

## 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 responsiveness in BTBR  $T^+ Itpr3^{tf}/J$  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.<sup>1</sup> The neurotransmitter dopamine (DA) has a pivotal modulatory contribution on executive functions, learning, reward and emotional processing,<sup>2,3</sup> all of which are impaired in ASD patients.<sup>4–6</sup> 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 expressions, spoken language and gestures.<sup>7,8</sup> Moreover, stimulus–reward association learning appears to be impaired in young and adult ASD patients, a deficit that correlates with clinical symptoms of social dysfunction.<sup>9,10</sup> Importantly, functional magnetic

resonance imaging (fMRI) studies have revealed blunted activation of the ventral striatum in individuals with ASD when processing either social or monetary rewards.<sup>11–13</sup> Consistent with these findings, alterations in genes encoding DA transporter (DAT<sup>14</sup>), receptors<sup>15,16</sup> and enzymes involved in DA metabolism have been recently linked to ASD.<sup>17</sup> Taken together, these initial studies highlight a reduced responsiveness 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.<sup>18</sup> 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.<sup>19</sup> Recent behavioural screenings have identified the inbred BTBR  $T^+ Itpr3^{tf}/J$  (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.<sup>20–22</sup> In an attempt to unravel the role and molecular

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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.<sup>23</sup> 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.<sup>24–26</sup> Briefly, mice were anaesthetized with isoflurane, intubated and artificially ventilated. A femoral artery was cannulated for compound administration, blood pressure monitoring, infusion of paralyzing agent and terminal blood gas sampling ( $p_a\text{CO}_2$  and  $p_a\text{O}_2$ ). Mean  $p_a\text{CO}_2$  and  $p_a\text{O}_2$  levels recorded in the two groups at the end of the experiment were  $25 \pm 1$  and  $23 \pm 2$  mm Hg, and  $283 \pm 22$  and  $287 \pm 6$ , respectively. No statistically significant intergroup difference in arterial blood gases was observed ( $P > 0.22$ , all comparisons, Student's *t*-test). fMRI data were acquired on a 7T Pharmascan (Bruker, Ettlingen, Germany) as recently described.<sup>24,27</sup> 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.<sup>28</sup> Co-centred 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: (a)  $\text{TR}_{\text{eff}} = 3000$  ms,  $\text{TE}_{\text{eff}} = 38$  ms, rapid acquisition relaxation-enhanced factor 8, FOV 40 mm,  $100 \times 100 \times 500$   $\mu\text{m}$  resolution (b) FLASH:  $\text{TR} = 288$  ms,  $\text{TE} = 3.1$  ms,  $\alpha = 30^\circ$ ; FOV 40 mm,  $180 \times 180 \times 600$   $\mu\text{m}$  resolution,  $\text{dt} = 60$  s,  $\text{Nr} = 50$ , corresponding to 50 min total acquisition time. Time series were sensitized to reflect alterations in cerebral blood volume (CBV) using  $5 \mu\text{l g}^{-1}$  of blood-pool contrast agent (Molday Ion, Biopal, Worcester, MA, USA). Fifteen minutes later each subject received an intraarterial injection of GBR-12909 dihydrochloride ( $5 \text{ mg kg}^{-1}$ ). A group of B6 mice challenged with vehicle (saline,  $n = 6$ ) served as baseline rCBV reference for the effect of GBR 12909. The dose of GBR-12909 was selected based on behavioural studies<sup>29</sup> and pilot fMRI tests performed in the lab. The fMRI response to GBR-12909 was mapped and quantified as previously described.<sup>24,27,30,31</sup> Briefly, fMRI time series were spatially normalized to a common reference space<sup>25</sup> and signal intensity changes were converted into fractional rCBV changes. rCBV time series before (8 min) and after drug or vehicle injections (25 min) were calculated and voxel-wise group statistics was performed using FEAT Version 5.63 with 0.5 mm spatial smoothing and using an input function that captured the gradual increase in rCBV signal observed upon GBR-12909 injection. Group comparisons were carried out using multilevel Bayesian inference, a *Z*-threshold  $> 1.6$  and a corrected cluster significance threshold of  $P = 0.001$ .

### Constant potential amperometry

Preparation and maintenance of corticostriatal slices have been previously described elsewhere.<sup>32,33</sup> Briefly, mice were anaesthetized with chloral hydrate and killed by decapitation. The brain was rapidly removed and mounted on an agar block submerged in cold artificial cerebrospinal fluid

(aCSF). Horizontal 350- $\mu\text{m}$  thick slices were maintained in artificial cerebrospinal fluid at  $32 \pm 0.5^\circ\text{C}$  for 45 min before recordings. A bipolar Ni/Cr insulated stimulating electrode and a carbon fibre electrode were gently positioned into the dorsal striatum, with an imposed voltage of 0.55 V.<sup>33</sup> Electrode calibration was performed at the end of each experiment in artificial cerebrospinal fluid containing DA ( $300 \text{ nM} - 3 \mu\text{M}$ ). Stimulations were carried out employing a single rectangular electrical pulse (80–500- $\mu\text{A}$ , 20–40  $\mu\text{s}$  duration) every 3–5 min. Once the DA-induced currents reached stability (typically after 5–6 successive stimulations), drugs were superfused onto the striatal slice. GBR 12909 and quinpirole were tested at concentrations of  $5 \mu\text{M}$  (30 min) and  $0.1 \mu\text{M}$  (5 min), respectively. GBR 12909 and quinpirole were tested in two separate studies (GBR 12909:  $n = 9$  B6 and  $n = 11$  BTBR; quinpirole:  $n = 9$  B6 and  $n = 9$  BTBR). No oxidation currents were detected when stimulations were performed at 0V, thus corroborating the specificity of the evoked dopaminergic response measured. Electric signals were digitized, converted in  $\mu\text{M}$  values. Statistical comparisons were performed with the Student's *t*-test and results were expressed as mean  $\pm$  s.e.m.

### Motor response to dopaminergic drugs

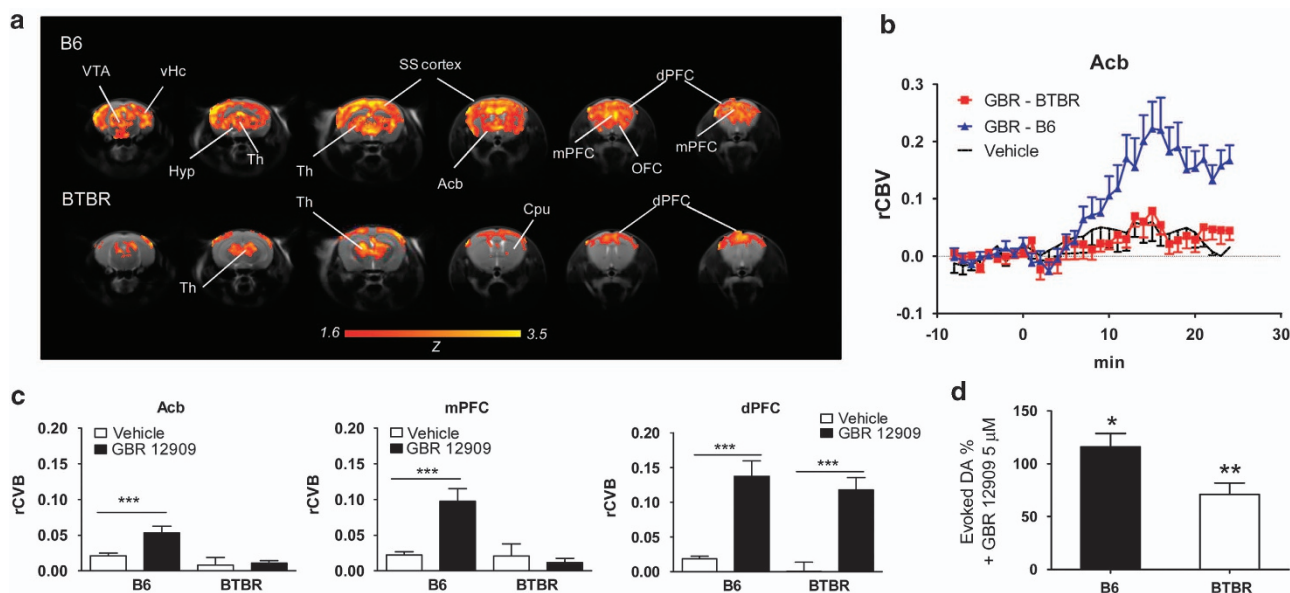
Motor responses to dopaminergic drugs were analysed as previously described.<sup>33</sup> To measure the effect induced by motor-stimulant drugs (for example, GBR 12909 and SKF 81297), mice were habituated to novel home-cages ( $35 \times 25 \times 30$  cm) for 60 min before the test session. GBR 12909 was administered intraperitoneally (i.p.;  $10 \text{ mg kg}^{-1}$ ; B6,  $n = 5$ ; BTBR,  $n = 6$ ;  $20 \text{ mg kg}^{-1}$ ; B6,  $n = 6$ ; BTBR,  $n = 6$ ). Two independent vehicle-treated groups were used to test the effect of  $10 \text{ mg kg}^{-1}$  (B6,  $n = 6$ ; BTBR,  $n = 6$ ) or  $20 \text{ mg kg}^{-1}$  GBR 12909 (B6,  $n = 5$ ; BTBR,  $n = 6$ ). Locomotor activity was recorded for 30 min. SKF 81297 was administered i.p. ( $1.25 \text{ mg kg}^{-1}$ ; B6,  $n = 7$ ; BTBR,  $n = 6$ ;  $2.5 \text{ mg kg}^{-1}$ ; B6,  $n = 6$ ; BTBR,  $n = 6$ ) and its effects were compared with a vehicle group (B6,  $n = 6$ ; BTBR,  $n = 6$ ). To assess the motor suppression effect induced by quinpirole, mice were analysed for 30 min in novel test cages after i.p. injection of this drug at a dose of  $0.25 \text{ mg kg}^{-1}$  (B6,  $n = 7$ ; BTBR,  $n = 6$ ), or vehicle (B6,  $n = 7$ ; BTBR,  $n = 6$ ). Motor activity was recorded by a computerized video tracking system (Videotrack, Viewpoint S.A., Champagne au Mont d'Or, France). GBR 12909-induced motor activity was analysed within strain by two-way analysis of variance (ANOVA; treatment  $\times$  time) with repeated measures. Total locomotion produced by GBR 12909, SKF 81297 or quinpirole treatment, was analysed by two-way ANOVA (strain  $\times$  treatment), followed by one-way ANOVA (treatment). For the analysis of SKF 81297-induced motor activity, one-way ANOVA was followed by Fisher's *post hoc* comparison. Statistical analyses were performed with StatView software (version 5.0.1.0; SAS, Cary, NC, USA).

### Haloperidol-induced catalepsy

Catalepsy was assessed using the bar test, as previously reported.<sup>34</sup> Sixty minutes before treatment, mice were singly placed in a novel cage to familiarize with the new environment. Animals were subsequently treated i.p. with  $0.5 \text{ mg kg}^{-1}$  haloperidol (B6,  $n = 4$ ; BTBR,  $n = 6$ ) or vehicle ( $n = 4$  per strain) and catalepsy measured after 120 min by gently placing their front limbs over a 4-cm-high horizontal bar. The intensity of catalepsy was assessed by measuring the time each mouse remained in this position, with the limbs completely immobile for a maximum of 300 s. Haloperidol-induced catalepsy was analysed by two-way ANOVA (strain  $\times$  treatment), followed by one-way ANOVA (treatment). Statistical analysis was performed with StatView software (version 5.0.1.0; SAS Institute).

### Western blotting

Western blotting was performed as previously described.<sup>33</sup> Briefly, mice were administered  $5 \text{ mg kg}^{-1}$  of SKF 81297,  $0.5 \text{ mg kg}^{-1}$  haloperidol or their respective vehicles i.p. and killed by decapitation 30 min later. The heads 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* reflects the *in vivo* situation.<sup>35,36</sup> Aliquots (2  $\mu\text{l}$ ) 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, Chalfont St Giles, UK). Membranes were immunoblotted overnight using selective antibodies



**Figure 1.** Compromised DA-mediated fMRI reactivity in BTBR mice. **(a)** fMRI activation (relative cerebral blood volume—rCBV) elicited by acute intravenous administration of the DA reuptake inhibitor in B6 (top,  $n = 10$ ) and BTBR mice (bottom,  $n = 10$ ). Yellow/orange areas indicate regions in which the drug elicited a significant fMRI response with respect to a control vehicle (saline) injection ( $Z$ -score  $> 1.6$ ,  $P < 0.001$ , cluster corrected). Note the lack of GBR 12909-induced fMRI response in several forebrain regions of BTBR mice, with a prominent involvement of ventro-striatal mesolimbic hippocampus and posterior parietal cortices. **(b)** Temporal evolution of the fMRI signal (CBV) in the nucleus accumbens upon injection of GBR 12909 in BTBR and control B6 mice. Basal fMRI signal in vehicle-administered B6 subjects is reported for reference ( $n = 6$ ). Drugs were administered at time 0. **(c)** Quantification of the fMRI changes produced by GBR 12909 administrations in B6 and BTBR mice ( $AUC_{0-20 \text{ min}}$  post injection). Basal fMRI signal in vehicle-administered B6 subjects is reported for reference (\*\*\* $P < 0.001$  vs vehicle,  $t$ -test) **(d)** DA-evoked CPA responses in the striatum of BTBR and B6 mice on co-incubation with GBR 12909 ( $5 \mu\text{M}$ ). The drug induced a robust elevation of extracellular DA levels (expressed as percentage of baseline) in both strains (\* $P < 0.05$ , \* $P < 0.01$ , vs baseline, paired  $t$ -test). AUC, area under the curve; CBV, cerebral blood volume; CPA, constant potential amperometry; Cpu, caudate putamen; DA, dopamine; dPFC, dorsal prefrontal cortex; fMRI, functional magnetic resonance imaging; Hyp, hypothalamus; mPFC, medial prefrontal cortex; OFC, orbito-frontal cortex; SS, somatosensory cortex; Th, thalamus; vHc, ventral hippocampus; VTA, ventral tegmental area.

against P-Ser845-GluR1 (1:500, PhosphoSolutions), P-Ser40-TH (1:1000, Millipore Biotechnology, Billerica, MA, USA) and P-Ser235/236-S6 (1:500, Cell Signaling Technology, Danvers, MA, USA). Blots were then incubated in horseradish peroxidase-conjugated secondary antibodies and target proteins were visualized by ECL detection (Thermo Scientific, Rockford, IL, USA), followed by quantification using Quantity One software (Bio-Rad). Antibodies against GluR1 (1:1000; Millipore Biotechnology), TH (1:1000; Millipore Biotechnology) and S6 (1:1000, Cell Signaling) that are not phosphorylation state specific were used to estimate the total amount of proteins. All optical density values were normalized to glyceraldehyde-3-phosphate dehydrogenase for variation in loading and transfer. Normalized values were then averaged and used as dependent variable. SKF 81297-induced P-Ser845-GluR1 levels were analysed by Student's  $t$ -test; results from haloperidol-induced P-Ser40-TH, P-Ser845-GluR1 and P-Ser235/236-S6 levels were analysed by two-way ANOVA (strain  $\times$  treatment), followed by one-way ANOVA (treatment).

#### In situ hybridization

Mice were killed and their brains quickly dissected, embedded in Tissue Tek (Sakura) and frozen at  $80^\circ\text{C}$ . Fourteen-micrometre cryostat sections were cut in the coronal plane and *in situ* hybridization analyses were performed according to protocols previously described.<sup>37</sup> Coronal sections along the rostro-caudal extent of striatum and substantia nigra from both B6 and BTBR male adult mice were hybridized with <sup>35</sup>S-labelled antisense riboprobes (*Drd1*, 1.3 Kb: B6  $n = 4$ , BTBR  $n = 7$ ; *Drd2*, 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.

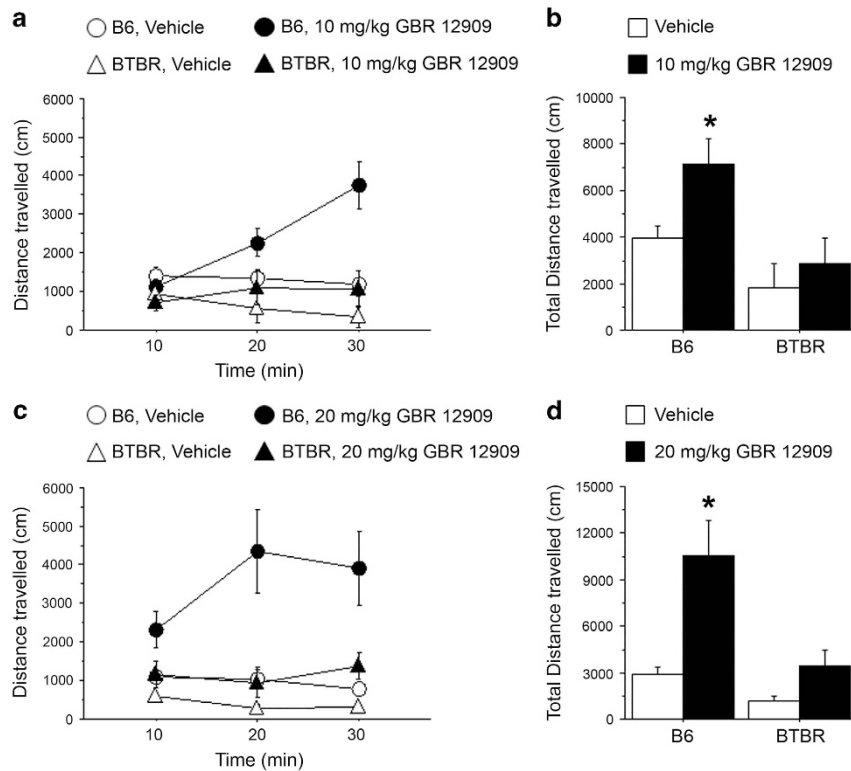
#### Image analysis and quantification

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-elicited fMRI responses have been demonstrated to serve as a plausible surrogate for mesolimbic dopaminergic reactivity in rodent species.<sup>38–40</sup> As human fMRI studies reported reduced reactivity of ventro-striatal DA terminals in individuals with ASD,<sup>11–13</sup> we employed pharmacological fMRI<sup>24,41</sup> 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 agents,<sup>24,39,42</sup> 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



**Figure 2.** Blunted GBR 12909-induced motor activity in BTBR mice. Motor activity induced by 10 mg kg<sup>-1</sup> and (a, b) and 20 mg kg<sup>-1</sup> (c, d) GBR 12909 in B6 (10 mg kg<sup>-1</sup>, n = 5; 20 mg kg<sup>-1</sup>, n = 6) and BTBR mice (n = 6 per dose). Two independent vehicle-treated groups were used to test the effect of 10 mg kg<sup>-1</sup> (n = 6 per strain) and 20 mg kg<sup>-1</sup> 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<sup>43</sup> (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<sup>44,45</sup> 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 additional mechanism, besides a slightly reduced DAT efficiency or tonic DA activity, which may contribute to the blunted dopaminergic responsiveness observed with fMRI.

Blunted GBR 12909-induced horizontal motor stimulation in BTBR mice

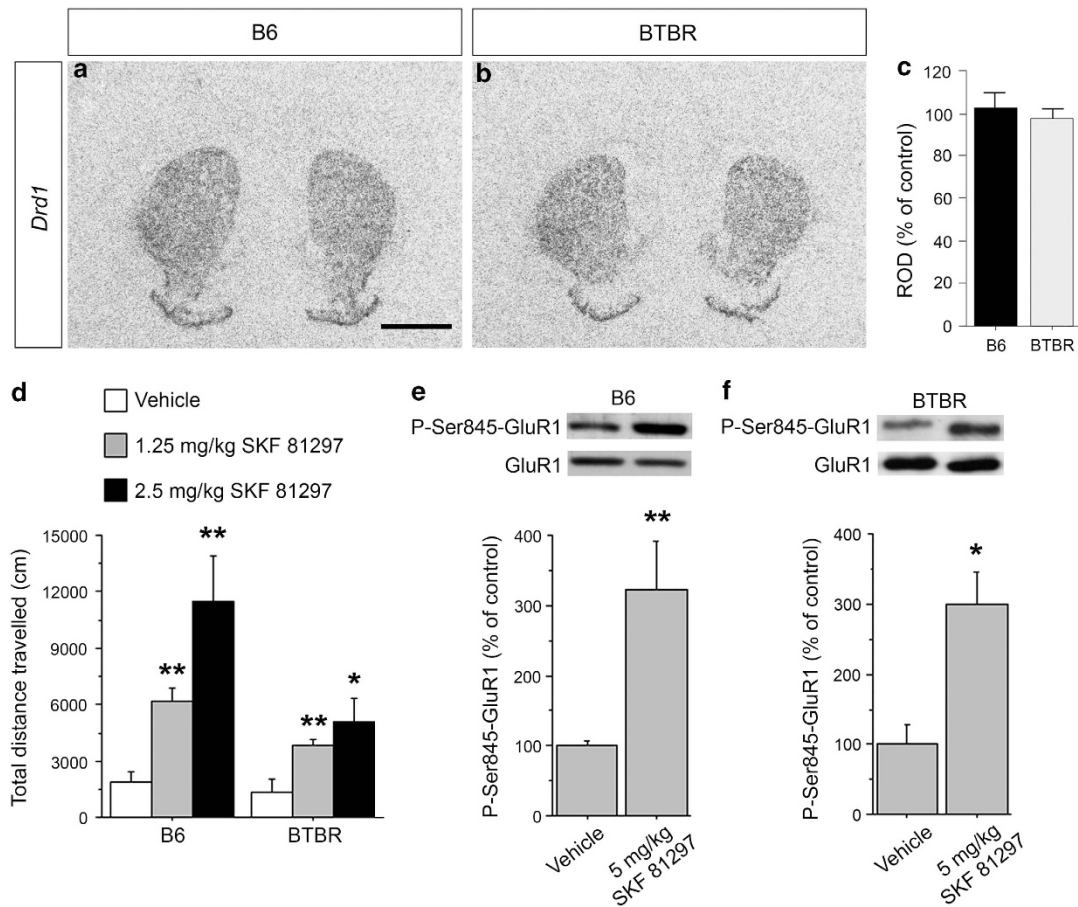
To assess the behavioural relevance of the alterations found with fMRI, we evaluated the stimulant *in vivo* effect of GBR 12909 on

the horizontal motor activity of BTBR and B6 mice, a behaviour dependent on DA neurotransmission in the nucleus accumbens.<sup>46,47</sup> Consistent with the imaging results, both the GBR 12909 doses tested (10 and 20 mg kg<sup>-1</sup> 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<sup>-1</sup>, B6: F<sub>(1,18)</sub> = 7.222, P = 0.0249; BTBR: F<sub>(1,20)</sub> = 0.482, P = 0.5031; Figure 2c; 20 mg kg<sup>-1</sup>, B6: F<sub>(1,18)</sub> = 9.313, P = 0.0138; BTBR: F<sub>(1,20)</sub> = 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<sup>-1</sup>: B6 F<sub>(1,9)</sub> = 7.222, P = 0.0249; BTBR, F<sub>(1,10)</sub> = 0.482, P = 0.5031; Figure 2d, 20 mg kg<sup>-1</sup>: B6, F<sub>(1,9)</sub> = 9.313, P = 0.0138; BTBR: F<sub>(1,10)</sub> = 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.<sup>48</sup> Drd1 mRNA expression levels were measured in the striatum of BTBR and B6 mice using *in situ* hybridization with a <sup>35</sup>S-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



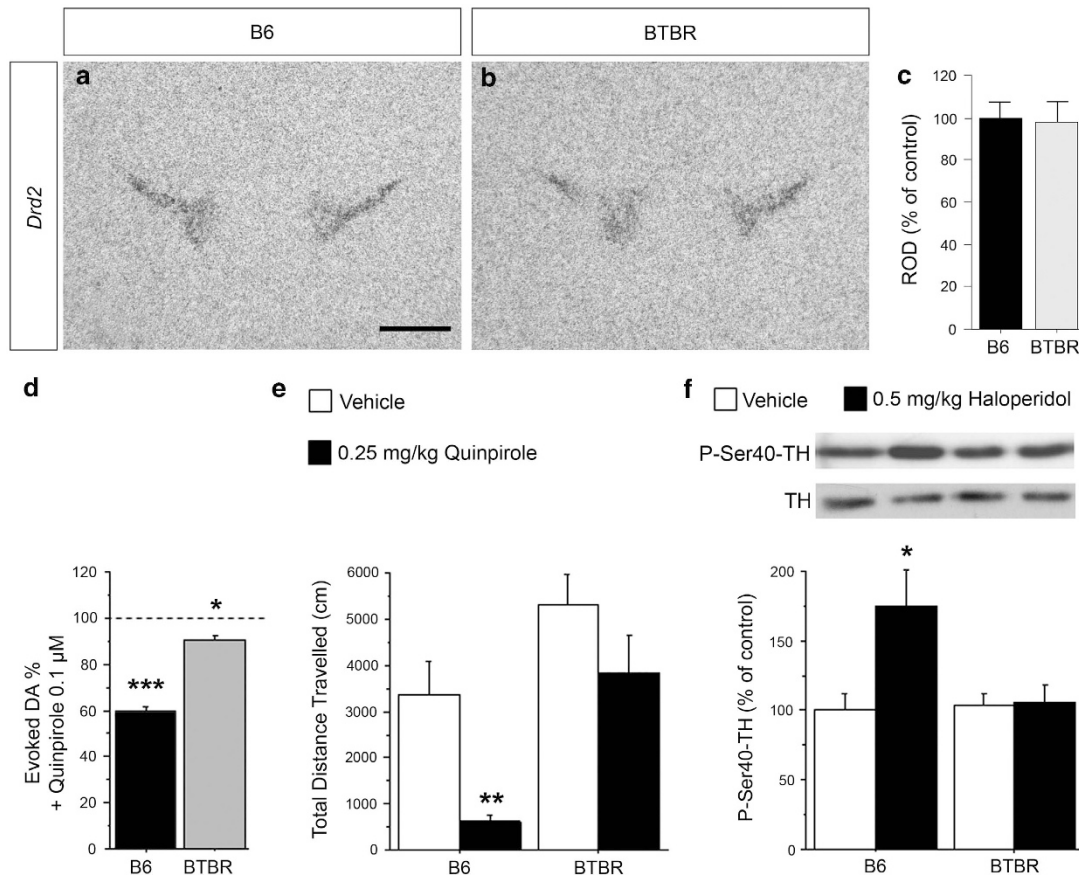
**Figure 3.** Preserved *Drd1*-mediated motor stimulation and signalling in BTBR mice. Representative images of coronal sections showing *Drd1* mRNA expression in the striatum of B6 (a) and BTBR (b) animals (scale bar, 1.5 mm). (c) Histogram showing *Drd1* mRNA levels in BTBR animals ( $n = 7$ ) obtained 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<sup>-1</sup> SKF 81297 in B6 (vehicle,  $n = 6$ ; 1.25 mg kg<sup>-1</sup>,  $n = 7$ ; 2.5 mg kg<sup>-1</sup>,  $n = 6$ ) and BTBR animals (vehicle,  $n = 5$ ; 1.25 mg kg<sup>-1</sup>,  $n = 6$ ; 2.5 mg kg<sup>-1</sup>,  $n = 6$ ). 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<sup>-1</sup> 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  $\pm$  s.e.m.

*in vivo*, by measuring motor activity elicited by selective agonist SKF 81297 administration (1.25 and 2.5 mg kg<sup>-1</sup>), as previously reported<sup>49</sup> (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.<sup>50,51</sup> 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,<sup>48</sup> as well as in reward processing and

stimulus discrimination,<sup>52</sup> a contribution that is believed to result from the opposing action of presynaptic and postsynaptic *Drd2* pools.<sup>53</sup> 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.<sup>48,49</sup> 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.<sup>49,54</sup> 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.<sup>49,54</sup> However, quinpirole-induced DA release inhibition in BTBR mice was only marginal, and greatly attenuated with respect to the one observed in B6 mice ( $90.5 \pm 2.96\%$  vs  $60.3 \pm 3.56\%$ ,  $n = 9$ , respectively;  $P = 0.00002$ , Student's *t*-test). In line with the dramatic *Drd2*-related differences



**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 overflow in both strains (B6,  $60 \pm 3.6\%$  of baseline,  $P < 0.00001$ , BTBR  $90.6 \pm 3.0\%$  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  $0.25 \text{ mg kg}^{-1}$  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  $0.5 \text{ mg kg}^{-1}$  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  $\pm$  s.e.m. DA, dopamine.

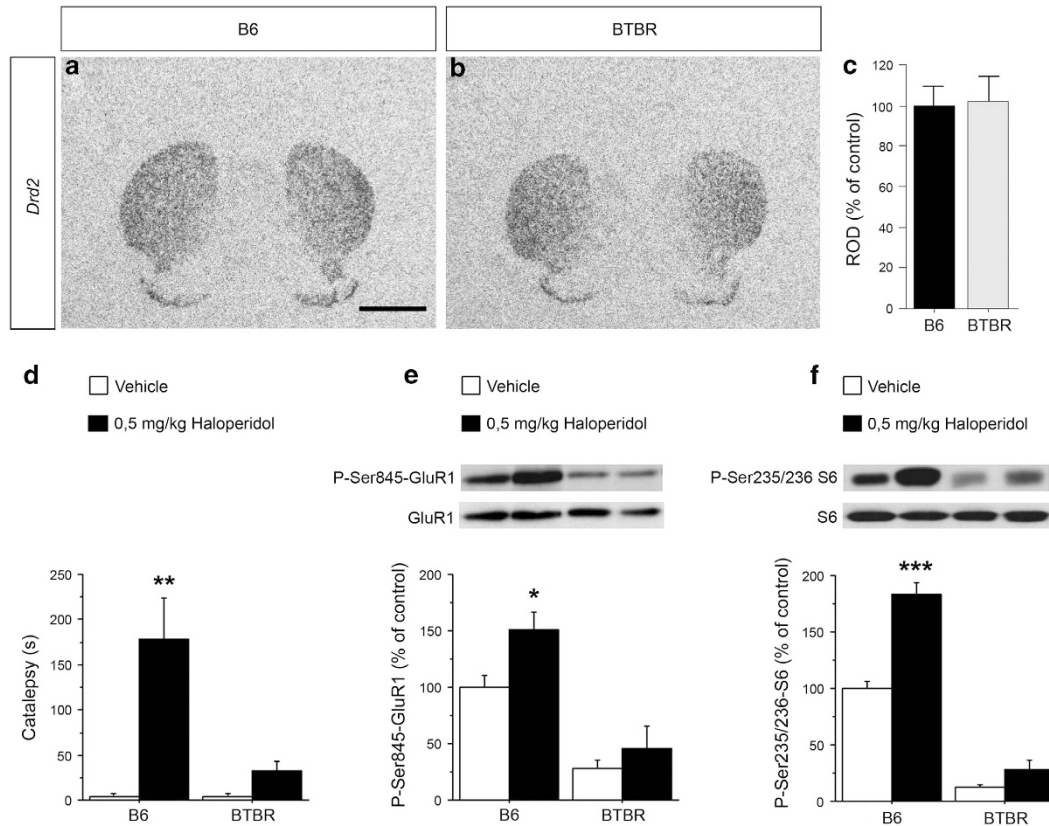
found between strains, quinpirole administration robustly inhibited locomotor activity in B6 mice, but failed to elicit significant motor responses in BTBR mice (one-way ANOVA: B6:  $F_{(1,12)} = 14.725$ ,  $P = 0.0024$ ; BTBR:  $F_{(1,10)} = 1.992$ ,  $P = 0.1885$ ; Figure 4e).

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,<sup>55,56</sup> blockade of presynaptic *Drd2* by haloperidol ( $0.5 \text{ mg kg}^{-1}$ ) significantly increases striatal P-Ser40-TH levels in B6 mice ( $F_{(1,7)} = 8.897$ ,  $P = 0.0204$ ). Interestingly, haloperidol failed to produce appreciable striatal P-Ser40-TH alterations in BTBR mice ( $F_{(1,7)} = 0.021$ ,  $P = 0.8893$ ; Figure 4f). Collectively, these results highlight remarkably blunted presynaptic *Drd2* signalling and function in BTBR animals.

#### 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.<sup>49</sup> As expected, robust haloperidol-induced catalepsy was observed in B6 mice (one-way ANOVA:  $F_{(1,6)} = 14.967$ ,  $P = 0.0083$ ; 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 hypofunctionality 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 adenylate 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).<sup>34,57</sup> As expected, a significant increase in haloperidol-dependent striatal P-Ser845-GluR1 levels was observed in B6 mice (one-way ANOVA:  $F_{(1,9)} = 7.732$ ,  $P = 0.0214$ ), 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<sup>58</sup> 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



**Figure 5.** Impaired *Drd2*-mediated postsynaptic reactivity and signalling in BTBR mice. Representative coronal sections showing *Drd2* mRNA expression in the striatum of B6 (a) and BTBR mice (b). The histogram (c) illustrates *Drd2* mRNA levels in BTBR ( $n=7$ ) after densitometric quantification of autoradiograms. Values are expressed as percentage variation with respect to B6 control mice ( $n=4$ ). (d) Cataleptic response induced by  $0.5 \text{ mg kg}^{-1}$  of haloperidol in B6 ( $n=4$ ; vehicle,  $n=4$ ) and BTBR animals ( $0.5 \text{ mg kg}^{-1}$ ,  $n=6$ ; vehicle,  $n=4$ ). Striatal PKA-dependent P-Ser845-GluR1 (e) and P-Ser235/236-S6 (f) protein levels in B6 and BTBR mice treated with  $0.5 \text{ mg kg}^{-1}$  haloperidol (P-Ser845-GluR1: B6,  $n=5$ ; BTBR,  $n=6$ ; P-Ser235/236-S6:  $n=6$  each strain) or vehicle (P-Ser845-GluR1: B6,  $n=5$ ; BTBR,  $n=4$ ; P-Ser235/236-S6:  $n=6$  each strain). The top panels in e and f show representative blots comparing the different strains and treatment groups. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ , compared with vehicle-treated group within strain (one-way analysis of variance). All values are expressed as mean  $\pm$  s.e.m. PKA, protein kinase A.

presence of an impaired A2aR intracellular signalling in BTBR mice. This hypothesis was tested by measuring the changes of phosphorylation levels of ribosomal protein S6 on Ser-235/236 in response of haloperidol ( $0.5 \text{ mg kg}^{-1}$ ) administration, a selective A2aR/PKA-related target associated to *Drd2* blockade.<sup>59</sup> Statistical analysis of the P-Ser-235/236-S6 levels (Figure 5f) revealed a blunted adenosine A2aR/PKA mediated signalling in the striatum of BTBR mice (one-way ANOVA:  $F_{(1,10)} = 2.784$ ,  $P = 0.1262$ ) whereas a significant phosphorylation increase was found in B6 animals ( $F_{(1,10)} = 45.079$ ,  $P < 0.0001$ ). Collectively these data corroborate the presence of profoundly dysfunctional D2dr- and A2aR-mediated neurotransmission in BTBR, and provide a plausible mechanistic explanation for the blunted striatal fMRI responses observed with GBR 12909 in these animals.

## 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.<sup>60</sup> 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<sup>22,61,62</sup> and communication deficits,<sup>63,64</sup> together with increased repetitive behaviours,<sup>20,65</sup> 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.<sup>66</sup> 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 analogously reduced functional responses in the reward circuits of individuals diagnosed with ASD when processing either social and monetary rewards.<sup>11–13</sup> 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<sup>17,67</sup> reuptake,<sup>14</sup> as well as all in the three major components of the D2-like DA receptor family<sup>15,16</sup> (that is, *Drd2*, *Drd3* and *Drd4* (ref. 68–70)) have been recently associated to ASD. Similarly, variation in A2aR have also been recently associated to ASD symptoms severity,<sup>70</sup> a finding that has been corroborated by recent *in silico* analyses of the molecular pathways regulated by

DA and involved in ASD.<sup>17</sup> The ability of drugs targeting Drd2 such as aripiprazole and risperidone to improve, albeit marginally, core ASD symptoms,<sup>12,71,72</sup> together with key contribution of Drd2 receptor in mediating social behaviour in humans<sup>73,74</sup> and pair-bonding animals, such as the prairie vole (*Microtus ochrogaster*),<sup>75</sup> 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. *Shank3B*-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.<sup>76</sup> 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 often associated to ASD symptoms, exhibit profoundly impaired striatal (mostly Drd1-mediated) DA neurotransmission, together with altered motor skills.<sup>77,78</sup> Similarly, mice carrying methyl-CpG-binding protein 2 (MECP2) mutations, an alteration that causes inheritable ASD-like symptoms show substantial reductions in striatal DA<sup>79</sup> 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 neurobehavioral 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.<sup>80</sup> 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.<sup>27</sup> 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 grey matter abnormalities), together with translationally relevant features, including the presence of reduced fronto-cortical perfusion, and acallosal development, two alterations that have been previously described in ASD subpopulations.<sup>81–83</sup> A recent study from Han *et al.*<sup>84</sup> 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,<sup>85</sup> 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 Barbas<sup>86</sup>). 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.<sup>8,10–13,87</sup> 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.<sup>13,18</sup> 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<sup>88,89</sup> suggests the presence of a general reinforcement 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,<sup>90</sup> 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,<sup>62,91</sup> 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.*<sup>91</sup> recently employed a behavioural flexibility test 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.<sup>92</sup> 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.<sup>93</sup> However, recent pharmacological studies did not highlight any effect of risperidone in improving sociability or normalizing self-grooming in BTBR mice.<sup>21,94</sup> 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,<sup>21</sup> 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.<sup>93,95</sup> Our results, together with the presence of altered reward processing in ASD populations suggest that this variability can reflect differential reactivity 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<sup>96</sup>) 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 GBR-12909-induced) prefrontal levels of DA, possibly involving Drd2-mediated dysregulation of long-range basolateral amygdala–prefrontal cortical pathways<sup>97</sup> could explain this observation. Alternatively, imbalances in Drd2-like receptor subtypes that exhibit low-level expression in frontal areas, such as the Drd4,<sup>98</sup> or regional differences in extrastriatal Drd1 receptors' density, could have a role in mediating this inter-strain 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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