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Density and function of central serotonin (5-HT) transporters, 5-HT_{1A} and 5-HT_{2A} receptors, and effects of their targeting on BTBR T+tf/J mouse social behavior

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Abstract

BTBR mice are potentially useful tools for autism research because their behavior parallels core social interaction impairments and restricted-repetitive behaviors. Altered regulation of central serotonin (5-HT) neurotransmission may underlie such behavioral deficits. To test this, we compared 5-HT transporter (SERT), 5-HT_{1A} and 5-HT_{2A} receptor densities among BTBR and C57 strains. Autoradiographic [^{3}H] cyanoimipramine (1nM) binding to SERT was 20–30% lower throughout the adult BTBR brain as compared to C57BL/10J mice. In hippocampal membrane homogenates, [^{3}H] citalopram maximal binding (B_{max}) to SERT was 95 ± 13 fmol/mg protein in BTBR and 171 ± 20 fmol/mg protein in C57BL/6J mice, and the BTBR dissociation constant (K_D) was 2.0 ± 0.3 nM versus 1.1 ± 0.2 in C57BL/6J mice. Hippocampal 5-HT_{1A} and 5-HT_{2A} receptor binding was similar among strains. However, 8-OH-DPAT-stimulated [^{35}S] GTPγS binding in the BTBR hippocampal CA_1 region was 28% higher, indicating elevated 5-HT_{1A} capacity to activate G-proteins. In BTBR mice, the SERT blocker, fluoxetine (10 mg/kg) and the 5-HT_{1A} receptor partial-agonist, buspirone (2 mg/kg) enhanced social interactions. The D_2/5-HT_2 receptor antagonist, risperidone (0.1 mg/kg) reduced marble burying, but failed to improve sociability. Overall, altered SERT and/or 5-HT_{1A} functionality in hippocampus could contribute to the relatively low sociability of BTBR mice.

Keywords: 5-HT_{1A} receptor, buspirone, CA_1 of hippocampus, fluoxetine, SERT, sociability.

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Autism spectrum disorders (ASDs) are complex developmental psychiatric conditions in which multiple genetic and environmental risk factors, and their interactions, appear to be involved. Dysfunction of several neurotransmitter systems have been implicated in ASD etiology (Bartlett *et al*. 2005; Pardo and Eberhart 2007). The serotonin (5-HT) system is chief among them and may underlie characteristic ASD social interaction impairments (Santangelo and Tsatsanis 2005; Brune *et al*. 2006; Lam *et al*. 2006; Moy *et al*. 2006). The 5-HT system plays many critical roles in brain development, and its perturbation in rodents can result in behavioral phenotypes reminiscent of autism (Whitaker-Azmitia 2005; Borue *et al*. 2007; Boylan *et al*. 2007; Murrin *et al*. 2007). About 20–45% of ASD patients have elevated platelet 5-HT levels, which might suppress 5-HT terminal formation if it also occurs early in brain development (Anderson *et al*. 1990; Whitaker-Azmitia 2005). At critical postnatal stages, capacity for brain 5-HT synthesis might be reduced, or peak 5-HT levels may be mistimed (Chandana *et al*. 2005). Such processes could impair 5-HT system function and impede therapeutic intervention persistently.

In some studies, ASD susceptibility appears to correlate with function-impairing polymorphisms of the 5-HT

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Abbreviations used: 5-HT, serotonin; 5-HTTLPR, SERT-linked promotor region polymorphism; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)-tetralin; ASD, autism spectrum disorder; BDNF, brain-derived neurotrophic factor; SERT, 5-HT transporter; SNP, single nucleotide polymorphism; SSRI, 5-HT reuptake inhibitor.

transporter (SERT), such as the short allele form of the SERT-linked promoter region polymorphism (5-HTTLPR) and 5-HT receptor genes (Chugani 2002; Sutcliffe *et al*. 2005; Cho *et al.* 2007; Orabona *et al*. 2009; Richardson-Jones *et al*. 2010). Consistent with this scenario, reduced cortical SERT density, as measured by tomography, has been reported in autistic children and adults (Makkonen *et al*. 2008; Nakamura *et al*. 2010). In other reports, the long allele form of 5-HTTLPR appears to be over-transmitted in autistic patients and/or is associated with increased aggression and repetitive behaviors in the disorder (Devlin *et al*. 2005; Brune *et al*. 2006). Thus, evidence from clinical studies suggests altered expression and/or function of the SERT may contribute to behavioral deficits in subpopulations of autistic patients.

From early juvenile stages (PD 21) in to adulthood, the inbred BTBR T+tf/J (BTBR) mouse exhibits deficits in play and social approach and engages in excessive self-grooming repetition relative to other strains, such as C57 or FVB lines, that can be analogized to core symptoms of ASD (Bolivar *et al*. 2007; Crawley 2007; Moy *et al.* 2007; Yang *et al*. 2007a; McFarlane *et al.* 2008). Aberrant social behaviors in BTBR mice must have some genetic basis, because they remain after cross-fostering with C57 mothers (Yang *et al*. 2007a; Benno *et al*. 2008). Furthermore, the behavioral response of BTBR mice to the SERT blocker citalopram is more pronounced than that of C57BL/6 mice (Crowley *et al*. 2006). Based on these findings, we hypothesized that altered SERT or 5-HT receptor function could contribute to the aberrant social behavior of BTBR mice, and compared ligand binding properties of its SERT, 5-HT_{1A} and 5-HT_{2A} receptors with more 'sociable' C57 strains.

Few effective therapeutic interventions are available for ASD, and hardly any of them improve social behavior. Selective 5-HT reuptake inhibitors (SSRIs) such as fluoxetine (Prozac) improve symptoms for some autistic patients, but they have limited effectiveness as a comprehensive ASD therapeutic, and their use in juveniles is controversial (Kirsch *et al*. 2008; Daws 2009; Henry *et al*. 2009; West *et al*. 2009; Richardson-Jones *et al*. 2010). Risperidone, a D_2/5-HT_2 antagonist, is often used to control aggression and self-injury in ASD, but it is less efficacious in some patient groups and does not enhance sociability (Marek *et al*., 2003; Dolzan *et al*. 2008; West *et al*. 2009). Buspirone, an anxiolytic 5-HT_{1A} partial agonist is reported to enhance rodent social interaction at low doses (File and Seth 2003). As the relatively low sociability of BTBR mice bears some face validity to autism, and its utility for pharmacological testing has been suggested (Moy *et al*. 2006), we examined the effects of acute fluoxetine, risperidone and buspirone administration on their social and repetitive behaviors. The combined neurochemical and behavioral approach of this study was designed to reveal how key regulators of 5-HT transmission in emotional centers of the brain could be involved in mammalian social interaction and repetitive behaviors.

Materials and methods

Animals

All animal procedures were performed in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committees of the University of Texas Health Science Center at San Antonio, TX (UTHSCSA), and William Paterson University, Wayne, NJ (WPU). Mice at both facilities were housed and bred under standard conditions: 12-h light/dark cycle, 20–22°C, *ad libitum* access to food (Teklad rodent diet, Harlan, Indianapolis, IN, USA) and water in ventilated racks with plastic housing cages lined with chipped or shaved wood bedding. Water was changed every 2 days and cages refreshed every 7–10 days. Adult (4-month-old) male BTBR and C57BL/10J mice used in autoradiography were second generation offspring from colony founders obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at WPU. Mice were killed by cervical dislocation and decapitation. Whole brains were removed, rinsed in saline and fresh–frozen on powdered dry ice and express shipped the same day from WPU to UTHSCSA for the quantitative autoradiography experiments. There were a total of eight BTBR and eight C57BL/10J mouse brains used in autoradiography.

C57BL/6J mice are often used as a control for BTBR mice in social interaction tests (e.g. McFarlane *et al.* 2008; Silverman *et al*. 2010), and their binding properties at brain 5-HT sites have been well-described because they are a common background strain for the SERT knock-out mouse (e.g. Li *et al*. 2000, 2003; Montañez *et al*. 2003). However, we compared SERT and 5-HT_{1A} and 5-HT_{2A} receptor-binding site densities of BTBR mice with C57BL/10J mice instead, because the literature indicated that 10J mice are better matched for brain size during juvenile development, and like BTBR mice they are also prone to hippocampal anatomy deficits (Wahlsten *et al.* 2003; Deacon *et al.* 2007; Kusek *et al*. 2007). Thus, C57BL/10J mice may more appropriate behavioral controls than C57BL/6J for BTBR mice for brain development studies.

Male BTBR mice used in behavior (3- to 4-month-old) and saturation binding (4-month-old) at UTHSCSA were second generation offspring from colony founders obtained from the Jackson Laboratory and bred in the UTHSCSA facility. Adult (4-month-old) C57BL/6J mice used in saturation binding and 6- to 8-week-old C57BL/10J and C57BL/6J used in social interaction testing were purchased directly from the Jackson Laboratory, and were housed in UTHSCSA facilities for at least one week prior to use in experiments. Mice were killed by cervical dislocation and decapitation, brains for saturation binding were rinsed in ice-cold saline, hippocampi were dissected out and used immediately in experiments.

Quantitative autoradiography

Tissue preparation

Brains from BTBR and C57BL/10J mice were coronally sectioned at a thickness of 20 μm in a cryostat (Leica, Bannockburn, IL, USA), at −20°C at the levels of prefrontal cortex, hippocampus, and dorsal raphe. Sections were thaw-mounted onto gelatin-coated microscope slides, desiccated and stored at −80°C until their use in binding assays. Sections were collected from eight animals per strain for all autoradiography studies.

Serotonin transporter

For SERT binding, the method of Kovachich *et al*. (1988) was used. Sections on slides were incubated for 18 h in 4°C 50 mM Tris, 120 mM NaCl, 5 mM KCl buffer containing 1 nM [^{3}H] cyanoimipramine (American Radiolabeled Chemicals, St Louis, MO, USA). Non-specific binding was defined by incubating adjacent sections in assay buffer containing 10 μM sertraline (Pfizer, Groton, CT, USA). A post-incubation wash was carried out in 4°C Tris–NaCl–KCl buffer for 1 h. [^{3}H] cyanoimipramine has high affinity and specificity for the SERT, and because of its slow dissociation under these conditions, it is a useful radioligand for high volume quantitative autoradiography experiments (Kovachich *et al.* 1988).

5-HT$_{1A}$ receptor

5-HT$_{1A}$ receptor binding was performed at 26°C in Tris–HCl buffer for 1 h using 2 nM [^{3}H] 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) (GE Healthcare, Piscataway, NJ, USA) as we have performed previously (Rossi *et al.* 2008). One micromolar WAY100,635 (Tocris, Ellisville, MO, USA) was added to the incubation solution to determine non-specific binding in adjacent sections on slides.

5-HT$_{1A}$ receptor agonist-stimulated GTPγS binding

For 5-HT$_{1A}$ receptor-stimulated [^{35}S] GTPγS binding, the methods of Rossi *et al*. (2008) were used. Brain sections on slides were equilibrated in buffer containing dithiothreitol (2 mM), then pre-incubated in buffer containing 2 mM GDP, and finally incubated in buffer containing 40 pM [^{35}S] GTPγS (Perkin-Elmer NEN, Boston, MA, USA) in the absence (basal) or presence (agonist-stimulated) of a maximal (E_{max}) concentration of 8-OH-DPAT (1 μM). Non-specific binding was defined under basal conditions with 10 μM GTPγS. All reagents were from Sigma.

5-HT$_{2A}$ receptor

5-HT$_{2A}$ binding involved incubating sections for 30 min in Tris–HCl buffer containing 1 nM [^{3}H] ketanserin (Perkin-Elmer NEN), with 100 nM prazosin, 100 nM pyrilamine, and 1 μM tetrabenazine to block non-5-HT$_{2A}$ binding as previously described (Valdez *et al*. 2002). Ten-micromolar methysergide was used to determine non-specific binding. All reagents were from Sigma.

Quantitative image analysis

From SERT, 5-HT$_{1A}$ and 5-HT$_{2A}$ assays, [^{3}H] labeled sections were opposed to Kodak Biomax MR film along with [^{3}H] calibration standards (American Radiolabeled Chemicals), calibrated to brain mash, for 6 weeks. For 8-OH-DPAT-stimulated GTPγS binding, [^{35}S] GTPγS-labeled sections were opposed to Kodak Biomax MR film along with [^{14}C] calibration standards (American Radiolabeled Chemicals) for 48 h.

Autoradiograms were captured with a digital imaging system: Nikon lens, Kaiser copy stand, 'Northern Lights' precision illuminator (all from InterFocus Imaging Ltd., Linton, UK), camera and frame grabber card (Scion Corporation, Frederick, MD, USA). Digital brain images were calibrated to units of fmol/mg protein for [^{3}H] ligands (per Geary *et al*. 1985) or nCi/mg for [^{35}S] ligands using [^{14}C] standards, and measured using NIH Image, version 1.47 (NIH, Bethesda, MD, USA) on a Macintosh with OS 9. Specific agonist-stimulated 5-HT$_{1A}$ [^{3}H]GTPγS binding was expressed as percent above basal binding.

Saturation radioligand binding to serotonin transporters in hippocampal homogenates

Saturation binding of [^{3}H] citalopram in membrane homogenate preparations from mouse hippocampi was performed following the methods of D'Amato *et al*. (1987), with minor modifications. Five independent, replicate experiments were performed to compare SERT saturation curves for each mouse strain. Fresh hippocampi were pooled from either two BTBR or two C57BL/6J mice (4-month-old males) for each preparation. Hippocampal membrane homogenates were incubated at 26°C for 1 h in buffer containing 0.1–12 nM of [^{3}H] citalopram (PerkinElmer). Non-specific binding was defined by 50 μM sertraline (Pfizer). Incubation was terminated by addition of 4 mL of buffer, pH 7.4 at 4°C, and rapid filtration under vacuum onto Whatman GF/B filter paper strips (Brandel, Gaithersburg, MD, USA) pre-soaked in 5% polyethyleneimine (Sigma). Filters were washed twice and radioactivity trapped on the filters was measured by liquid scintillation counting. Binding data were analyzed by non-linear regression using DeltaGraph software (Red Rock Software, Salt Lake City, UT, USA). Unlike [^{3}H] cyanoimipramine binding (Kovachich *et al.* 1988), [^{3}H] citalopram binding requires neither an extended incubation at 4°C nor a 1-h post-incubation wash, and is therefore better suited for homogenate binding experiments (D'Amato *et al*. 1987).

Behavioral tests following acute treatment with drugs affecting the serotonergic system

Social interaction

Male BTBR mice, bred in the UTHSCSA-LAR facility, and C57BL/6J and C57BL/10J mice purchased from Jackson laboratory and housed for one week in the UTHSCSA-LAR facility were utilized at 3 months of age as subjects in social interaction, social sniff and social novelty behavior tests. BTBR mice do not exhibit any preference for a stranger mouse over a novel object in these sociability tests, which have been extensively validated in previous studies (Moy *et al.* 2004, 2007, 2009; Nadler *et al*. 2004; Yang *et al*. 2007a, Yang *et al*. 2007b; McFarlane *et al*. 2008; Ryan *et al*. 2010; Silverman *et al*. 2010). 'Stranger' mice, male mice of the same strain and age as the subjects but with different parents and no prior contact with the subject mice, were habituated to wire cup cages in arenas during three 30-min exposures conducted over two days prior to testing. Risperidone (0.1 mg/kg), buspirone (2 mg/kg) and fluoxetine (10 mg/kg), from Sigma Chemical Co., were dissolved in saline with the aid of heat and/or sonication as necessary, and administered at 26°C to BTBR mice by intraperitoneal (i.p.) injection 30 min prior to commencing the test protocols. Drug doses were selected based on prior studies in mice to produce behavioral effects without sedation (Holmes *et al*. 2002; File and Seth 2003; Dulawa *et al*. 2004; Wang *et al*. 2007; Silverman *et al*. 2010). C57BL/6J and C57BL/10J mice were not injected prior to testing, but the testing procedure was otherwise the same.

Social interaction and social sniff tests were conducted between 09:00 and 16:00 hours under dim red light (16 lux), because similar baseline results were obtained for BTBR mice irrespective of whether conducted in light versus dark phase of housing light cycles (Yang *et al*. 2007b). We utilized four custom-made three-chambered

rectangular plastic testing arenas, with dimensions and properties similar to arenas described in Moy *et al*. (2007), including slide-in doors and transparent interior walls. Before testing, subject mice were introduced into the central chamber of the empty arena first with doors to side compartments closed for 10 min, then with the doors opened so the subjects could explore the entire arena for another 10 min. Subjects were then confined in the central chamber, while either an empty wire cup-cage or cup cage containing a novel stranger mouse (stranger 1), which the subject had no prior contact with, were introduced into opposite ends of the arena. The doors were re-opened for the subject to explore the testing arena, novel cage and stranger for 10 min of testing.

Following the social interaction test, subjects were again confined to the central chamber, while a new stranger mouse (stranger 2) was placed under the empty cup cage for the social novelty test. The original stranger (stranger 1) remained under the same cup cage in the same end of the arena. The doors were opened for another 10 min testing session. Behavior in the testing arenas was filmed from above with digital camera (R742 Photosmart, Hewlett Packard, Palo Alto, CA, USA) mounted on a tripod (Targus, Anaheim, CA, USA). Chamber entries and social sniff time (sniffing of a stranger by the subject mouse) was monitored by observers unaware of treatments.

Marble burying

Marble burying behavior was assessed after social novelty, utilizing previously described general procedures for this test in mice (Matsushita *et al*. 2005; Bruins-Slot *et al*. 2008). At 70 min post-injection, subjects were introduced into a clean, sterilized large plastic rat housing cage filled with bedding to a depth of 5 cm and topped with 16 blue marbles evenly spaced apart in three rows of 5–6 marbles and topped with a filter lid for 30 min. The number of marbles buried by each mouse was tallied at the conclusion of the test. Buried is defined by having over two thirds of the total top surface of the marble covered by bedding.

Statistical analyses

For autoradiography, one-way MANOVA (when multiple brain regions were measured) or ANOVA (for single brain regions) statistical comparisons were performed. Wilks λ- or *F*-values reaching significance ($p < 0.05$) were evaluated *post hoc* by Newman–Keul's test. For satuaration analysis, maximal binding (B_{max}) and dissociation constant (K_D) values were compared by one-way ANOVA, and Tukey's HSD *post hoc* test. Comparison of the social interaction and social novelty behavior among drug treatment groups was performed using repeated measures ANOVA, with one way ANOVA and Fishers LSD *post hoc* analyses where significant main effects or interactions were observed. Social sniff time and marble burying were also compared among groups by ANOVA and Fisher's LSD *post hoc*. Statistical analyses were performed using STATISTICA software (StatSoft, Tulsa, OK, USA).

Results

Serotonin transporter binding in BTBR and C57 brains

Serotonin transporter density was significantly reduced by 20–30% in BTBR mice relative to C57BL/10J mice in most brain regions measured [Wilks' $\lambda(10,5) = 0.08$, $p = 0.03$, $F(1,14) \geq 8$, Newman–Keul's *post hoc* $p < 0.05$, $n = 8$]. Representative autoradiograms illustrating [^{3}H] cyanoimipramine binding in brain terminal fields from 4-month-old male BTBR and C57BL/10J mice are shown in Fig. 1. Specific SERT-binding densities in several brain regions for BTBR and C57BL/10J mice are shown in Table 1.

SERT saturation binding to [^{3}H] citalopram in BTBR hippocampal homogenates revealed maximal binding (B_{max}) of 95.4 ± 13.2 fmol/mg protein and a dissociation constant (K_D) of 2.0 ± 0.3 nM that differed significantly from the B_{max} and K_D of C57BL/6J hippocampi (171.2 ± 20 fmol/mg protein and 1.1 ± 0.2 nM) [$F(1,8) \geq 8.4$, $p < 0.025$ for B_{max} and K_D], as Fig. 2 shows.

Serotonin 5-HT$_{1A}$ receptor binding and agonist-stimulated G-protein coupling

The density of 5-HT$_{1A}$ receptors in brain, as measured by the binding of [^{3}H] 8-OH-DPAT, did not differ significantly between BTBR and C57BL/10J strains in any of the 10 regions wherein we examined SERT binding [$\lambda(10,5) = 0.2$, $p = 0.24$], and no trends were observed. For example, BTBR versus C57BL/10 5-HT$_{1A}$-binding density in the dentate gyrus of hippocampus was 348 ± 22 versus 370 ± 20 fmol/mg protein, in the ventromedial hypothalamus it was 123 ± 9 versus 117 ± 6 fmol/mg protein, in the medial prefrontal cortex it was 149 ± 9 versus 160 ± 12 fmol/mg protein, and

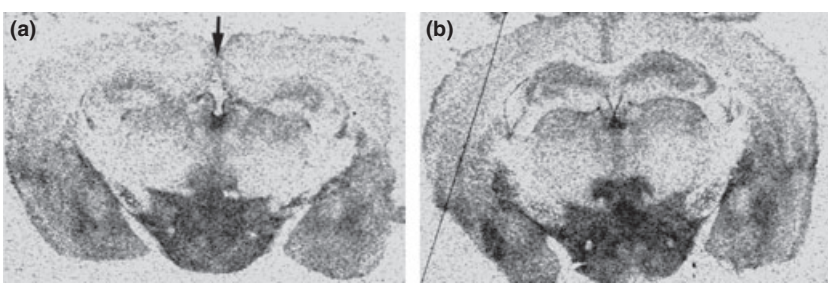

Fig. 1 Representative autoradiograms of [^{3}H] cyanoimipramine (1 nM) binding to serotonin transporters in (a) BTBR and (b) C57BL/10J adult male mouse brains. Shown are autoradiograms of total binding. Note the missing corpus callosum and hippocampal commissure in BTBR brain (arrow), as described by Wahlsten *et al.* (2003).

Table 1 Binding of [^{3}H] cyanoimipramine (1 nM) to serotonin transporters in brain regions of adult male BTBR and C57BL/10J mice. Non-specific binding was defined in the presence of 10 μM sertraline, and was < 10% of total binding

Brain region Specific binding	BTBR fmol/mg pr.	C57BL/10J fmol/mg pr.
Medial prefrontal cortex	435 ± 22[a]	515 ± 32
Parietal cortex	323 ± 11*	402 ± 25
Caudate putamen	367 ± 23	402 ± 38
Hippocampus		
CA1	359 ± 25*	540 ± 28
CA2	400 ± 29*	528 ± 34
CA3	429 ± 19*	608 ± 36
Dentate Gyrus	472 ± 35*	630 ± 36
Basolateral nu. amygdala	865 ± 16*	1073 ± 35
Ventromed. hypothal. nu.	968 ± 39*	1182 ± 68
Dorsal raphe nu.	1342 ± 47*	1675 ± 60

[a]Mean ± standard error, $n = 8$. *Significantly less than C57BL/10J ($p < 0.05$).

in the dorsal raphe nucleus it was 392 ± 20 versus 376 ± 30 fmol/mg protein.

However, 8-OH-DPAT-stimulated [^{35}S] GTPγS binding in the CA1 region of hippocampus was significantly higher in BTBR than C57BL/10J mice [$F(1,14) = 5.8$, Newman–Keul's *post hoc* $p < 0.05$, $n = 8$]. CA1 basal binding for BTBR was 149 ± 9 versus 151 ± 10 tissue equivalent values

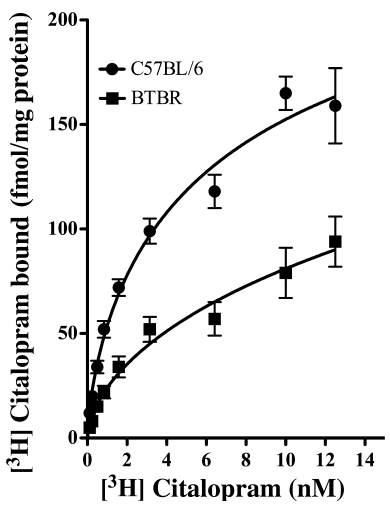

Fig. 2 Specific binding of [^{3}H] citalopram to SERT in hippocampal membrane homogenates from C57BL/6J and BTBR mice. Membrane preparations were incubated with increasing concentrations of [^{3}H] citalopram. Specific binding was obtained by subtracting non-specific binding from total binding at each ligand concentration. Experiments were performed in triplicate, with hippocampal homogenates pooled from two mice per replicate, $n = 5$ replicates.

(nCi/g) in C57BL/10J mice, and 8-OH-DPAT-stimulated [^{35}S] GTPγS binding in this region was 28% higher in BTBR mice than in C57BL/10J mice. No other significant differences were observed in 8-OH-DPAT-stimulated [^{35}S] GTPγS binding in any of the ten other brain regions measured [$\lambda(11,4) = 0.2$, $p = 0.45$], but there was a non-significant trend for 8-OH-DPAT-stimulated binding to be higher in other regions of the dorsal hippocampus. In the dorsal raphe nucleus, there was no difference in basal binding (394 ± 29 vs. 348 ± 31 nCi/g) or 8-OH-DPAT-stimulated [^{35}S] GTPγS binding (31 ± 7% vs. 28 ± 6% above basal) among BTBR or C57BL/10J mice, respectively. Figure 3 illustrates the relationship between 5-HT$_{1A}$ receptor density and 8-OH-DPAT stimulated G-protein coupling in the CA1 region of the hippocampus.

Serotonin 5-HT$_{2A}$ receptor binding

There was no significant difference observed in [^{3}H] ketanserin binding to 5-HT$_{2A}$ sites between BTBR and C57BL/10J adult mice in any region measured [$F(1,14) < 0.8$, $p > 0.39$, $n = 8$]. Representative 5-HT$_{2A}$ receptor densities for BTBR and C57BL/10J mice were 112 ± 24 versus 96 ± 15 fmol/mg protein in the CA1 region of the hippocampus, 74 ± 17 versus 92 ± 10 fmol/mg protein in the ventromedial hypothalamus, and 345 ± 32 versus 372 ± 38 fmol/mg protein in layer IV of the frontal-parietal cortex.

Behavioral test outcomes following acute drug administration in BTBR mice

In the social interaction test, there was a significant drug treatment x social preference interaction effect in the repeated measures ANOVA [$F(3,28) = 3.5$, $p = 0.03$]. Saline-treated BTBR controls exhibited no preference for social interaction; they spent significantly less time in the box with the stranger mouse and more time in the arena box containing the empty cage than either risperidone- or buspirone-treated mice [$F(3,28) = 3.13$, $p < 0.05$, indicated by ** in Fig. 4a]. Although risperidone treatment reduced dwelling in the novel cage box as compared with saline-treated controls, it failed to improve the lack of preference for sociability in BTBR mice because they instead spent more time in the center box. Buspirone and fluoxetine treatments significantly increased preference for sociability, defined as spending proportionally more time in the arena box containing a novel stranger [$F(3,28) = 3.24$, $p < 0.05$ signified by * in Fig. 4a]. Both buspirone and fluoxetine treatments significantly increased the amount of time spent sniffing the stranger mouse over saline treated control times [$F(3,28) = 9$, $p < 0.01$, Fig. 4b], whereas risperidone treatment failed to do so. The number of box entries did not differ for any drug treatment group [$F(3,28) = 0.9$, $p = 0.42$], the mean was 37 ± 4 entries for all groups.

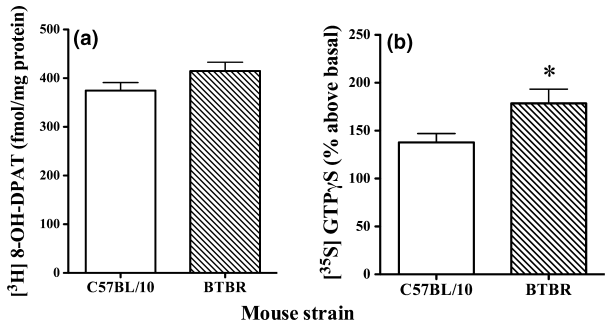

Fig. 3 5-HT_{1A} receptor binding and function in CA1 region of hippocampus of adult BTBR and C57BL/10J mice. (a) The specific binding of [^{3}H] 8-OH-DPAT (2 nM) to 5-HT_{1A} receptors. (b) [^{35}S]GTPγS binding stimulated by the agonist 8-OH-DPAT (1 μM). Specific binding is expressed as % above basal. Bars represent mean and lines standard error of the mean, $n = 8$. *Significantly higher 8-OH-DPAT-induced stimulation than in C57BL/10J.

In the social novelty test, there was a significant drug treatment effect in the repeated measures ANOVA [$F(3,28) = 3.2$, $p = 0.04$]. BTBR mice in all drug-treatment groups exhibited a trend toward reduced preference for social novelty as compared with the saline control group [$F(3,28) = 2.4$, $p = 0.08$]. This is because they spent proportionately less time in the box containing the new mouse (stranger 2) relative to the time they spent in the box containing the original stranger mouse (stranger 1) (Fig. 4c, $^*p < 0.05$). Although time spent in the arena center is not typically included in sociability data analysis (Moy *et al.* 2007), we observed that risperidone-treated mice spent significantly more time in the arena center than the other treatment groups [$F(3,28) = 3.18$, $p = 0.039$, Fisher's LSD $p < 0.05$]. These mice were not sedated by risperidone (0.1 mg/kg) treatment, all of them were active and explored the central arena, engaged in self-grooming, sniffing, head movements, and entered the two side chambers. They just spent more of the test time occupying the central arena.

In the marble burying test, there was no significant difference in number of marbles buried among BTBR mice in all drug treatment groups [$F(3,28) = 1.8$, $p = 0.17$], when all mice were included. However, one of the eight saline-treated mice was an outlier that did not bury any marbles. When this control was dropped, the risperidone-treated mice buried significantly fewer marbles than the saline-treated controls [$F(3,27) = 2.9$, $p < 0.05$, Fig. 4d].

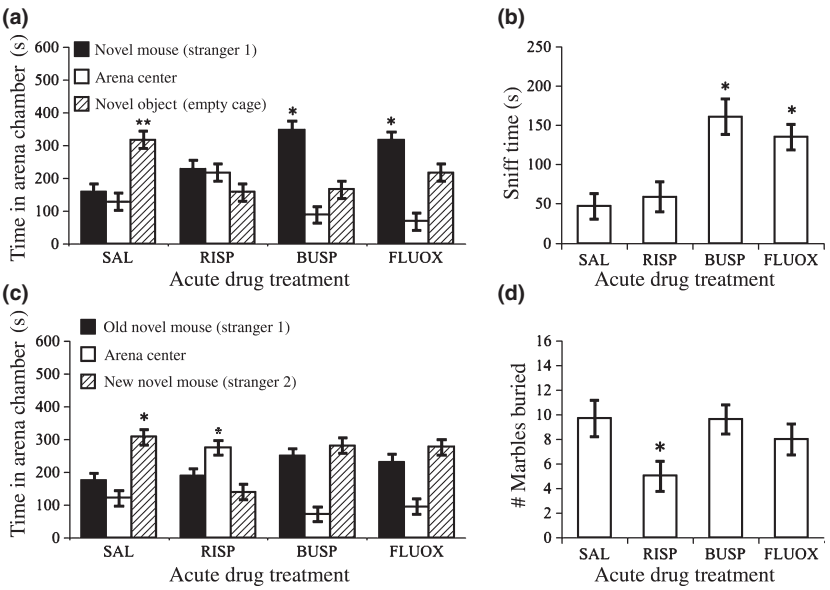

Fig. 4 Effect of acute drug treatments on the behavior of BTBR mice in tasks relevant to core behavioral symptoms of ASD. The effects of acute saline (SAL), risperidone (RISP, 0.1 mg/kg), buspirone (BUSP, 2 mg/kg) or fluoxetine (FLUOX, 10 mg/kg) administration on BTBR mouse behavior. $n = 8$ per treatment. (a) Buspirone and fluoxetine increased sociability in BTBR mice. Time spent by subjects in the arena box containing the stranger mouse was significantly greater than the time spent in the box containing a novel empty cage ($^*p < 0.05$). Saline-treated BTBR mice exhibited no preference for sociability and spent significantly more time in the box with the novel object and less time in the box with the stranger than either buspirone- or fluoxetine-treated mice ($^{**}p < 0.05$). (b) Buspirone and fluoxetine treatments increased the time spent by BTBR mice sniffing the stranger mouse in the social interaction test relative to saline-treated controls ($^*p < 0.05$). (c) In the test for social novelty, only the saline-treated mice spent proportionally more time in the box containing the new mouse (stranger 2) relative to the box containing the old novel mouse (stranger 1) ($^*p < 0.05$). (d) Marble burying by BTBR mice was reduced by risperidone treatment ($^*p < 0.05$).

Outcomes of behavioral tests comparing C57BL/6J and C57BL/10J mice

For the social interaction test, there was a significant main effect of strain [$F(1,14) = 16.7$, $p = 0.001$], but not of the repeated factor (no proportional preference for social novelty for either strain) or the interaction in the repeated-measures ANOVA [$F(1,14) \leq 0.43$, $p > 0.5$]. C57BL/10J mice spent significantly less time than C57BL/6J mice in the box containing the stranger mouse [$F(1,14) = 4.3$, $p = 0.05$]. Interestingly, the C57BL/10J mice also spent significantly more time in the middle of the testing arena than the C57BL/6J mice [$F(1,14) = 16.7$, $p = 0.001$], and made fewer box entries [47 ± 4 vs. 68 ± 4 entries, $F(1,14) = 16$, $p = 0.001$] than C57BL/6J mice (Fig. 5a). There was no difference among strains in the amount of time spent sniffing the stranger mice in the social interaction test [$F(1,14) = 0.097$, $p = 0.75$, Fig. 5b], but as compared with saline-treated BTBR mice (Fig. 4b) both C57 strains spent far more time sniffing the stranger mice in this test. In the preference for social novelty test, there was no effect of strain or interaction [$F(1,14) \leq 2.3$, $p > 0.15$], but there was a significant repeated measures factor, which indicates that both mice spent more time in the box containing the new stranger (stranger 2) versus the old stranger that remained in the arena from the social interaction test (stranger 1) [$F(1,14) = 9.1$, $p = 0.01$]. There was no significant difference between the C57 strains in the amount of time spent in each of the three boxes of the testing arena in the social novelty test (Fig. 5c). There was no difference among the C57 strains in the number of marbles buried [$F(1,14) = 0.9$, $p > 0.3$], as shown in Fig. 5d.

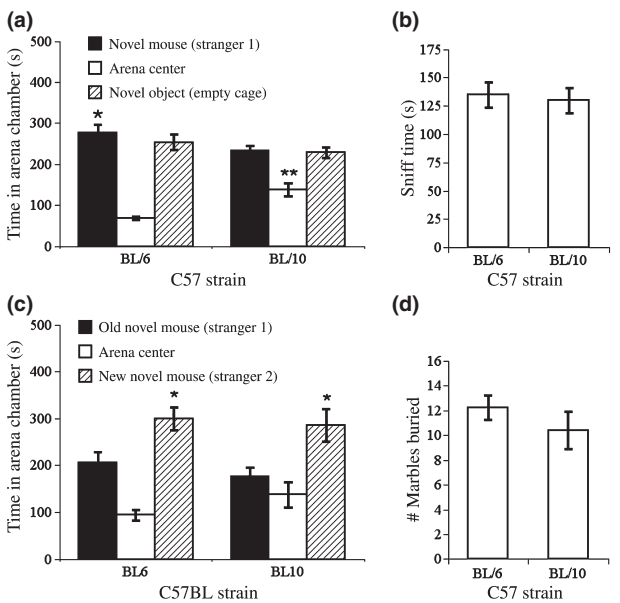

Fig. 5 C57 strain comparison for behavior in tasks relevant to core ASD symptoms. The social interaction, social novelty and marble burying behavior of 8 week old C57BL/10J and C57BL/6J mice were compared, $n = 8$ per strain. (a) C57BL/6J mice exhibited a significant preference for social novelty that was not shared by C57BL/10J mice (*$p < 0.001$). This difference was largely caused by an increase in time spent in the middle chamber of the testing arena, which was significantly greater in C57BL/10J mice (**$p < 0.05$). (b) Time spent sniffing a novel stranger mouse (C57BL/6J) did not differ among C57 subject strains. (c) In the test for social novelty, both C57 strains exhibited a significant preference for social novelty, because subject mice spent proportionally more time in the box containing the new mouse (stranger 2) relative to the box containing the old novel mouse (stranger 1) (*$p < 0.01$). (d) C57BL/6J and C57BL/10J mice buried a similar number of blue marbles with wood-chip bedding over 30 min in this test of compulsive behavior.

Discussion

BTBR mice exhibit sociability deficits and engage in repetitive behaviors that are analogous to core behavioral symptoms of autism (Bolivar *et al*. 2007; McFarlane *et al.* 2008), and have been used in other studies aiming to characterize the effects of potential or extant therapeutic interventions on social behavior and other relevant behaviors (Silverman *et al*. 2010; Chadman 2010). The present study compared SERT and 5-HT_{2A} and 5-HT_{1A} receptor-binding properties in BTBR and C57 mice, and the effects of single-dose acute drug treatments targeting those sites on BTBR performance in tests of sociability and compulsive behavior. A major finding was that differences in SERT expression and 5-HT_{1A} function occur among BTBR and C57 strains. These might contribute to the social behavior impairments of BTBR mice via differential regulation of 5-HT neurotransmission.

Binding properties of the BTBR SERT and behavioral effects of its blockade

Various genetic polymorphisms affecting SERT structure and function, including 5-HTTLPR and rare variants, have been linked to or are associated with autism susceptibility (Sutcliffe *et al*. 2005; Brune *et al*. 2006; Veenstra-Vanderweele *et al*. 2009; Raznahan *et al*. 2009). Assuming that one or more SERT polymorphisms might also occur in BTBR mice, we measured SERT density in various regions of the brain by quantitative autoradiography using [^{3}H] cyanoimipramine, and in hippocampal membrane homogenates with [^{3}H] citalopram, and found it to be about 20–40% lower throughout the brain than the SERT of C57 mice. The observed relative reduction in SERT binding in BTBR mice may be due in part to differential affinity of SERTs among these strains for the SSRI citalopram. The affinity of the BTBR SERT for [^{3}H] citalopram ($K_D = 2 \pm 0.3$ nM) was roughly half as strong as that of the C57BL/6J mouse SERT ($K_D = 1.1 + 0.2$ nM), because drug-ligand affinities are inversely proportional to their dissociation constant (K_D) values. Although differential affinity may account for some share of the lower SERT density found in BTBR mice, our

findings also suggest that BTBR SERT density is also lower than the SERT density in C57 mouse brain. This is because at radioligand concentrations producing maximal SERT binding in both strains, the lower density of BTBR SERT expression relative to C57 mice remains. Our [^{3}H] citalopram homogenate saturation binding data also show that hippocampal SERT B_{max} in BTBR is roughly 40% lower than in C57BL/6J mice. As lower SERT density in BTBR versus C57 brains occurred with use of two different SERT-specific radioligands and binding techniques, we believe that it is not an artifact. Western blot or mRNA analysis might be used to compare SERT expression between these strains in future studies.

Differences in hormonal regulation of SERT expression could contribute to the lower SERT binding found in BTBR mice relative to C57 strains. BTBR mice have elevated baseline serum corticosterone levels, and stress induces an exaggerated increase in the level of this steroid hormone above baseline as compared with C57BL/6 mice (Benno *et al*. 2009). Elevated glucocorticoids have been shown to produce age and duration-dependent effects on SERT expression, since acute exposure to dexamethasone in neonatal rats increased SERT expression, but sub-chronic exposure 20-month-old rats decreased SERT expression as measured by [^{3}H] paroxetine (McGrath *et al*. 1997; Slotkin *et al*. 1997). In 3-month-old adrenalectomized rats, high subdermal levels of corticosterone administered sub-chronically also reduced the density of SERT and 5-HT$_{1A}$-binding sites (Maines *et al*. 1999). However, sub-chronic oral administration of corticosterone failed to reduce hippocampal [^{3}H] 5-HT uptake at 10 nM in rats, or alter the potency of citalopram to block it (Fernandez *et al*. 2001). Likewise, the modest difference in SERT expression alone is unlikely to account for the social behavior impairments of BTBR mice, which were also improved by acute exposure to the SSRI fluoxetine. In addition to higher corticosterone, plasma progesterone and its metabolite 5α-pregnan-3α-ol-20-one are elevated in BTBR serum and lower in the cerebellum as compared with C57BL/6 mice (Frye and Llaneza 2010). Hence, the modest relative reductions in BTBR SERT density may result from inherently high corticosterone levels, along with other hormones that may regulate SERT.

Our findings of lower BTBR SERT expression and SSRI affinity can also be related to a pair of concurrent single nucleotide polymorphism (SNP) haplotypes for the SERT gene [Glu39 → Gly plus Arg152 → Lys, called ER (native form) and GK (mutant)] that differ among C57 and other strains including BTBR and 129S, wherein GK impairs C57 SERT capacity to take up 5-HT (Carneiro *et al*. 2009). In that study, there was a non-significant trend toward C57BL/10 mice (GK) having slightly higher midbrain [^{3}H] paroxetine binding than ER strains. Prefrontal cortex and striatal tissue 5-HT content in BTBR and C57BL/6 mice is not significantly different (Onaivi *et al*. 2010). Hence, we postulated that SERT expression might be elevated in C57 mice to accommodate its impaired functionality relative to higher efficiency ER SERT of BTBR mice. Studies are underway to compare BTBR and C57 SERT capacity for 5-HT uptake *in vivo* and *in vitro*.

Despite potentially reduced SERT density and its lower affinity for SSRIs, SERT blockade appears to improve social behavior in BTBR mice at 10 mg/kg, as seen in the present study and in Chadman (2010). In tail suspension tests, BTBR mice were also more responsive to SSRI treatments than C57BL/6 mice (Crowley *et al*. 2006). Use of SSRIs has been modestly effective for treatment of repetitive and compulsive behaviors in some autism patients, but they do not generally improve sociability, and are ineffective in patients with impaired SERT function (Kirsch *et al.* 2008; Henry *et al.* 2009; West *et al.* 2009). SERT knock out (−/−) mice, like BTBR, 129S and a few other strains, also exhibit impaired social interaction behavior relative to SERT wild-type (+/+) mice (Moy *et al*. 2009). Most human carriers of common polymorphisms impairing SERT function are heterozygous, so SERT heterozygous (+/−) mice are more realistic models of human SERT abnormalities, yet SERT +/− mice are relatively social, with similar baseline behavior to SERT +/+ mice (Moy *et al*. 2009). However, stressed SERT +/− mice, and adult SERT +/+ and SERT +/− pups of SERT +/− dams stressed during pregnancy exhibit significant impairments in subsequent sociability tests, and increased anxiety (Bartolomucci *et al.* 2010; Jones *et al*. 2010; Jansen *et al.* 2010). It would be of interest to see if stressed SERT +/− mouse social behavior would also be improved by fluoxetine. Such an outcome seems unlikely, given that human carriers of SERT polymorphisms tend also to be non-responsive to SSRI treatments (Henry *et al*. 2009). Given the 40–60% prevalence of common SERT polymorphisms in human populations (Lesch *et al*. 1996; Gelernter *et al*. 1997; Noskova *et al*. 2008), increased susceptibility of 5-HTTLPR short allele carriers toward depression following stressful life events (Caspi *et al*. 2003), and the association of SERT polymorphisms with autism susceptibility (Sutcliffe *et al*. 2005; Brune *et al*. 2006; Veenstra-Vanderweele *et al.* 2009; Raznahan *et al.* 2009), clearly alternative drug targets to the SERT are needed for this sizable subpopulation of psychiatric patients.

The BTBR 5-HT$_{2A}$ receptor, ligand binding and antagonist effects on behavior

The 5-HT$_2$ receptor has also been identified as a potential candidate gene for autism in some populations (Veenstra-VanderWeele *et al*. 2002; Cho *et al*. 2007). The density of this receptor did not differ among BTBR and C57BL/10J brains in the brain regions measured for this study. Risperidone, a D$_2$/5-HT$_2$ antagonist which is commonly used to control aggression and self-injury in ASD, is not particularly effective at improving sociability in autistic patients (West *et al*. 2009). The present data are in agreement with this, as

evidenced by failure of risperidone at 0.1 mg/kg to increase time spent by BTBR mice in the proximity of a stranger mouse. It is notable that Silverman *et al.* (2010) and Chadman (2010) also found that risperidone failed to improve social interaction at all doses tested, and both laboratories found that this drug suppressed locomotor activity in BTBR mice at higher doses.

Risperidone treatment (0.1 mg/kg) significantly reduced marble burying, whereas neither 2 mg/kg buspirone or 10 mg/kg fluoxetine treatments altered this parameter in BTBR mice. Mouse marble burying behavior is thought to be indicative of drug efficacy for management of obsessive compulsive behavior, and is reduced by 5-HT_{2A} antagonists such as risperidone and haloperidol, and by 5-HT_{1A} full agonists such as 8-OH-DPAT (Matsushita *et al*. 2005; Bruins-Slot *et al*. 2008; Thomas *et al*. 2009). Functional interactions between these 5-HT receptor subtypes may modulate stereotyped behaviors, as evidenced by the observation that increased 5-HT availability potentiates 5-HT_{2A} receptor-mediated head twitch through pre-synaptic 5-HT_{1A} autoreceptor blockade (Fox *et al*. 2010). Although we found no difference in 5-HT_{2A} receptor expression among strains, we cannot rule out the possibility that functional 5-HT_{2A} receptor alterations may influence this behavior.

G-protein coupling to the BTBR 5-HT_{1A} receptor, and behavioral effects of buspirone

Consistent with observations in brain tissue from young adults with ASDs (Blatt *et al*. 2001), we found no difference in serotonin 5-HT_{1A} receptor density between BTBR and C57BL/10J mice. However, our finding of enhanced agonist-stimulated 5-HT_{1A} [^{35}S] GTPγS binding to G-proteins in CA1 of hippocampus, but not in the dorsal raphe of BTBR mice indicates a heightened potential for post-synaptic responsiveness, with no apparent effect on autoreceptor functionality. Given that BTBR mice have elevated baseline corticosterone levels, we anticipated reduced G-protein coupling capacity in the dorsal raphe, because chronic administration of corticosterone reduced 5-HT_{1A} agonist-stimulated [^{35}S] GTPγS binding in dorsal raphe of adult wild-type littermates of brain-derived neurotrophic factor (BDNF) knock-out mice (Hensler *et al*. 2007). However, in that same study, corticosterone-treated BDNF knock-out mice did not exhibit a reduction in agonist-stimulated [^{35}S] GTPγS binding in the raphe. Hence, the effects of corticosterone on 5-HT_{1A} receptor functional capacity may depend on levels of BDNF expression, and timing and duration of exposure, among other factors.

There is still little known about factors mediating a relationship between 5-HT_{1A} receptors in the CA1 of hippocampus and social interaction behavior, particularly in the three chambered mouse social interaction tests, in which anxiety state must play some role (Crawley 2007). The dorsal hippocampus appears to be involved in this behavior, since agonism of 5-HT_{1A} receptors in this region produces anxiogenic effects and decreased social interaction in open arena rat social interaction tests (File and Seth 2003). We found that the atypical anxiolytic buspirone, at a dose of 2 mg/kg, significantly improved sociability in BTBR mice. Buspirone acts as a partial agonist at hippocampal 5-HT_{1A} receptors, and its actions at 2 mg/kg are likely to be mediated through partial blockade of postsynaptic receptors and full agonist activity at presynaptic 5-HT1A autoreceptors (Yocca 1990; File and Seth 2003). While buspirone has not been extensively studied as a treatment for autism symptoms, it reduced hyperactivity and stereotyped behaviors in three autistic children and reduced aggression in one autistic woman with no adverse effects (Realmuto *et al*. 1989; Brahm *et al*. 2008). Perhaps buspirone should be tested further for its therapeutic potential to improve social interaction behavior in autism.

Sociability behavioral test components and C57 mice as normative controls

In the present study, BTBR social interaction behavior was improved by fluoxetine and buspirone treatments, but not by risperidone. However, all drugs administered in this study reduced BTBR's preference for social novelty in the test, which immediately followed the social interaction test. Whether this outcome is indicative of a property conveying therapeutic benefit remains unclear. Preference of C57BL/6J mice for social novelty is abolished if locations of the old and new strangers are switched (Pearson *et al*. 2010). We did not explore alternative stranger locations in our BTBR social novelty tests, the original strangers were in the same location from the prior social interaction test, as per Moy *et al*. (2004, 2007, 2009).

Because we observed little difference among C57BL/6J and C57BL/10J mice in social interaction in a visual burrow system (Benno *et al*. 2008), we expected their behavior in the three-chambered social interaction test to be similar. For some components of the test, this was true, for example, we found that the time engaged in social sniff of stranger mice did not differ among the C57 strains in the social interaction test, and their behavior in the social novelty test was similar. However, we found that C57BL/10J mice tended to spend more time in the middle chamber of the arena, and made significantly fewer chamber entries than the C57BL/6J mice, because they were generally less ambulatory in the social interaction test. In this respect, C57BL/10J mice (47 ± 4 box entries) and BTBR mice (37 ± 4 box entries) are both slower and less exploratory than C57BL/6J mice (68 ± 4 box entries) in the social interaction arena. Based on this parameter, C57BL/10J mice might be considered better controls for sociability than BTBR mice. However, we also found that C57BL/10J failed to exhibit significant preference for sociability (chambers with novel mice over empty cages) in social interaction tests, whereas C57BL/6 mice did. How-

ever, the significant 'preference for sociability' displayed by the C57BL/6 mice in our hands was modest as compared to C57BL/10J although it was dramatically different from the apparent preference for the empty cage chamber exhibited by our saline-treated BTBR mice. It has been suggested that other strains, such as the FVB strain may be better standard controls for sociability than C57BL/6J mice (Bolivar *et al*. 2007; Moy *et al*. 2007).

Other possible influential factors for stereotypical BTBR behavior

BTBR mice lack a corpus callosum (Wahlsten *et al*. 2003), and comparisons of the impact of cross-hemispheric connectivity (or lack thereof) on mouse social behavior in different strains has yielded inconsistent results (Fairless *et al*. 2008; Yang *et al*. 2009). These abnormalities in forebrain development in mice are controlled by genes in two areas on the X chromosome and occur in all BTBR mice (Kusek *et al*. 2007; MacPherson *et al*. 2008). C57BL/10J mice perform differently in cognition, nest construction and motor function tests as compared with C57BL/6J mice, and they exhibit a range of disruptions in corpus callosum structure, albeit none as severe as those observed in BTBR mice (Wahlsten *et al*. 2003; Deacon *et al*. 2007). In BTBR mice activity-dependent reversal of long-term potentiation occurs rapidly, and contextual fear memory is impaired, but object recognition remains normal or is enhanced as compared with C57BL/6 mice (MacPherson *et al*. 2008). Studies of people congenitally lacking a corpus callosum commonly report social immaturity, social incompetence, literal-mindedness and limited empathy (Paul *et al*. 2004; Symington *et al*. 2010). It is not clear how this level of sophistication in communication can be assessed in the context of the three chambered social interaction test for mice. However, the absent corpus callosum and reduced hippocampal commisure may contribute to the typical performance of BTBR mice in sociability tests.

In the present study, we have shown that BTBR mice exhibit neurochemical and behavioral properties indicative of altered serotonergic neurotransmission that might contribute to their impaired social behavior. Of course, other factors we have not explored herein may also be involved. For example, excess or mistimed serotonin exposure during brain development may contribute to or interact with previously described neuroanatomical abnormalities in the BTBR brain to contribute to its behavioral deficiencies (Wahlsten et al. 2003; Whitaker-Azmitia 2005; Borue *et al*. 2007; Boylan *et al*. 2007; MacPherson *et al*. 2008; Pascucci *et al*. 2008). BTBR mice and all 129S-dervied strains have a 25-bp deletion in the *Disrupted In Schizophrenia 1* (*Disc1*) gene, with potential effects on neurogenesis and dendritic spine growth in the hippocampus and other limbic areas relevant to social behavior (Clapcote and Roder 2006, 2007; Duan *et al,* 2007; Chubb *et al*. 2008; Jackson Laboratory 2008; Ayhan *et al*. 2010). Hence, some of the reduced SERT density we observe in BTBR versus C57 mice may be due to different organization of the 5-HT neuron outgrowths or network in the brain.

Other genetic factors and/or interactions could also alter the protein expression and/or ligand-binding properties of other monoamine transporters or receptors, key metabolic enzymes or peptide hormones (e.g. Mortensen *et al*. 2001; Murphy *et al*. 2003; Wang and Lewis 2009). Indeed, additional functional coding SNPs that differ among BTBR and C57BL/6 mice have been found, including one altering the enzyme kynurine 3-hydroxylase, two affecting FAD binding, and one affecting the mitochondrial transmembrane region (McFarlane *et al*. 2008). As C57 and BTBR mice are products of over 70 years of differential recombination and inbreeding, other mutations or combinations of fixed alleles affecting this locus or its gene expression could have accumulated, as such lines are likely to differ by several hundred SNPs (Witmer *et al*. 2003; Petkov *et al*. 2004). Some, but clearly not all, of these factors may warrant further attention.

In conclusion, BTBR mice are among several possible translational research tools with potential utility for examining the neuropathology of disorders wherein sociability impairment is prevalent, and in which novel therapeutic targets for the improved treatment of such disorders can be tested. Taken together, our findings suggest that altered serotonin system function is evident in BTBR mice and may contribute to its unusual behavioral repertoire.

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