty parameters of tested BTBR mice, and the effects observed for DOZ were similar to those witnessed with ST-713.

Recent studies revealed that dysfunction of dopaminergic neurotransmitter system and low levels of brain DA are present in BTBR mice [2,67]. Therefore, there is a wide clinical use of drugs that mainly target the D2Rs [20]. Moreover, the DS has strongly been associated with social behavior, attentional skills and perception, and motor activity, while development's abnormalities in these areas have all been linked to ASD as well [15,16]. In our study, the deteriorating effects observed with the antipsychotic CPZ on sociability features were obliterated by co-administration with ST-713, which also bears the structure of CPZ, indicating that ST-713 may have modulated the dopaminergic neurotransmission system in a constructive way, or its procognitive effect as a H3R antagonist counteracted the negative effects observed with CPZ when administered alone, or the promoted release of neurotransmitters such as ACh played a crucial role in this context of observed behavioral enhancements. Furthermore, ST-713 displayed the same trend to ameliorate the behavioral deficits of tested BTBR mice in the repetitive and compulsive-like performances. The MB and NS tests showed that ST-713 (5 and 10 mg/kg) and DOZ promoted paramount behavioral progresses, compared to the effects observed with the lower dose (ST-713, 2.5 mg/kg). The observed effects for ST-713 in MB and NS tests were entirely reversed when SCO or RAM were co-administered, indicating that cholinergic as well as histaminergic neurotransmissions are, also, involved in the effects observed for ST-713 on the repetitive and compulsive-like performances of tested animals. The mechanism by which the repetitive/compulsive behavior is improved following systemic administration with ST-713 may be back traced to its nature of multiple-active H3R and D2/D3R antagonist, which is able to modulate the release of different neurotransmitters besides HA and DA, such as ACh and 5-HT, in several specific brain areas. Interestingly, the reference antipsychotic drug CPZ failed to mitigate the increased repetitive behaviors in MB test, however, significantly reduced the compulsive/aggressive features of tested animals in NS test. Open field is a widely accepted test to determine behaviors based on mutual exploration and aversion against open and bright areas [68]. Thus, this test was well suited for assessing the effect of ST-713 on anxiety and psychomotor activity in BTBR mice. In comparison to C57 and other inbred mice strains, BTBR mice demonstrate an inconstant profile of anxiety-like behaviors [68]. In the current study and as compared to C57, BTBR spent more time in the center of the arena in OF test, which indicated low levels of anxiety and impulsive behaviors. This observation comprehends previous preclinical observations in rodents, and may be elucidated with the stress conditions exhibited on BTBR mice during the OF test [47,69]. Interestingly, ST-713 with all doses was able to restore these abnormal levels of anxiety in OF test. Hence, our findings suggested that, a dysregulation between HA, ACh, and DA level might associate with abnormal levels of anxiety and impulsive behavior observed in tested BTBR mice. This theory is supported by a previous preclinical report, in which ACh level decline in the prefrontal cortex region of BTBR mice exhibited attention deficit and impulsive behaviors [70]. Furthermore, DOZ and all doses of ST-713 totally restored the abnormal anxiety levels of treated BTBR mice in OF test. However, reference drug CPZ failed to alter the abnormal anxiety-like features of tested animals. In addition, ST-713 (2.5, 5, and 10 mg/kg) and reference drugs CPZ as well as DOZ failed to alter the increase in total distance

travelled or time spent in the periphery, indicating that the doses used by the test compound ST-713 and reference drugs were not able to influence locomotor activity of treated BTBR mice. This finding is important to exclude any confounding factors, such as alteration of locomotor activity, which may provide false positive results in regards to the ST-713-provided enhancements on sociability, social novelty, repetitive, or compulsive/aggressive features.

Numerous previous reports provided evidences of incessant neuroinflammatory processes in several brain regions including microglial activation in individuals diagnosed with ASD and numerous other neuropsychiatric diseases [6]. Evidence indicated that, prolonged microglial activation resulted in increased inflammatory mediators, which lead to a loss of synaptic connections and to the neuronal cell death [71]. These findings were also in harmony with our previous observations that additional CNS disruptions are involved in the behavioral disorders [3,4]. Also, several earlier preclinical reports indicated that hippocampal as well as cerebellar neuroinflammations, including numerous proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and TGF-β, may modify several social parameters in adult mice, as hippocampus and cerebellum are involved in executive and cognitive functions [6,58,72–74]. Moreover, NF-κB was found to play a key role in inflammation, through its ability to induce transcription of pro-inflammatory genes [75,76]. Accordingly, previous studies have revealed that the expression of NF-κB was increased in activated microglia in experimental rodents and also postmortem in patients diagnosed with ASD and SCH [75,76]. In addition, upregulation of iNOS and inducible cyclooxygenase (COX-2) was found to lead to an increase of nitric oxide and prostaglandins, respectively. These substances have a toxic effect in neurons, and consequently were evidenced in ASD as well as SCH [71,77–79]. Consistent with previous studies that reported dysregulation of proinflammatory cytokines in BTBR mice brains [3–5,10,30,31,54,58,80,81], our observations confirmed a remarkably higher expression of NF-κB p65, COX-2, and iNOS in the hippocampus and the cerebellum of BTBR compared to C57. However, ST-713 (5 mg) significantly mitigated the increased levels of assessed cytokines, demonstrating the attenuating effects of ST-713 on the expression of NF-κB p65, COX-2, and iNOS. The current findings are significant and comprehend previous reports in which HA deficiency in histidine decarboxylase knock out mice has been shown to produce significantly higher levels of susceptibility to an inflammatory challenges, that further highlights the significance of brain HA in regulating the levels of proinflammatory cytokines in the brain [82]. These reports fit well with our current results that implicate the involvement of histaminergic neurotransmission in brain. Therefore, the overall neuroprotective effects observed for ST-713 could be explained with the ability of ST-713 to promote the release of brain HA, ACh and DA, as neuroprotective action may be subsequently responsible for the improvement in ASD-like behaviors in BTBR mice.

5. Conclusions

The observed results demonstrate that simultaneous targeting of the CNS histaminergic and dopaminergic neurotransmissions by ST-713 is highly beneficial for palliation of several ASD-like features, namely ASD-like social deficits and repetitive/compulsive behaviors. Moreover, the multi-active novel compound ST-713 mitigated the increased levels of several hippocampal as well as cerebellar proinflammatory cytokines of tested BTBR mice. However, further *in-vivo* assessments on brain levels of HA, ACh, and DA in BTBR mice following systemic treatments of test compound ST-713 as well as reference drugs are still necessary to clarify whether multiple-active compounds, e.g. ST-713, is superior to a standard H3R antagonists/inverse agonists, e.g. pitolisant, or AChEIs, such as DOZ, when applied alone.

Funding

The Office of Graduate Studies and Research of UAE University as well as Zayed-Center for Health Sciences are thanked for the support provided to BS with funds (31R233 and 31R244). The authors also acknowledge the partial support of DFG INST 208/664-1 FUGG and COST Actions CA15135, CA18133, and CA18240, which were kindly provided to HS.

CRediT authorship contribution statement

Bassem Sadek was responsible for the study concept, design, and acquisition and analysis of animal data. **Nermin Eissa and Mohammed Al Awad** conducted behavioral and biochemical experiments. **Karthikkumar Venkatachalam and Petrilla Jayaprakash** were responsible for biochemical assessments and analysis. **Sicheng Zhong, Frauke Stölting, and Holger Stark** were responsible for the synthesis and pharmacological in vitro characterization of test compound ST-713.

Declaration of conflicting interests

The authors declare no conflict of interest.

Author contributions

All authors have read and agreed to the published version of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111517.

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