**ORIGINAL ARTICLE**

**Dysfunctional dopaminergic neurotransmission in asocial BTBR mice**

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Autism spectrum disorders (ASD) are neurodevelopmental conditions characterized by pronounced social and communication deficits and stereotyped behaviours. Recent psychosocial and neuroimaging studies have highlighted reward-processing deficits and reduced dopamine (DA) mesolimbic circuit reactivity in ASD patients. However, the neurobiological and molecular determinants of these deficits remain undetermined. Mouse models recapitulating ASD-like phenotypes could help generate hypotheses about the origin and neurophysiological underpinnings of clinically relevant traits. Here we used functional magnetic resonance imaging (fMRI), behavioural and molecular readouts to probe dopamine neurotransmission responsivity in BTBR T+/Itpr3tf−/J mice (BTBR), an inbred mouse line widely used to model ASD-like symptoms owing to its robust social and communication deficits, and high level of repetitive stereotyped behaviours. C57BL/6J (B6) mice were used as normosocial reference comparators. DA reuptake inhibition with GBR 12909 produced significant striatal DA release in both strains, but failed to elicit fMRI activation in widespread forebrain areas of BTBR mice, including mesolimbic reward and striatal terminals. In addition, BTBR mice exhibited no appreciable motor responses to GBR 12909. DA D1 receptor-dependent behavioural and signalling responses were found to be unaltered in BTBR mice, whereas dramatic reductions in pre- and postsynaptic DA D2 and adenosine A2A receptor function was observed in these animals. Overall these results document profoundly compromised DA D2-mediated neurotransmission in BTBR mice, a finding that is likely to have a role in the distinctive social and behavioural deficits exhibited by these mice. Our results call for a deeper investigation of the role of dopaminergic dysfunction in mouse lines exhibiting ASD-like phenotypes, and possibly in ASD patient populations.

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**INTRODUCTION**

Autism spectrum disorders (ASD) are neurodevelopmental conditions characterized by social and communication deficits and repetitive behaviours. Abnormalities in neurotransmitter pathways have been associated to ASD, with evidence for a possible implication of glutamatergic, GABAergic and serotonergic imbalances.1 The neurotransmitter dopamine (DA) has a pivotal modulatory contribution on executive functions, learning, reward and emotional processing,1–3 all of which are impaired in ASD patients.4–6 In spite of this, the contribution of DA and the cellular mediators that regulate its function in ASD have received little attention. The results of recent psychosocial, neuroimaging and genetic research call for a reconsideration of the role of this neurotransmitter in ASD. Individuals with ASD show reduced responsiveness to reward stimuli, a feature that appears to be especially prominent with social reinforcers such as facial interactions, impaired communication and repetitive stereotypic behaviours.20–22 In an attempt to unravel the role and molecular resonance imaging (fMRI) studies have revealed blunted activation of the ventral striatum in individuals with ASD when processing either social or monetary rewards.11–13 Consistent with these findings, alterations in genes encoding DA transporter (DAT14), receptors15,16 and enzymes involved in DA metabolism have been recently linked to ASD.17 Taken together, these initial studies highlight a reduced responsivity of mesolimbic DA reward circuit as a reliable neuroimaging endophenotype for ASD, and suggest that DA neurotransmission imbalances could underlie the diminished incentive salience to reward observed in ASD patients.18 However, the cellular and molecular substrates underlying these deficits remain largely undetermined.

While ASD may be uniquely human, mouse models have proven useful in generating hypotheses about the genetic and neurobiological underpinnings of the disorder.19 Recent behavioural screenings have identified the inbred BTBR T+/Itpr3tf−/J (BTBR) mouse strain as a popular preclinical model of ASD, owing to its robust and pronounced deficits in reciprocal social interactions, impaired communication and repetitive stereotypic behaviours.20–22 In an attempt to unravel the role and molecular...
determinants of dopaminergic dysfunction in ASD, here we employed fMRI, behavioural as well as *in vitro* pharmacological and molecular readouts to probe DA function and responsiveness in BTBR mice. Age-matched, normosocial C57BL/6J (B6) mice were used as reference comparators. 23 Our approach revealed dramatically blunted DA D2 receptor (Drd2r) and adenosine A2A (A2AR) neurotransmission in BTBR strain, a finding that could play a role in the social deficits exhibited by these animals, and that is consistent with emerging evidence of a putative contribution of altered dopaminergic reactivity in ASD.

**MATERIALS AND METHODS**

Adult mice of the inbred strains BTBR and B6 were purchased from the Jackson Laboratory (Bar Harbour, ME, USA). All research involving animals were carried out in accordance with the European directive 86/609/EEC governing animal welfare and protection. Animal research protocols were also reviewed and consented to by a local animal care committee.

**Drugs**

GBR 12909 dihydrochloride was obtained from Tocris Bioscience (Bristol, UK). R(-)-SKF 81297, (-)-quinpirole dihydrochloride and haloperidol were obtained from Sigma (St Louis, MO, USA). Haloperidol was dissolved in a solution of 10% acetic acid in saline. GBR 12909, SKF 81297 and quinpirole were dissolved in saline solution.

**Pharmacological fMRI (phMRI)**

fMRI experiments were performed in adult male BTBR (n = 10) and C57BL/6 (B6, n = 10) mice as recently described. 24,26 Briefly, mice were anaesthetized with isofluorane, intubated and artificially ventilated. A femoral artery was cannulated for compound administration, blood pressure monitoring, infusion of paralysing agent and terminal blood gas sampling (pCO2 and pO2). pCO2 levels rose from 35 to 40 mmHg and pO2 was maintained at 150 mmHg. In the two supers (B6 and the end of the experiment) were 25 ± 1 and 23 ± 2 mm Hg, and 283 ± 22 and 287 ± 6, respectively. No statistically significant intergroup difference in arterial blood gases was observed (P > 0.22, all comparisons, Student's t-test). Functional data were acquired on a 7T Pharmascan (Bruker, Ettlingen, Germany) as recently described. 24,27 A 72-mm birdcage resonator was used for radiofrequency transmit and a Bruker quadrature ‘Mouse Brain coil’, placed dorsally on the skull of the animal, was used for radiofrequency receive. 28 Co-registered anatomical and fMRI images were acquired using a rapid acquisition relaxation-enhanced and a fast low-angle shot MRI sequence, respectively with the following imaging parameters:

\[ T\text{R} = 3000 \text{ms, } T\text{E} = 38 \text{ms, rapid acquisition relaxation-enhanced factor 8, FOV 40 mm, 100 × 100 × 500 μm resolution} \]

and

\[ T\text{R} = 228 \text{ms, } T\text{E} = 3.1 \text{ms, } α = 30^\circ, \text{FOV 40 mm, 180 × 180 × 600 μm resolution, duration } 60\text{s} , \text{N} = 50, \text{total acquisition time } 30 \text{min, blood pool-contrast agent} \]

The brains of the animals were fixed in 4% paraformaldehyde for 24 h and sectioned in 50-μm-thick slices. Histological preparations were stained for tyrosine hydroxylase and TH immunoreactivity and TH positive cells were counted as previously described. 29

**Western blotting**

Western blotting was performed as previously described. 32 Briefly, mice were administered 5 mg kg\(^{-1}\) of SKF 81297, 0.5 mg kg\(^{-1}\) haloperidol or their respective vehicle i.p. and killed by decapitation 30 min later. The brains were immediately immersed in liquid nitrogen for 5–6 s, the brains were removed and the striatal dissected within 20 s on an ice-cold surface, sonicated in 1% SDS and boiled for 10 min. This extraction procedure prevents protein phosphorylation and dephosphorylation, hence ensuring that the level of phosphoproteins measured *ex vivo* reflect the *in vivo* situation. 33,34 Aliquots (2 μl) of the homogenate were used for the protein determination by Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA, USA). Equal amounts of total proteins for each sample were loaded onto 12% polyacrylamide gels. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred onto PVDF (polyvinylidene difluoride) membranes (GE Healthcare, Chantont St Giles, UK). Membranes were immunoblotted overnight using selective antibodies.
In situ hybridization

Mice were killed and their brains quickly dissected, embedded in Tissue Tek (Sakura) and frozen at 80 °C. Fourteen-micrometre cryostat sections were cut in the coronal plane and in situ hybridization analyses were performed according to protocols previously described.15 Coronal sections along the rostro-caudal extent of striatum and substantia nigra from both B6 and BTBR male adult mice were hybridized with 35S-labelled antisense riboprobes (Ord1, 1.3 Kb; B6 n = 4, BTBR n = 7; Ord2, 0.4 Kb; B6 n = 4, BTBR n = 7). Hybridized sections were exposed to Biomax MR X-ray films (Kodak, Rochester, NY, USA) for 2–8 days.

Image analysis and quantification

For densitometric analyses, brain sections cut throughout the rostro-caudal extent of the striatum (n = 20) and substantia nigra (n = 5) were used. Images of autoradiography films resulting from radioactive in situ hybridization experiments were scanned at a resolution of 1200 d.p.i. Optical density was evaluated in striatum and substantia nigra of B6 and BTBR adult male mice. Background optical density values were determined in structures of the same section devoid of specific signal and subtracted for correction to obtain the relative optical density value. Results were expressed as percentage variation of mRNA expression in control animals. Data were analysed by Student’s t-test.

RESULTS

Lack of ventro-striatal DA-induced fMRI responses in BTBR mice

DA-elicted fMRI responses have been demonstrated to serve as a plausible surrogate for mesolimbic dopaminergic reactivity in rodent species.38–40 As human fMRI studies reported reduced reactivity of ventro-striatal DA terminals in individuals with ASD,11–13 we employed pharmacological fMRIs24,41 to map the functional response elicited by selective DAT inhibitor GBR 12909 in BTBR and B6 strain. Consistent with previous fMRI studies with DA-releasing agents24,39,42 GBR 12909 elicited widespread and robust activation of cortical and subcortical regions in B6 mice with respect to vehicle (Figure 1a, b and c; Z > 1.6, P < 0.01, corrected vs vehicle), with a clear and robust involvement of mesolimbic DA terminals such as the nucleus accumbens and medial prefrontal cortex (Figure 1a, b and c). Interestingly, in BTBR mice the drug did not produce detectable fMRI responses in the aforementioned striatal and mesolimbic dopaminergic areas, but elicited only a focal pattern of activation that comprised the thalamus, motor and somatosensory regions and the dorsal (but not medial) prefrontal cortex. The magnitude of GBR 12909-induced fMRI response in these areas was comparable to that observed in B6 mice (Supplementary Figure S1), thus arguing against the presence of generalized vascular or metabolic disturbance in these animals. Lack of appreciable GBR 12909-induced fMRI activation was also found in hippocampal regions, as against the presence of generalized vascular or metabolic disturbance in these animals. Lack of appreciable GBR 12909-induced fMRI activation was also found in hippocampal regions, as
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Figure 2. Blunted GBR 12909-induced motor activity in BTBR mice. Motor activity induced by 10 mg kg$^{-1}$ and (a, b) and 20 mg kg$^{-1}$ (c, d) GBR 12909 in B6 (10 mg kg$^{-1}$, n = 5; 20 mg kg$^{-1}$, n = 6) and BTBR mice (n = 6 per dose). Two independent vehicle-treated groups were used to test the effect of 10 mg kg$^{-1}$ (n = 6 per strain) and 20 mg kg$^{-1}$ GBR 12909 (C57BL/6J, n = 5; BTBR, n = 6). Locomotion was expressed as distance travelled, measured every 10 min over a 30-min session and presented as time course (a, c) or total activity (b, d). All values are expressed as mean ± s.e.m. *P < 0.05, compared with vehicle-treated group within strain (one-way analysis of variance).

well as in postero-parietal and latero-cortical regions of BTBR mice. We detected no significant confounding effect of genotype on anaesthesia during fMRI as assessed by the magnitude of mean arterial blood pressure, a sensitive indicator of anaesthesia depth in rodents$^{14}$ (P = 0.17). GBR administration produced a short-lived (~8 min) blood pressure decrease (~6.0 ± 6.3 mm Hg and ~22.50 ± 5.1 mm Hg in BTBR and B6, respectively) that was, however, well within the autoregulation window under halothane anaesthesia$^{44,45}$ and temporally uncorrelated with the fMRI responses (the latter lasting >25 min), thus arguing against a peripheral origin of the discrepant fMRI responses mapped in the two strains.

To investigate the origin of the discrepant dopaminergic fMRI reactivity in the two strains, constant potential amperometry was employed to detect striatal alterations in the levels of extracellular DA, following incubation with the DAT blocker GBR 12909 (Figure 1d). Interestingly, both strains exhibited significant evoked DA responses with respect to baseline (129 ± 21% and 82 ± 5.4% of baseline, in B6 and BTBR mice, P = 0.03 and P = 0.003, respectively, paired t-test), although the effect appeared to be slightly lower in BTBR mice (P = 0.04, Student’s t-test). The presence of robust GBR 12909-evoked DA release in both strain argues against a primary contribution of DAT-mediated dysfunction to the discrepant fMRI signals recorded, as total lack of striatal fMRI responses was observed in BTBR mice. This finding suggests the presence of an additional mechanism, besides a slightly reduced DAT efficiency or tonic DA activity, which may contribute to the blunted dopaminergic responsivity observed with fMRI.

Blunted GBR 12909-induced horizontal motor stimulation in BTBR mice
To assess the behavioural relevance of the alterations found with fMRI, we evaluated the stimulant in vivo effect of GBR 12909 on the horizontal motor activity of BTBR and B6 mice, a behaviour dependent on DA neurotransmission in the nucleus accumbens. Consistent with the imaging results, both the GBR 12909 doses tested (10 and 20 mg kg$^{-1}$ i.p.) produced robust and sustained horizontal motor responses in B6 mice, but failed to elicit significant forward locomotor activity in BTBR mice (Figure 2a; two-way ANOVA with repeated measures, treatment effect: 10 mg kg$^{-1}$, B6: F(1,18) = 7.222, P = 0.0249; BTBR: F(1,20) = 0.482, P = 0.5031; Figure 2c; 20 mg kg$^{-1}$, B6: F(1,18) = 9.313, P = 0.0138; BTBR: F(1,20) = 4.892, P = 0.0514). Analogous results were obtained when the effect was expressed as cumulative motor activity over the experimental time-window examined (0–30 min; Figure 2b, one-way ANOVA, 10 mg kg$^{-1}$: B6 F(1,18) = 7.222, P = 0.0249; BTBR: F(1,19) = 0.482, P = 0.5031; Figure 2d, 20 mg kg$^{-1}$: B6, F(1,18) = 9.313, P = 0.0138; BTBR: F(1,19) = 4.892, P = 0.0514). Together with the fMRI findings, these behavioural data corroborate the presence of a severe striatal DA hypo-responsiveness in BTBR mice.

Comparable DA D1 receptor-mediated behaviour and signalling in BTBR and B6 mice
The lack of consistent fMRI and behavioural responses to GBR 12909 in BTBR mice, despite the ability of the compound to robustly augment DA levels in the striatum of these animals, led us to investigate the expression and functionality of pre- and postsynaptic DA receptors.

The DA D1 receptor (Drd1) is among the most important postsynaptic effectors of DA function. Drd1 mRNA expression levels were measured in the striatum of BTBR and B6 mice using in situ hybridization with a 31S-radiolabelled antisense riboprobe. Densitometric analyses revealed comparable levels of Drd1 in BTBR and B6 mice (P = 0.51, Student’s t-test; Figure 3a, b and c). The functional reactivity of Drd1 in both strains was next probed
Preserved Drd1-mediated motor stimulation and signalling in BTBR mice. Representative images of coronal sections showing Drd1 mRNA expression in the striatum of B6 (a) and BTBR (b) animals (scale bar, 1.5 mm). (c) Histogram showing Drd1 mRNA levels in BTBR animals (n = 7) obtained after densitometric quantification of autoradiograms expressed as percentage variation as compared with B6 control animals (n = 4). (d) Motor activity induced by 1.25 and 2.5 mg kg⁻¹ SKF 81297 in B6 (vehicle, n = 6; 1.25 mg kg⁻¹, n = 7; 2.5 mg kg⁻¹, n = 6) and BTBR animals (vehicle, n = 5; 1.25 mg kg⁻¹, n = 6; 2.5 mg kg⁻¹, n = 6). Locomotion is expressed as the total distance travelled (in cm). *P < 0.05, **P < 0.01, compared with vehicle-treated group within strain (Fisher’s post hoc analysis). P-Ser845-GluR1 protein levels determined by western blotting in the striatum of B6 (e) and BTBR mice (f) treated with 5 mg kg⁻¹ SKF 81297 (B6, n = 8; BTBR, n = 3) or vehicle (B6, n = 9; BTBR, n = 3). The top panels in e and f show representative blots comparing the different strains. *P < 0.05, **P < 0.01, compared with vehicle, Student’s t-test (f and e, respectively). All values are expressed as mean ± s.e.m.

**Figure 3.** Preserved Drd1-mediated motor stimulation and signalling in BTBR mice. in vivo, by measuring motor activity elicited by selective agonist SKF 81297 administration (1.25 and 2.5 mg kg⁻¹), as previously reported⁴⁹ (Figure 3d). A comparable dose-dependent increase in locomotor activity was observed in both strains (one-way ANOVA: B6, F(2,14) = 5.148, P = 0.0211; BTBR, F(2,16) = 10.341, P = 0.0013), corroborating the presence of a functionally reactive Drd1 receptor pool in BTBR mice.

This notion was further confirmed by investigations of the signalling efficiency of Drd1/cyclic adenosine monophosphate/protein kinase A (PKA)-dependent phosphorylation of GluR1 subunit at Ser845.⁵⁰,⁵¹ The results of this assay revealed that SKF 81297 administration triggered comparable Drd1-dependent P-Ser845-GluR1 levels increase in the striatum of both B6 (Figure 3e) and BTBR (Figure 3f) mice with respect to vehicle-treated animals (B6, P < 0.01; BTBR, P < 0.05; Student’s t-test). Collectively, these findings point at comparable Drd1 responsivity in BTBR and B6 mice, and argue against a primary contribution of Drd1 alterations in the lack of GBR-induced fMRI and motor-stimulant responses observed in BTBR animals.

Blunted presynaptic DA D2 receptor function in BTBR mice

DA D2 receptors (Drd2) have a crucial role in mediating the stimulating effects of DA,⁴⁸ as well as in reward processing and stimulus discrimination,⁵² a contribution that is believed to result from the opposing action of presynaptic and postsynaptic Drd2 pools.⁵³ Based on these considerations, we investigated the state of Drd2 mRNA expression and functional responsivity at presynaptic sites in BTBR and B6 mice. mRNA expression levels of Drd2 in the substantia nigra was measured in B6 and BTBR mice using in situ hybridization (Figure 4). This measure reflects the mRNA distribution and occurrence of presynaptically localized Drd2 autoreceptors, a receptor pool that provides negative feedback mechanism, which adjusts neuronal firing rate, synthesis and DA release.⁴⁸,⁴⁹ No difference in the intensity of autoradiographic signals (Figure 4a, b and c) was observed between strains (P = 0.60, Student’s t-test). We next probed the functional state of presynaptic Drd2 receptors in controlling DA release.⁴⁹,⁵⁴ This assay was performed by using constant potential amperometry measurement in the presence of the selective Drd2-like agonist, quinpirole (Figure 4d). In line with previous observations, quinpirole robustly depressed striatal DA release in B6 mice (P < 0.0001, paired t-test, Figure 4d), an effect mediated by activity at presynaptic Drd2 receptors.⁴⁹,⁵⁵ However, quinpirole-induced DA release inhibition in BTRB mice was only marginal, and greatly attenuated with respect to the one observed in B6 mice (90.5 ± 2.96% vs 60.3 ± 3.56%, n = 9, respectively; P = 0.00002, Student’s t-test). In line with the dramatic Drd2-related differences
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found between strains, quinpirole administration robustly inhibited locomotor activity in B6 mice, but failed to elicit significant motor responses in BTBR mice (one-way ANOVA: B6: F_{(1,12)} = 14.725, P = 0.0024; BTBR: F_{(1,10)} = 1.992, P = 0.1885; Figure 4c).

We next assayed intracellular signalling cascade mediated by Drd2 autoreceptors in regulating DA synthesis by measuring phosphorylation levels at Ser40 of striatal tyrosine hydroxylase (P-Ser40-TH). In accordance with previous studies,^{55,56} blockade of presynaptic Drd2 by haloperidol (0.5 mg kg\(^{-1}\)) quinpirole in B6 (vehicle, n = 7; quinpirole, n = 7) and BTBR mice (vehicle, n = 6; quinpirole, n = 6). Locomotion is expressed as total distance travelled (in cm). (f) Striatal P-Ser40-TH protein levels in B6 and BTBR mice treated with 0.5 mg kg\(^{-1}\) haloperidol (B6, n = 3; BTBR, n = 4) or vehicle (B6, n = 6; BTBR, n = 5). The top panels in f show representative blots comparing the different strains and treatment groups. *P < 0.05, **P < 0.01, compared with vehicle-treated group within strain (one-way analysis of variance; f and e, respectively). All values are expressed as mean ± s.e.m. DA, dopamine.

Blunted postsynaptic DA D2 receptor function in BTBR mice

We next investigated postsynaptic Drd2 mRNA expression and function in BTBR and B6 mice. Consistent with previous measurements in the substantia nigra, Drd2 mRNA levels in the striatum and nucleus accumbens appeared comparable in the two strains (P = 0.89; Student’s t-test; Figure 5a, b and c). To probe the functional responsivity of postsynaptic Drd2 receptors, we measured in the two cohorts the cataleptic effect produced by the Drd2-like antagonist haloperidol.49 As expected, robust haloperidol-induced catalepsy was observed in B6 mice (one-way ANOVA: F_{(1,8)} = 14.967, P = 0.0083; Figure 5d). Interestingly only marginal effects were found in BTBR mice (F_{(1,8)} = 5.228, P = 0.0516; Figure 5d) thus highlighting a significant hypo-functionality of postsynaptic Drd2 receptors in these animals. We next investigated the efficiency of postsynaptic Drd2 signalling at MSN sites. By blocking the negative control exerted by Drd2 activity upon adenilate cyclase, the use of haloperidol can unmask adenosine A2aR-dependent modulation of cyclic adenosine monophosphate/PKA-dependent phosphorylation of GluR1 on Serine 845 (P-Ser845-GluR1).^{54,57} As expected, a significant increase in haloperidol-dependent striatal P-Ser845-GluR1 levels was observed in B6 mice (one-way ANOVA: F_{(1,8)} = 7.732, P = 0.0214), whereas in BTBR animals phosphoprotein levels were unaltered compared with vehicle-treated controls (F_{(1,8)} = 0.484, P = 0.5064) (Figure 5e). Because basal P-Ser845-GluR1 levels are concomitantly affected by Drd1 and A2aR receptor activation^{58} and as Drd1-mediated signalling appeared to be functional in BTBR (Figure 3f), the discrepant PKA-related phosphoprotein levels observed in the two strains (Figure 5f) led us to investigate the

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**Figure 4.** Impaired Drd2-mediated presynaptic responsivity and signalling in BTBR mice. Representative images of coronal section showing Drd2 mRNA expression at the level of the substantia nigra of B6 (a) and BTBR animals (b, scale bar, 1 mm). (c) Histogram of Drd2 mRNA levels in BTBR animals (n = 7) obtained after densitometric quantification of autoradiograms expressed as percentage variation as compared with B6 control animals (n = 4). (d) Normalized amperometric responses in the presence of the Drd2 agonist quinpirole (100 nM). The drug depressed DA over time in both strains (B6, 60 ± 3.6% of baseline, P < 0.00001, BTBR 90.6 ± 3.0% of baseline, P < 0.013, paired t-test), although the effect in BTBR appeared greatly attenuated compared with the one observed in control B6 subjects (**P < 0.05, ***P < 0.01 vs baseline, paired t-test). (e) Motor activity induced by 0.25 mg kg\(^{-1}\) quinpirole in B6 (vehicle, n = 7; quinpirole, n = 7) and BTBR mice (vehicle, n = 6; quinpirole, n = 6). Locomotion is expressed as total distance travelled (in cm). (f) Striatal P-Ser40-TH protein levels in B6 and BTBR mice treated with 0.5 mg kg\(^{-1}\) haloperidol (B6, n = 3; BTBR, n = 4) or vehicle (B6, n = 6; BTBR, n = 5). The top panels in f show representative blots comparing the different strains and treatment groups. *P < 0.05, **P < 0.01, compared with vehicle-treated group within strain (one-way analysis of variance; f and e, respectively). All values are expressed as mean ± s.e.m. DA, dopamine.
The mesolimbic DA systems is composed of midbrain dopaminergic projections that originate in the ventral tegmental area, and extend alongside the ventral axis of the brain to project to forebrain areas of BTBR mice, including mesolimbic terminals in the ventral striatum, are of particular significance, because recent human neuroimaging research has consistently revealed analogous reduced functional responses in the reward circuits of individuals diagnosed with ASD when processing either social and monetary rewards. Although a direct extrapolation of our findings to a multifactorial and possibly human-specific neurodevelopmental conditions such as ASD requires extreme caution, it is tempting to speculate that functional dopaminergic deficits analogous to those identified in the present study could have a contributing role in the reward hypo-responsivity associated to the disorder. Recent genetic association studies are consistent with this view, as alterations in genes encoding enzymes involved in DA metabolism, such as DA neurotransmission and the specific observation of a blunted fMRI reactivity in several forebrain areas of BTBR mice, including mesolimbic terminals in the ventral striatum, are of particular significance, because recent human neuroimaging research has consistently revealed analogous reduced functional responses in the reward circuits of individuals diagnosed with ASD when processing either social and monetary rewards.

**DISCUSSION**

The mesolimbic DA systems is composed of midbrain dopaminergic projections that originate in the ventral tegmental area, and extend alongside the ventral axis of the brain to project to structures closely associated with the limbic system, most prominently the nucleus accumbens and the prefrontal cortex. Our work documents the presence of substantially blunted striatal and mesolimbic DA neurotransmission in BTBR mice, an effect associated to dysfunctional pre- and postsynaptic Drd2 signalling, with a plausible contribution of striatal adenosine A2AR alterations. BTBR mice display a distinctive behavioural profile, characterized by robust social communication deficits, together with increased repetitive behaviours, which collectively have promoted a widespread use of the model in preclinical research to mimic symptoms and generate novel hypotheses about the origin and components of neurodevelopmental disorders such as ASD. Within this framework, the presence of dysfunctional Drd2 neurotransmission and the specific observation of a blunted fMRI reactivity in several forebrain areas of BTBR mice, including mesolimbic terminals in the ventral striatum, are of particular significance, because recent human neuroimaging research has consistently revealed analogous reduced functional responses in the reward circuits of individuals diagnosed with ASD when processing either social and monetary rewards. Although a direct extrapolation of our findings to a multifactorial and possibly human-specific neurodevelopmental conditions such as ASD requires extreme caution, it is tempting to speculate that functional dopaminergic deficits analogous to those identified in the present study could have a contributing role in the reward hypo-responsivity associated to the disorder. Recent genetic association studies are consistent with this view, as alterations in genes encoding enzymes involved in DA metabolism, such as DA neurotransmission and the specific observation of a blunted fMRI reactivity in several forebrain areas of BTBR mice, including mesolimbic terminals in the ventral striatum, are of particular significance, because recent human neuroimaging research has consistently revealed analogous reduced functional responses in the reward circuits of individuals diagnosed with ASD when processing either social and monetary rewards.
DA and involved in ASD. The ability of drugs targeting Drd2 such as aripiprazole and risperidone to improve, albeit marginally, core ASD symptoms, together with key contribution of Drd2 receptor in mediating social behaviour in humans and pair-bonding animals, such as the prairie vole (Microtus ochrogaster), provide indirect support to this view. Evidence of compromised striatal neurotransmission has also been recently provided in mouse lines modelling alterations in ASD candidate genes, such as the postsynaptic protein SHANK3. Shank3 KO mice display morphoanatomical, cellular and electrophysiological alterations in striatal medium spiny neurons, which are thought to be responsible for the high levels of repetitive grooming in these animals. Collectively, these studies are consistent with a possible contribution of DA imbalances in ASD, a phenomenon that is likely to be of multifactorial and polygenic origin, but that may converge to determine dysreactivity of the mesolimbic reward systems, possibly (but not exclusively) via imbalances in Drd2-mediated neurotransmission. Neuroimaging techniques (for example, positron emitting tomography) using, for example, Drd2 sensitive tracer could be employed in clinical ASD populations to further investigate the receptorial and neurochemical determinants of reward hyposensitivity observed in ASD. It should also be noted that profound DA alterations have also been identified in mouse lines modelling inheritable forms of ASD. For example, mice recapitulating genetic alterations at the basis of Fragile X syndrome, an inheritable form of intellectual disability otherwise associated to ASD symptoms, exhibit profound impairments in striatal (mostly Drd1-mediated) DA neurotransmission, together with altered motor skills. Similarly, mice carrying methyl-CpG-binding protein 2 (MECP2) mutations, an alteration that causes inheritable ASD-like symptoms show substantial reductions in striatal DA and motor deficits. Although these dopaminergic alterations cannot be directly related to our findings in BTBR mice, where no motor deficits have been described, the presence of robust (albeit heterogeneous) DA deficits in mouse models of high construct validity for ASD is of interest and lends indirect support to the involvement of dopaminergic dysfunction in ASD.

Our results have also implications for the preclinical use of asocial BTBR mice in preclinical research to model idiopathic ASD. The BTBR line has been selected out of a thorough neurobehavioural characterization of different mouse lines owing to its high face validity, that is, its ability to reliably and robustly express phenotypes (for example, social and communication deficits) reminiscent of clinical manifestations of ASD. In an attempt to assess and somewhat ‘objectify’ the translational relevance of the approach, we and others have recently undertaken research efforts aimed to assess the construct validity of the model and probe its ability to reproduce neuroanatomical and pathophysiological determinants of ASD. To this purpose, we used MRI to investigate in BTBR anatomical and functional neuroimaging readouts that have been extensively used in ASD patient populations. Our results highlighted a composite picture, with unspecific traits that do not appear to be representative of typical neuroimaging findings in ASD (for example, widespread cortical thinning) and a stronger, albeit heterogeneous, involvement of saliently relevant features, including the presence of reduced fronto-cortical perfusion, and acallosal development, two alterations that have been previously described in ASD subpopulations. A recent study from Han et al. reported the presence of constitutively reduced inhibitory neurotransmission in neocortical areas of BTBR mice, a finding that has been observed in multiple monogenic mouse models of ASD and that is consistent with increasing clinical evidence of altered neural connectivity in excitatory and inhibitory cortical circuits in ASD patients (reviewed in Zikopoulos and Barbá). Our present findings expand these initial investigations by highlighting the presence of mesolimbic DA hyporeactivity in asocial BTBR mice, a feature that has been recently described in fMRI and psychosocial studies of ASD. Further research is, however, warranted to assess the exact behavioural and translational significance of these results. For example, it has been suggested that hyporeactivity of reward systems in ASD patients could preferentially affect social stimuli. The extent to which these alterations affect goal-oriented behaviour and social reward processing in BTBR is at the moment unclear, and the lack of behavioural readouts in this work prevents a direct extrapolation of our results to ASD-specific behavioural domains. Nonetheless, the recent description of reduced motivation in food- and social-reinforced operant tasks in BTBR mice suggests the presence of a general reinforce-ment deficit that would be largely consistent with the abnormal dopaminergic reactivity documented by our data. However, impaired gustatory signal transduction along with reduced motivation for appetitive food stimuli have also been observed in this strain, thus questioning the significance of reward probing in this strain via the use of food reinforcers. Additional experiments involving non-gustative reinforcers (for example, pharmacological or sexual stimuli) could be employed to probe the behavioural specificity (for example, social vs general motivation) of the reward deficits exhibited by this strain. Importantly, the DA dysreactivity observed in BTBR is likely to have a role in the cognitive flexibility deficits recently described in this mouse line, a behavioural trait that serves as a plausible proxy for the restricted interests and repetitive behaviour associated to ASD. To assess this, Amodeo et al. recently employed flexibility tests in which reward is presented in a probabilistic manner. Converging animal and human data suggest that performance in cognitive flexibility tests is inversely proportional to striatal Drd2 availability, and impaired by administration of Drd2 antagonists. The presence of impairments in striatal Drd2 neurotransmission in BTBR mice is therefore of great interest as it underscores a plausible substrate for these deficits of high translational relevance. This feature also provides a plausible mechanistic explanation for the lack of pharmacological effect of risperidone in BTBR mice. Risperidone is an FDA-approved serotonin 2A and Drd2 antagonist that has proven useful in mitigating irritability, hyperactivity and stereotypy in young ASD patients. However, recent pharmacological studies did not highlight any effect of risperidone in improving socialability or normalizing self-grooming in BTBR mice. This feature does not necessarily undermine the translational (and predictive) relevance of BTBR mice to model idiopathic ASD for two reasons. First, risperidone is not used to generally treat all forms of ASD, but only those characterized by pronounced irritability, aggression or self-injurious behaviour. Because BTBR mice generally show very low levels of aggression and do not develop any apparent health problems related to their high self-grooming, they do not appear to model self-injurious behaviour or other components of irritability relevant to risperidone-treated patient populations. Moreover, the clinical benefits of risperidone in improving social or communication skills exhibit a considerable variability across studies and patient populations. Our results, together with the presence of altered reward processing in ASD and variable variability, abnormally low sensitivity of measure of Drd2 receptor function across ASD populations, a hypothesis that could be assessed by correlating clinical response to risperidone with Drd2 function/availability.

Interestingly, our fMRI data in BTBR mice revealed a possible dysregulation of additional, nonstriatal DA targets/circuits whose contribution remains, at present, not known. By means of example, fMRI mapping highlighted reduced DA-mediated responsivity in widespread forebrain areas including the hippocampus, the posterior somatosensory cortices and the medial prefrontal cortex. The lack of fMRI responses in areas characterized by high Drd1 and low (albeit nonnegligible) Drd2 density such as the medial prefrontal cortex was unexpected, as pharmacological and behavioural studies demonstrated comparable striatal
DrD1 reactivity in BTBR and B6 mice. The reason for such a discrepant prefrontal responsivity in the two strains is not clear. The presence of altered tonic (or GBR12909-induced) prefrontal levels of DA, possibly involving DrD2-mediated disruption of long-range basolateral amygdala–prefrontal cortical pathways, could explain this observation. Alternatively, imbalances in DrD2-like receptor subtypes that exhibit low-level expression in frontal areas, such as the DrD4, or regional differences in extrastriatal DrD1 receptors’ density, could have a role in mediating this interstrain difference. Further investigations are warranted to uncover the origin and assess the translational significance of these findings.

In conclusion, we describe dysfunctional DrD2 neurotransmission and blunted mesolimbic reactivity in BTBR mice, a finding that could have a role in the social deficits exhibited by these mice, and that is consistent with emerging preclinical and clinical evidence pointing at a putative pathophysiological contribution of altered dopaminergic reactivity in neurodevelopmental disorders characterized by impaired social function, like ASD.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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