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$GABA_B$ Receptor Agonist R-Baclofen Reverses Social Deficits and Reduces Repetitive Behavior in Two Mouse Models of Autism

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Autism spectrum disorder (ASD) is diagnosed by two core behavioral criteria, unusual reciprocal social interactions and communication, and stereotyped, repetitive behaviors with restricted interests. Excitatory/inhibitory imbalance is a prominent hypothesis for the etiology of autism. The selective $GABA_B$ receptor agonist R-baclofen previously reversed social deficits and reduced repetitive behaviors in a mouse model of Fragile X syndrome, and Arbaclofen improved some clinical symptoms in some Fragile X and ASD patients. To evaluate R-baclofen in a broader range of mouse models of ASD, we tested both the R-baclofen enantiomer and the less potent S-baclofen enantiomer in two inbred strains of mice that display low sociability and/or high repetitive or stereotyped behaviors. R-baclofen treatment reversed social approach deficits in BTBR T+ Itpr3tf/J (BTBR), reduced repetitive self-grooming and high marble burying scores in BTBR, and reduced stereotyped jumping in C58/J (C58), at nonsedating doses. S-baclofen produced minimal effects at the same doses. These findings encourage investigations of R-baclofen in other preclinical model systems. Additional clinical studies may be warranted to further evaluate the hypothesis that the $GABA_B$ receptor represents a promising pharmacological target for treating appropriately stratified subsets of individuals with ASD.

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INTRODUCTION

Autism is a neurodevelopmental disorder diagnosed by deficits in two core behavioral domains: (1) unusual reciprocal social interactions and impaired social communication, and (2) stereotyped, repetitive behaviors with restricted interests. One prominent hypothesis, consistent with comorbid seizures and anxiety in autism spectrum disorder (ASD), is an imbalance of excitatory/inhibitory neurotransmission (Bourgeron, 2009; Geschwind and Levitt, 2007; Gogolla *et al*, 2009; LeBlanc and Fagiolini, 2011; Rubenstein and Merzenich, 2003). Reduced GABAergic neurotransmission and fewer GABAergic interneurons in mouse models with targeted mutations in risk genes for ASD, *in vivo* spectroscopy, and electrophysiological biomarkers of lower GABA activity in affected individuals support the hypothesis that elevating GABAergic activity may offer a therapeutic target for treating some components of ASD (Blatt and Fatemi, 2011; Eagleson *et al*, 2010; Gaetz *et al*, 2014; Han *et al*, 2012, 2014; Harada *et al*, 2011; Mori *et al*, 2012; Oberman, 2012; Sgado *et al*, 2013). One therapeutic strategy targeting the $GABA_B$ receptor subtype evaluated STX209 (Arbaclofen), the selective $GABA_B$ enantiomer, in clinical trials for Fragile X syndrome, Fragile X with an autism diagnosis, and ASD. A phase 2 clinical trial detected improvements on Aberrant Behavior Checklist (ABC)-Social Avoidance scores (Berry-Kravis *et al*, 2012) in Fragile X patients. An open-label trial of STX209 in patients with ASD not associated with Fragile X showed beneficial effects on ABC-irritability social withdrawal scale, and on the social responsiveness scale (Erickson *et al*, 2014).

Baclofen (β-p-chlorophenyl-GABA) is a GABA analog that acts at the $GABA_B$ receptor and reduces glutamate release (Bowery, 1993; Bowery *et al*, 1983; Henderson *et al*, 2012; Kang *et al*, 2012). STX209 and racemic baclofen administered to *Fmr1* knockout mice restored protein synthesis, corrected their increased dendritic spine density, and reduced audiogenic seizures (Henderson *et al*, 2012).

BTBR T+ Itpr3tf/J (BTBR) is an inbred strain of mice that exhibits robust, well-replicated impairments in social interactions, minimal vocalizations in social settings, high levels of repetitive self-grooming and digging, and cognitive deficits (Amodeo *et al*, 2012; Bolivar *et al*, 2007; Chadman, 2011; Gould *et al*, 2011; McFarlane *et al*, 2008; McTighe *et al*, 2013; Pearson *et al*, 2011, 2012; Pobbe *et al*, 2010; Scattoni *et al*, 2008; Silverman *et al*, 2013a, b; Yang *et al*, 2007b). Reduced spontaneous GABAergic neurotransmission in BTBR was recently reported (Gogolla *et al*, 2014; Han *et al*, 2014). C58/J (C58) is an independent inbred strain of mice that displays robust, well-replicated stereotyped vertical

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jumping and responds to mGluR5 antagonist treatment (Silverman *et al*, 2012), although endogenous GABA systems have not yet been explored in this strain.

We employed BTBR and C58 to test the hypothesis that R-baclofen could ameliorate autism-relevant behaviors in mouse models of autism. Rescue of social approach in BTBR, and reductions in repetitive behaviors in both BTBR and C58, were detected after acute, systemic R-baclofen treatment. In contrast, the less active enantiomer S-baclofen was less potent or inactive in both strains, using dose comparisons consistent with the literature (Bowery *et al*, 1983; Drew *et al*, 1984; Paredes and Agmo, 1989). These preclinical results lend support to further investigations of $GABA_B$ agonists as a pharmacological target for treating core diagnostic symptoms of ASD.

MATERIALS AND METHODS

Mice

Breeding pairs of C57BL/6J (B6), BTBR T+ Itpr3tf/J (BTBR), C58/J (C58), and 129/SvImJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred as harem trios in a conventional mouse vivarium at the University of California Davis School of Medicine in Sacramento. After weaning, juveniles were housed by sex and strain in Tecniplast cages in groups of two to four per cage. Cages were housed in ventilated racks in a temperature-controlled (68–72 °F) and humidity-controlled (~25%) colony room, on a 12-h circadian cycle, lights on from 0700 to 1900 h. Standard rodent chow and tap water were available *ad libitum*. In addition to standard bedding, a Nestlet square, shredded brown paper, and a cardboard tube (Jonesville Corporation, Jonesville, MI) were provided in each cage. Previous studies in our laboratory documented no sex differences in either BTBR or B6 on sociability or self-grooming assays (McFarlane *et al*, 2008; Silverman *et al*, 2010a; Yang *et al*, 2007a, b). Therefore, male and female mice were used in all studies in approximately equal proportions. Behavioral testing arenas were cleaned with 70% ethanol between test subjects. At least 5 min between cleaning and the start of the next session was allowed for ethanol evaporation and odor dissipation. All procedures were conducted in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by UC Davis Institute Animal Care and Use Committee (Protocol no. 16839).

Drug Administration

R- and S-baclofen hydrochloride (Sigma Aldrich, St Louis, MO) were acutely administered intraperitoneally in a 10 ml/kg injection volume of 0.9% physiological saline vehicle in the first cohort of B6 and BTBR mice. In accordance with the literature on the relative *in vivo* potencies of the two enantiomers (Henderson *et al*, 2012; Paredes and Agmo, 1989), R-baclofen was tested at doses of 1.0, 3.0, and 5.0 mg/kg, whereas S-baclofen was tested at doses of 0.1, 1.0, 3.0, and 10.0 mg/kg. In accordance with t_{max} values and preliminary findings in open field locomotion (Supplementary Figure S6), compounds were administered 60 min before the start of each behavioral assay (Henderson *et al*, 2012). Experimental design was between-subjects, with a 1-week washout period. Each cohort 1 mouse received a randomized single acute dose of R-baclofen hydrochloride (1.0, 3.0, and 5.0 mg/kg) or vehicle and was tested on one task per week. An identical design was employed for the R-baclofen replication cohort 2. A third cohort of B6 and BTBR received a single acute dose of S-baclofen hydrochloride (0.1, 1.0, 3.0, and 10.0 mg/kg) or vehicle before testing on social approach. Cohort 3 was subsequently treated with only the two highest doses of S-baclofen or vehicle before testing on self-grooming and marble burying.

Behavioral Testing

Behavioral assessment of BTBR and of the control strain B6 that displays normal sociability and low repetitive behaviors was conducted at ages 8–12 weeks, body weights 25–35 g, during the light phase of the circadian cycle. Order of testing was open field locomotion (week 1), social approach (week 2), self-groom (week 3), and marble burying (week 4). A second cohort was used to conduct replication. A third cohort of BTBR and B6 mice was used for behavioral testing following low dose S-baclofen hydrochloride or saline. C58 mice were similarly tested for responses to both R- and S-baclofen. The behavioral task order for C58 was open field locomotion (week 1), observations of spontaneous vertical jumping and self-grooming (week 2), and social approach (week 3). This order of testing was designed to focus on the main phenotype of C58, high stereotyped jumping, based on our previous unpublished findings of normal sociability in C58. A second cohort of C58 mice was used to conduct a replication of repetitive jumping after R-baclofen treatment. A third cohort of C58 mice was used for evaluating effects of the two highest doses of S-baclofen. Drug doses and digital videotapes of the behavioral sessions were coded by an independent investigator to ensure that the raters were blind to the treatment condition.

Behavioral Scoring

Self-grooming in BTBR and stereotyped jumping in C58. Mice were scored for spontaneous self-grooming and jumping as previously described (Silverman *et al*, 2010a, 2012). Each mouse was individually placed into a standard mouse cage, illuminated at ~40 lux. Cages were empty to eliminate digging in the bedding, a potentially competing behavior. After a 10-min habituation period in the test cage, each mouse was scored by a trained observer uninformed of the drug treatment using Noldus Observer event recording (Noldus Observer 8.0XT, Leesburg, VA), using parameters of cumulative time spent grooming all body regions, or cumulative bouts of jumping, during a 10-min session.

Marble burying assay. Repetitive marble burying was measured as previously described (Henderson *et al*, 2012; Thomas *et al*, 2009, 2012). Twenty black glass marbles (15 mm in diameter) were arranged in a symmetrical 4 × 5-cm grid on top of 2–3 cm deep bedding in clean, standard mouse cages (27 × 16.5 × 12.5 cm) with a filter top lid. Each mouse was placed in the center of the cage for a 30-min exploration period, after which the number of marbles buried was tallied by the investigator. 'Buried' was

defined as >50% covered by bedding (Thomas *et al*, 2009). Testing was performed under dim light (~15 lux).

Open field locomotion. General exploratory locomotion in a novel open field environment was assayed as previously described (Silverman *et al*, 2010b; Flannery *et al*, 2014). Open field activity was considered an essential control for direct drug effects on physical activity, for example, sedation or hyperactivity (Silverman *et al*, 2010a, 2012, 2013a), that could confound the interpretation of results from the self-grooming, marble burying, and social approach tasks. The testing room was illuminated at ~40 lux.

Sociability. Social approach was tested in an automated three-chambered apparatus using methods similar to those previously described (McFarlane *et al*, 2008; Silverman *et al*, 2010b, 2011, 2012, 2013a; Yang *et al*, 2011). Newly automated Ethovision XT videotracking software (Version 9.0, Noldus Information Technologies, Leesburg, VA) and modified nonreflective materials for the chambers were employed to maximize throughput. The updated apparatus (40 cm × 60 cm × 23 cm) was a rectangular, three-chambered box made from matte white finished acrylic (P95 White, Tap Plastics, Sacramento, CA). Opaque retractable doors (12 cm × 33 cm) were designed to create optimal entryways between chambers (5 cm × 10 cm) while providing maximal manual division of compartments. Three zones, defined using the EthoVision XT software, detected time in each chamber for each phase of the assay. Zones were defined as the annulus extending 2 cm from each novel object or novel mouse enclosure (inverted wire cup, Galaxy Cup, Kitchen Plus, http://www.kitchenplus.com). Direction of the head, facing toward the cup enclosure, defined sniff time. A top-mounted infrared sensitive camera (Ikegami ICD-49, B&H Photo, New York, NY) was positioned directly above every two 3-chambered units. Infrared lighting (Nightvisionexperts.com) provided uniform, low-level illumination. The subject mouse was first contained in the center chamber for 10 min, then explored all three empty chambers during a 10 min habituation session, and then explored the three chambers containing a novel object in one side chamber and a novel mouse in the other side chamber. Lack of innate side preference was confirmed during the initial 10 min of habituation to the entire arena. Novel stimulus mice were 129Sv/ImJ, a relatively inactive strain, aged 10–14 weeks, and matched to the subject mice by sex. Number of entries into the side chambers served as a within-task control for levels of general exploratory locomotion. In addition to the automated Ethovision videotracking scoring method, a trained rater scored the same videos using Noldus Observer XT event coding software (Noldus Information Technologies). Direct comparison of scores obtained with automated videotracking *versus* human observation of videos is presented in Supplementary Figure S7.

Statistical Analysis

Repeated Measures ANOVA (~ paired *t*-test) was used to analyze three-chambered social approach data. Comparisons of time spent in the chamber with the novel mouse *versus* time spent in the chamber with the novel object were conducted within each drug treatment group and within each strain. Similarly, time sniffing the novel mouse *versus* time sniffing the novel object were compared within each drug treatment group and within each strain. This statistical approach is consistent with our original development and validation of the three-chambered social approach task over 10 years ago (Nadler *et al*, 2004) and with data obtained in testing hundreds of cohorts of mice using this assay (Brielmaier *et al*, 2014; Chadman *et al*, 2008; Crawley *et al*, 2007; Ey *et al*, 2012; Moy *et al*, 2007; Silverman *et al*, 2012; Wohr *et al*, 2013; Yang *et al*, 2012). The absolute number of seconds spent with the novel mouse is highly variable across cohorts of the same genotype or strain, and is therefore not a sufficiently stable parameter for biologically meaningful comparisons across genotypes or across treatment groups. This assay primarily provides a yes-or-no comparison of mean group time with the novel mouse *versus* mean group time with the novel object. If this comparison is significant, then the group displays sociability. If it is not, the group does not display sociability. As described in our previous publications (Brielmaier *et al*, 2014; Chadman *et al*, 2008; Ey *et al*, 2012; Silverman *et al*, 2010b, 2012; Wohr *et al*, 2013; Yang *et al*, 2011, 2012) and others (Clipperton-Allen and Page, 2014; Kerr *et al*, 2013; Tsai *et al*, 2012), this within-group comparison is valid within a genotype, or within a drug treatment dose, but not across genotypes nor across drug treatment groups. Center chamber times are shown in the graphs only to visually display the absence of treatment effects on time spent in the central starting area that could indicate sedation or hyperactivity. For number of chamber entries during social approach, drug effects were compared within each strain by a separate between-groups drug × entries ANOVA. In cases where the overall ANOVA for drug was significant on number of entries, the treatment factor for each strain was further analyzed with Dunnett's *post hoc* test to compare each drug dose group with its vehicle control group.

Self-grooming and marble burying were analyzed using one-way ANOVA within strain for drug dose, using Statistica 10.0 software (Statsoft, www.statsoft.com). Repetitive vertical jumping was analyzed using a one-way ANOVA for drug dose. In cases where the overall ANOVA for the drug was significant, *post hoc* analysis was performed with Dunnett's *post hoc* test to compare each drug dose with its vehicle control.

Open field data were analyzed with a repeated measures ANOVA using a between-groups factor of drug within strain, and a within-group factor of time course, for the parameters of total distance, horizontal activity, or center time. Comparisons with a significant ANOVA were followed by Dunnett's *post hoc* analysis, using SigmaPlot version 12.0 (Systat, San Jose, CA) to identify treatment group differences.

RESULTS

R-Baclofen Increased Sociability in BTBR in the Three-Chambered Social Approach Task

Figure 1 illustrates the sociability scores from the automated three-chambered social approach task following a single dose of R-baclofen or saline vehicle (i.p.) in B6 and BTBR mice.

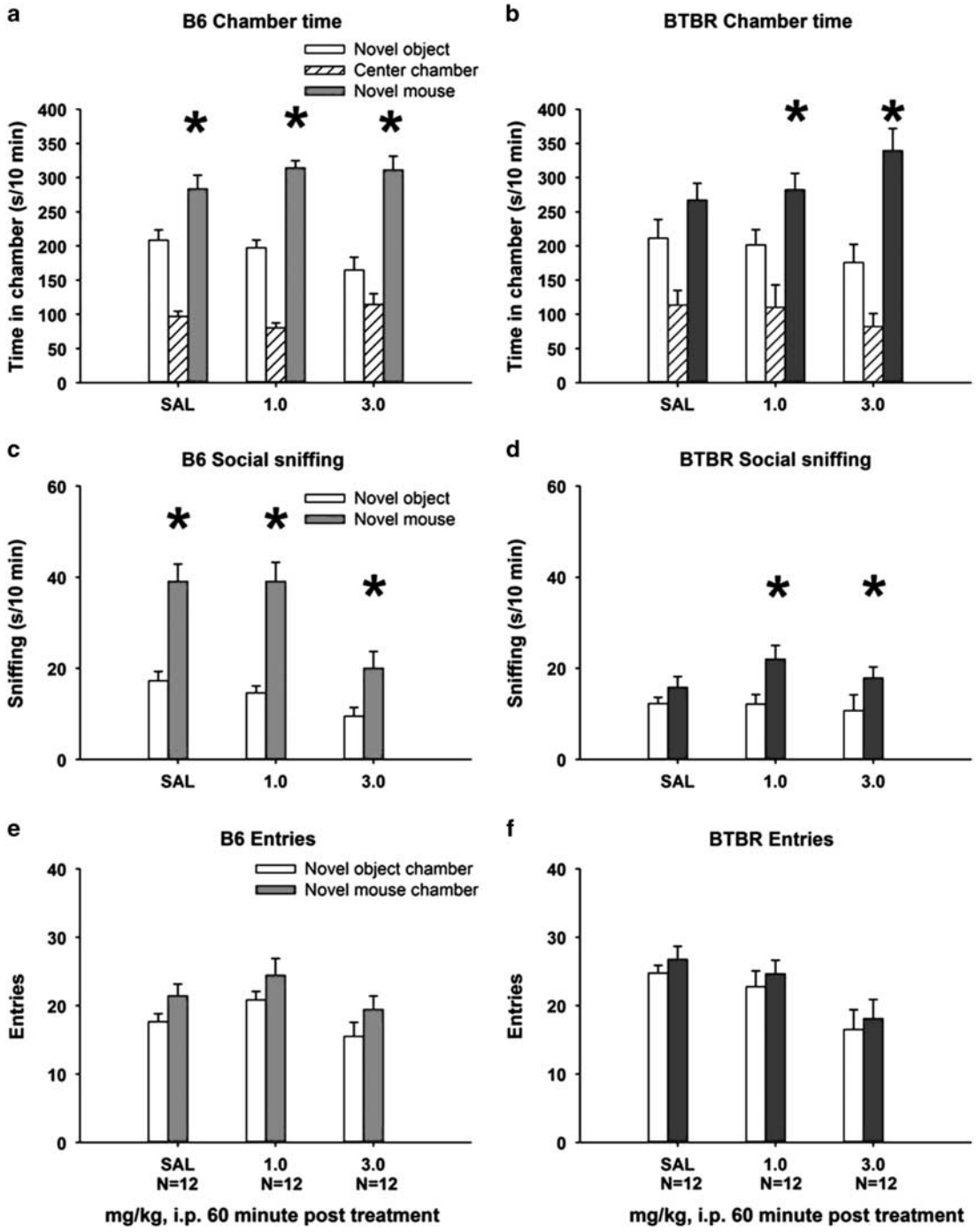

Figure 1 R-baclofen rescued social approach deficits in the BTBR mouse model of autism. R-baclofen or saline vehicle was administered acutely, 60 min before the three-chambered social approach test session. (a) B6 mice displayed normal sociability on the chamber time parameter, spending more time in the side chamber with the novel mouse as compared with the side chamber with the novel object, after treatment with vehicle and at each dose of R-baclofen. (b) BTBR mice exhibited characteristic lack of sociability on the chamber time parameter after vehicle treatment, spending approximately equal time in the side chamber with the novel mouse and the side chamber with the novel object. R-baclofen at doses of 1 and 3 mg/kg reversed the sociability deficits in BTBR. (c) Automated sniffing was measured by Noldus Ethovision 9.0XT software using settings that included (1) multiple body point dynamic subtraction detection, (2) the subject's nose in a discrete zone that surrounded the novel mouse or object, and (3) the subject's head oriented toward the novel mouse or object. B6 mice treated with vehicle or R-baclofen exhibited characteristic sociability on the directed sniffing parameter. (d) BTBR exhibited its characteristic lack of sociability on the directed sniffing parameter, that is, did not spend more time sniffing the novel mouse *versus* the novel object, after vehicle treatment. R-baclofen at doses of 1 and 3 mg/kg reversed the social sniffing deficits in BTBR. Number of entries into the side chambers was unaffected by R-baclofen treatment in (e) B6 and (f) BTBR, indicating the absence of confounding hyper- or hypo-exploratory locomotion during the social approach task. *$P<0.05$, novel mouse *versus* novel object. See Supplementary Figure S1 for the replication of R-baclofen social approach in B6 and BTBR in cohort 2.

Significant sociability was detected in B6, but not BTBR, in the saline vehicle groups, consistent with previous reports (Figure 1a, B6 saline: $F_{(1, 11)} = 11.11$, $p < 0.05$; Figure 1b, BTBR saline: $F_{(1, 11)} = 1.44$, NS). B6 continued to exhibit significantly more time in the chamber with the novel mouse than time in the chamber with the novel object for all doses of R-baclofen (Figure 1a, B6 1 mg/kg: $F_{(1, 11)} = 31.17$, $p < 0.001$; B6 3 mg/kg: $F_{(1, 11)} = 16.56$, $p < 0.01$), providing evidence for no deleterious actions of R-baclofen at these doses on normal sociability. R-baclofen, at doses of 1 and 3 mg/kg, reversed the sociability deficit in BTBR on the chamber time parameter (Figure 1b, BTBR 1 mg/kg: $F_{(1, 11)} = 6.32$, $p < 0.05$; BTBR 3 mg/kg: $F_{(1, 11)} = 8.12$, $p < 0.02$).

Social sniffing was defined as time spent within 2 cm of the wire cup, with the head facing the wire cup containing the stimulus mouse, as compared with the time spent sniffing the novel object, using the same body point detection settings. B6 subject mice displayed significant sociability on social sniffing in all saline and R-baclofen groups (Figure 1c, B6 saline: $F_{(1, 11)} = 40.41$, $p < 0.001$; B6 1 mg/kg: $F_{(1, 11)} = 7.36$, $p < 0.05$; B6 3 mg/kg: $F_{(1, 11)} = 10.35$, $p < 0.01$). BTBR failed to display significant sociability on social sniffing time (Figure 1d, BTBR saline: $F_{(1, 11)} = 3.81$, NS), as previously reported for BTBR at baseline and with various vehicles (Chadman, 2011; Pobbe *et al*, 2011; Silverman *et al*, 2010a, 2012, 2013b). R-baclofen reversed the low social sniffing in BTBR (Figure 1d, BTBR 1 mg/kg: $F_{(1, 11)} = 26.75$, $p < 0.01$; BTBR 3 mg/kg: $F_{(1, 11)} = 8.66$, $p < 0.02$). Replication with a second independent cohort of B6 and BTBR yielded similar findings (Supplementary Figure S1).

Previous research suggested that time spent sniffing the novel mouse is a more direct and sensitive measure of sociability than the chamber time parameter (Fairless *et al*, 2011; Yang *et al*, 2011). To be sure that social sniffing using the new automated scoring method was consistent with manual observer scoring methods previously employed, we compared scores from the automated directed sniffing software, including proximity and directional components, and manual scoring of digital videos by a trained observer uninformed of drug treatment. The same direction of drug effects, along with the expected variability in absolute number of seconds, were obtained for social sniffing with both methods (Supplementary Figure S2).

Number of entries into the side chambers was not affected by R-baclofen in B6 (Figure 1e, $F_{(2, 33)} = 2.57$, NS) or BTBR (Figure 1f, $F_{(2, 33)} = 2.58$, NS), indicating that R-baclofen administration had no effect on general exploratory activity throughout the three-chambered apparatus during the social approach assay. No innate side preference was present in B6 ($F_{(2, 33)} = 0.54$, NS) or BTBR ($F_{(2, 33)} = 0.36$, NS) during the task.

Normal sociability was seen in C58, consistent with our recent unpublished findings with other cohorts of C58, but in contrast to previous findings in another laboratory environment (Ryan *et al*, 2010). No deleterious effects of R-baclofen on a cohort of C58 subject mice were detected in the three-chambered social approach task (Supplementary Figure S3).

Additional data related to sociability using a single dose of R-baclofen in the male–female social interaction assay that simultaneously collects male ultrasonic vocalization emissions are presented in Supplementary Figure S4.

Additional control data that revealed no alteration in olfactory abilities by R-baclofen on a cohort of B6 and BTBR subject mice are presented in Supplementary Figure S5.

R-Baclofen Reduced Repetitive Behavior in BTBR and Stereotyped Behavior in C58

Figure 2a–d illustrates self-grooming and marble burying for B6 and BTBR. BTBR mice treated with saline displayed higher self-grooming times ($F_{(1, 23)} = 7.84$, $p < 0.02$) and buried a greater number of marbles ($F_{(1, 22)} = 16.91$, $p < 0.001$) in observational assays compared with control B6 treated with saline, consistent with earlier findings from our group and others (Silverman *et al*, 2010a, 2012; Amodeo *et al*, 2012. R-baclofen had no significant effect on self-grooming scores in B6 (Figure 2a, $F_{(2, 33)} = 1.25$, NS) or on marble burying (Figure 2c, $F_{(2, 33)} = 0.12$, NS). A significant reduction on self-grooming scores in BTBR treated with R-baclofen was detected (Figure 2b, $F_{(2, 33)} = 3.53$, $p < 0.05$, vehicle *versus* drug post hoc comparison $p = 0.02$). R-baclofen similarly reduced the number of marbles buried in BTBR (Figure 2d, $F_{(2, 33)} = 18.73$, $p < 0.001$, vehicle *versus* drug post hoc comparison $p = 0.001$).

Figure 2e illustrates stereotyped jumping in C58 mice treated with saline or R-baclofen. R-baclofen significantly reduced jumping ($F_{(3, 35)} = 3.47$, $p < 0.03$) at the 1 and 3 mg/kg doses, vehicle *versus* drug post hoc comparison with saline: 1 mg/kg $p = 0.043$, 3 mg/kg $p = 0.019$).

Replication in a second cohort is shown in Supplementary Figure S6.

R-Baclofen Did Not Produce Sedation or Hyperactivity or Anxiolytic-Like Responses in B6 or BTBR

Figure 3a,b,d and e illustrates the lack of effect of R-baclofen on open field exploratory locomotion in B6 and BTBR, tested 60 min after drug or saline administration. In B6 and BTBR, habituation to the novel environment was significant for total distance traversed in the novel open field (Figure 3a, B6: $F_{(5, 33)} = 65.01$, $p < 0.001$; Figure 3b, BTBR: $F_{(5, 33)} = 86.52$, $p < 0.001$). In B6 and BTBR, R-baclofen did not significantly affect total distance traversed (Figure 3a, B6: $F_{(2, 33)} = 1.12$, NS; Figure 3b, BTBR: $F_{,(2, 33)} = 1.27$, NS) or horizontal activity (Figure 3d, B6: $F_{(2, 33)} = 1.26$, NS; Figure 3e, BTBR: $F_{(2, 33)} = 1.77$, NS). No dose × distance traveled interactions were detected (B6: $F_{(10, 33)} = 0.99$, NS; BTBR: $F_{(10, 33)} = 0.52$, NS).

Figure 3c and f illustrates sedating effects of the highest dose of R-baclofen on open field exploratory locomotion in C58, tested 60 min after drug or saline administration. Similar to B6 and BTBR, in C58, habituation to the novel environment was significant for total distance traversed in the novel open field (Figure 3c, C58: $F_{(5, 38)} = 7.89$, $p < 0.001$). Moderate sedation was detected in C58, at the 3 mg/kg dose, as compared with saline (Figure 3c, C58 total distance: $F_{(3, 38)} = 5.72$, $p < 0.05$; Figure 3f, C58 horizontal activity: $F_{(3, 38)} = 11.64$, $p < 0.001$).

Figure 3g–i illustrates the absence of anxiolytic-like effects of R-baclofen using center time in an open field in B6, BTBR, and C58, tested 60 minutes after drug or saline administration. R-baclofen at 3 mg/kg reduced time in the center of the open field arena in B6 (Figure 3g, B6:

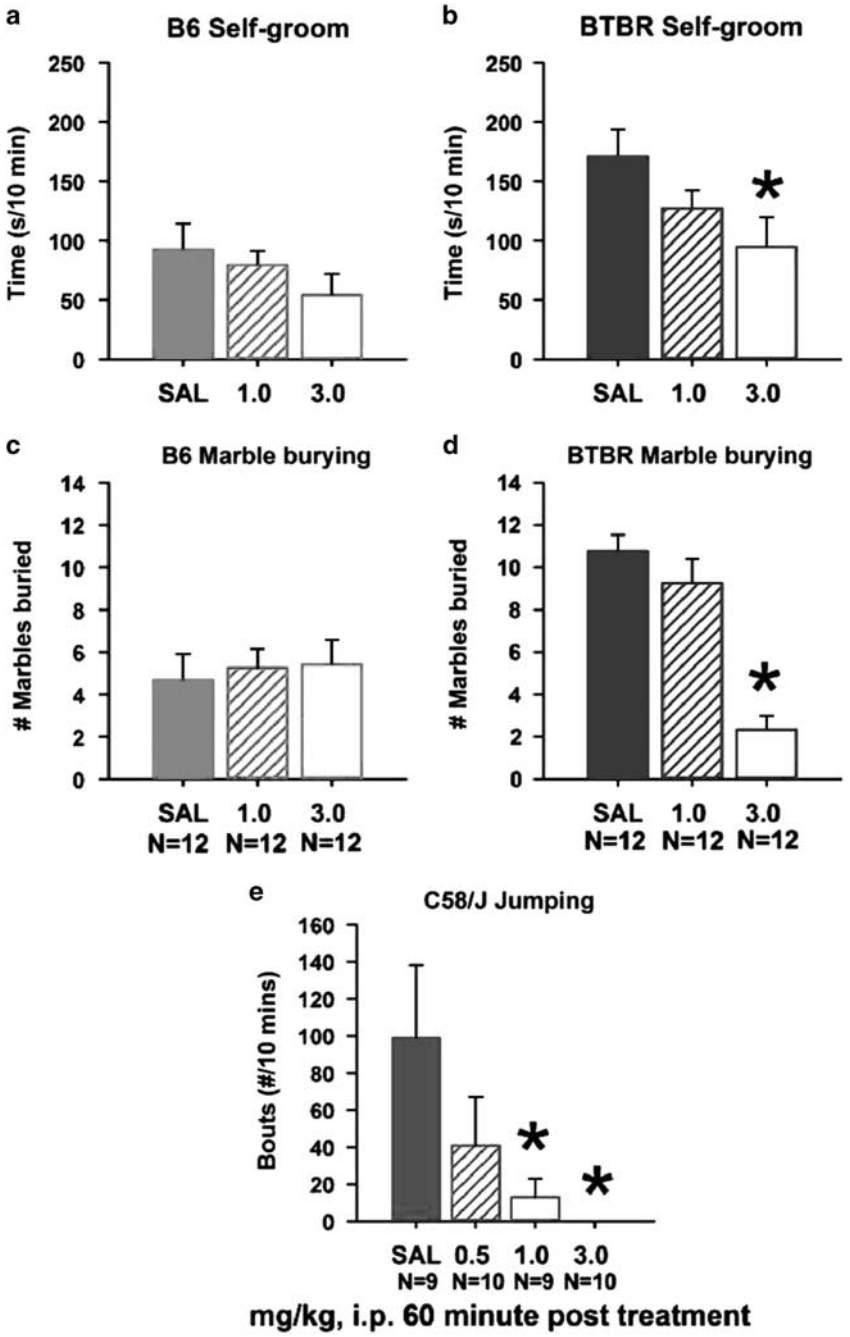

Figure 2 R-baclofen reduced repetitive behaviors in BTBR and stereotyped jumping in C58/J. Cumulative time spent engaged in repetitive behaviors, including self-grooming behavior, number of marbles buried, and number of vertical jumps during a session was scored by investigators blind to drug treatment. (a) B6 mice displayed their normally low levels of self-grooming after administration of saline or R-baclofen. (b) High levels of repetitive self-grooming in BTBR were reduced by R-baclofen at the highest dose, 3 mg/kg. $^*P<0.05$, by one-way ANOVA followed by Dunnett's *post hoc*, R-baclofen compared with saline. (c) B6 mice displayed their normally low levels of marble burying after treatment with saline or R-baclofen. (d) High levels of marble burying in BTBR mice were reduced by R-baclofen at the 3 mg/kg dose. $^*P<0.05$, by one-way ANOVA followed by Dunnett's *post hoc*, R-baclofen compared with saline. (e) Stereotyped vertical jumping in C58/J mice was significantly reduced by R-baclofen treatment at doses of 1 and 3 mg/kg. $^*P<0.05$, by one-way ANOVA followed by Dunnett's *post hoc*, R-baclofen compared with saline. See Supplementary Figure S2 for the R-baclofen repetitive behavior assays in cohort 2 of B6, BTBR, and C58/J.

$F_{(2,33)}=6.30$, $p<0.05$, vehicle *versus* drug *post hoc* comparison $p=0.006$) and in C58/J (Figure 3i, C58: $F_{(3,38)}=7.66$, $p<0.001$, vehicle *versus* drug post hoc comparison $p=0.0001$). R-baclofen had no effect on time in the center of the open field arena in BTBR (Figure 3h, BTBR: $F_{(2,33)}=0.89$, NS). As increases in center time would indicate anxiolytic actions, and the present data show only decreases in center time consistent with sedation at the highest dose in B6 and in C58, these center time findings indicate that R-baclofen is not producing anxiolytic effects, consistent

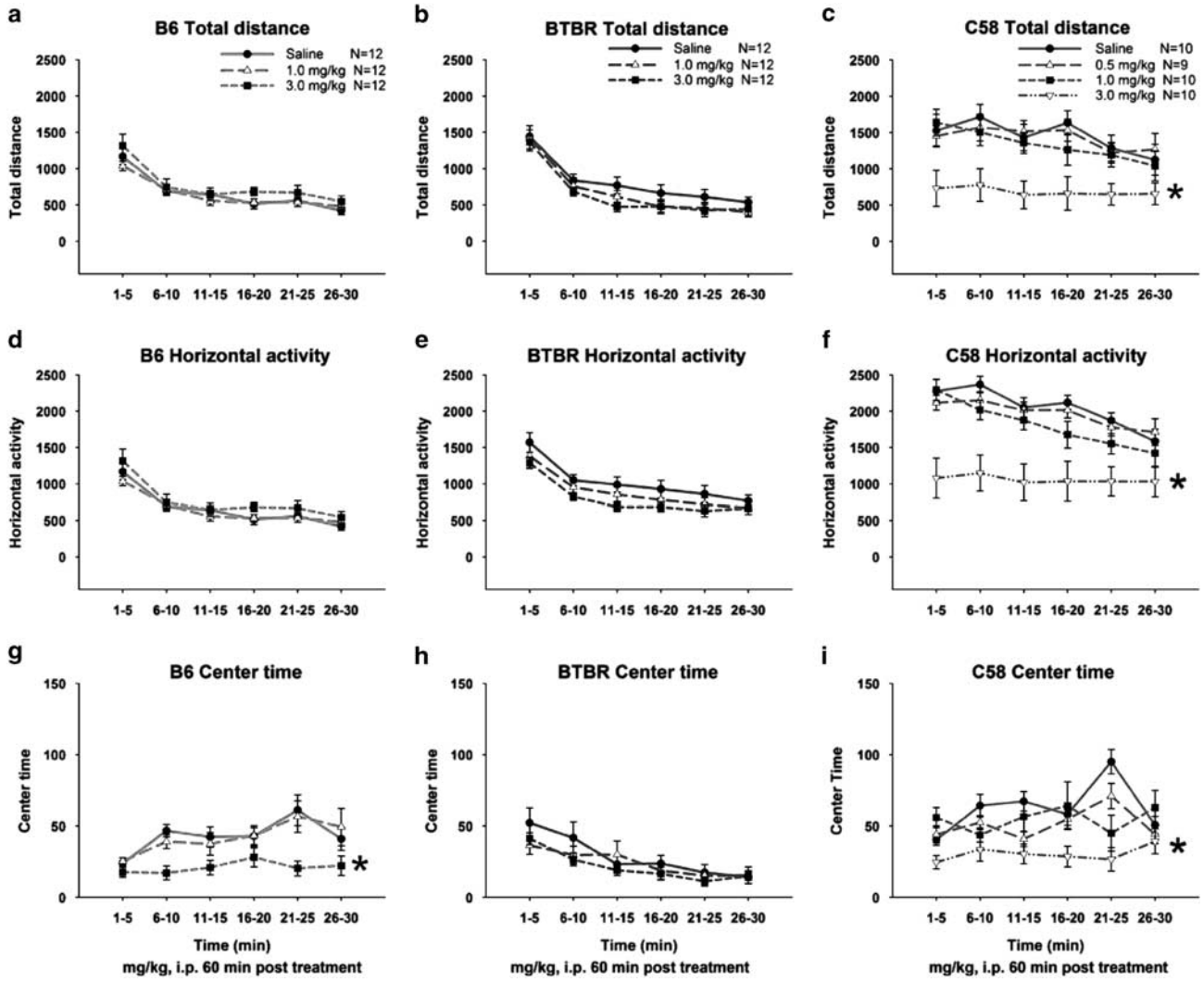

Figure 3 R-baclofen did not induce hyperactivity or sedation in B6 or BTBR or an increase of time spent in the center of the open field in B6, BTBR, or C58/J. Exploratory locomotion and time spent in the center of the arena were measured by total distance traversed, horizontal activity, and center time parameters across a 30 min test session in an Accuscan open field in B6, BTBR, and C58/J following administration of saline vehicle or R-baclofen. Total distance was unaffected by R-baclofen treatment in (a) B6 and (b) BTBR. Horizontal activity was unaltered by R-baclofen treatment in (d) B6 and (e) BTBR. R-baclofen reduced time in the center of the open field arena in B6, at the 3 mg/kg dose (g) but had no effect on center time in BTBR (h). R-baclofen treatment reduced total distance traversed, horizontal activity, and center time in C58/J at the 3 mg/kg dose (c, f, i). Data are shown in 5-min time bins. $^*P < 0.05$, drug by repeated measures ANOVA (over time), followed by Dunnett's *post hoc* as compared with saline. Based on these open field results, social and repetitive behavior tests were initiated at 60 min after treatment to avoid potential sedative confounds. See Supplementary Figure S7 for additional post-treatment intervals in the open field used to select the treatment regimen.

with findings on two specific anxiety-related tasks shown in Supplementary Figures S8 and S9.

No anxiolytic effects of R-baclofen on a cohort of B6 and BTBR subject mice were detected in the elevated plus-maze or the light↔dark exploration assays (Supplementary Figures S8 and S9), suggesting that R-baclofen reduces repetitive behaviors through a non-anxiolytic mechanism.

When administered at a shorter time interval before testing, 30 min, R-baclofen produced some sedative effects during open field locomotion in B6 at the highest dose tested, 5 mg/kg, and at both 3 and 5 mg/kg in BTBR (Supplementary Figure S7). These data in combination with published t_{max} values for Arbaclofen (Henderson *et al*, 2012), determined our choice of the 60 min pretreatment interval for the sociability, repetitive, and stereotypy assays.

S-Baclofen Was Less Effective on Autism-Relevant Behaviors in BTBR and C58

Figure 4 illustrates the sociability scores in B6 and BTBR mice treated with the less active enantiomer S-baclofen. Sociability was significant in B6 but not BTBR on chamber time (Figure 4a, B6 saline: $F_{(1,9)} = 36.19$, $p < 0.001$; Figure 4b, BTBR saline: $F_{(1,11)} = 0.83$, NS). S-baclofen had no effect on chamber time in B6 (Figure 4a, B6 0.1 mg/kg: $F_{(1,11)} = 6.28$, $p < 0.05$; B6 1 mg/kg: $F_{(1,11)} = 6.34$, $p < 0.05$; B6 3 mg/kg: $F_{(1,9)} = 12.36$, $p < 0.01$; B6 10.0 mg/kg: $F_{(1,9)} = 37.2$, $p < 0.01$).

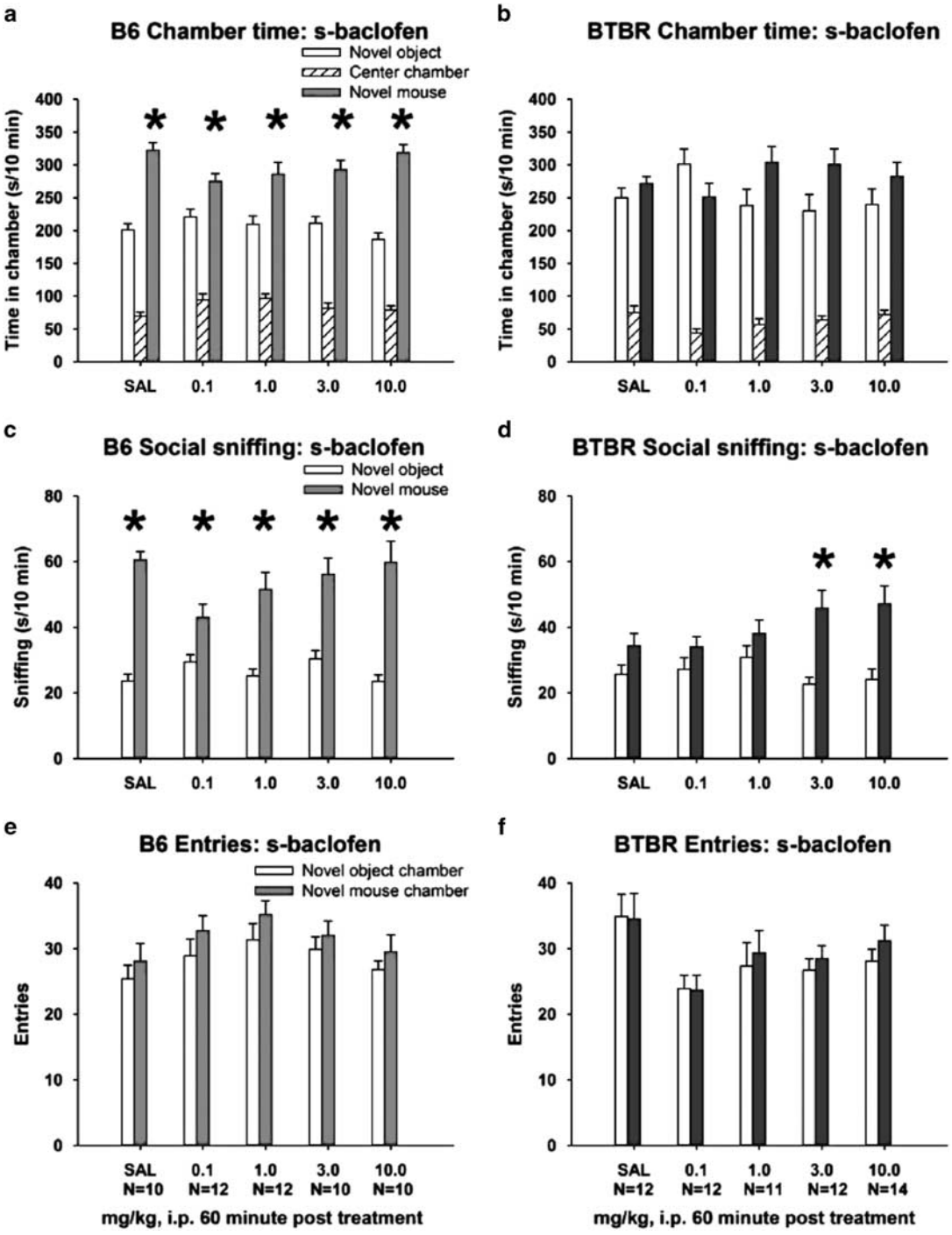

Figure 4 The less potent enantiomer S-baclofen was less effective on social approach in the BTBR mouse model of autism. S-baclofen was administered acutely 60 min before the test session. Vehicle control subject mice received saline. (a) B6 mice displayed normal sociability on the chamber time parameter, spending more time in the side chamber with the novel mouse as compared with the side chamber with the novel object, after treatment with saline and at each dose of S-baclofen. (b) BTBR mice exhibited its characteristic lack of sociability on the chamber time parameter after treatment with saline. BTBR did not spend more time in the side chamber with the novel mouse as compared with the side chamber with the novel object, after treatment with saline and at each dose of S-baclofen. (c) B6 mice treated with saline or S-baclofen exhibited characteristic sociability on the directed sniffing parameter, as described in the Materials and Methods and Figure 1. (b) BTBR exhibited its characteristic lack of sociability and did not spend more time sniffing the novel mouse *versus* the novel object, after treatment with saline. Although 1 mg/kg of R-baclofen improved sociability on the sniffing parameter in BTBR, higher doses of S-baclofen (3 and 10 mg/kg) were required to reverse the directed social sniffing deficits in BTBR. Number of entries into the side chambers was unaffected by S-baclofen treatment in (e) B6 and (f) BTBR, indicating the absence of confounding increased hyper- or hypo-exploratory locomotion during the social approach task. *$P<0.05$, novel mouse *versus* novel object. Absence of effects of S-baclofen on repetitive behavior assays in B6, BTBR, and C58 are illustrated in Supplementary Material Figure S10. Supplementary Material Figure S11 confirmed a lack of confounding sedative or activating effects at the doses of S-baclofen tested in open field locomotion.

S-baclofen did not reverse the lack of sociability on chamber time in BTBR (Figure 4b, BTBR 0.1 mg/kg: $F_{(1, 11)} = 1.37$, NS; BTBR 1 mg/kg: $F_{(1, 10)} = 1.86$, NS; BTBR 3 mg/kg: $F_{(1, 11)} = 2.23$, NS; BTBR 10.0 mg/kg: $F_{(1, 13)} = 0.92$, NS). Social sniffing was unaffected by S-baclofen in B6 (Figure 4c, B6 saline: $F_{(1, 9)} = 244.59$, $p < 0.001$; B6 0.1 mg/kg: $F_{(1, 11)} = 6.63$, $p < 0.05$; B6 1 mg/kg: $F_{(1, 11)} = 19.16$, $p < 0.005$; B6 3 mg/kg: $F_{(1, 9)} = 32.75$, $p < 0.001$; B6 10.0 mg/kg: $F_{(1, 9)} = 25.39$, $p < 0.001$). S-baclofen increased sniffing time in BTBR at doses of 3 and 10 mg/kg (Figure 4d, BTBR 3 mg/kg: $F_{(1, 11)} = 11.76$, $p < 0.006$; BTBR 10.0 mg/kg: $F_{(1, 13)} = 12.73$, $p < 0.005$), but not at 0.1 and 1 mg/kg or vehicle (Figure 4d, BTBR saline: $F_{(1, 11)} = 3.50$, NS; BTBR 0.1 mg/kg: $F_{(1, 11)} = 2.70$, NS; BTBR 1 mg/kg: $F_{(1, 10)} = 1.25$, NS), consistent with its reported lower potency as compared with R-baclofen.

Transitions into the chambers were not altered by S-baclofen in B6 (Figure 4e, $F_{(4, 49)} = 1.47$, NS) or BTBR (Figure 4f, $F_{(4, 56)} = 2.39$, NS), indicating that the drug administration had no effect on exploratory activity during the social approach assay.

Repetitive self-grooming and marble burying in BTBR and vertical jumping in C58 were unaffected by S-baclofen treatment (Supplementary Figure S10). No sedation was detected in B6 at the two higher doses of S-baclofen in open field locomotion (Supplementary Figure S11).

DISCUSSION

Autism is a multifaceted neurodevelopmental disorder with high variability in symptom presentation and biomarkers across individuals. Extensive consortium studies of ASD have revealed copy number variants and single gene mutations in GABA receptor subunit genes across a relative large percentage of cases of ASD and in comorbid syndromes that meet diagnostic criteria for ASD (Conant *et al*, 2014; Hogart *et al*, 2009; Kim *et al*, 2008; Ma *et al*, 2005; McCauley *et al*, 2004; Nurmi *et al*, 2001; Piton *et al*, 2013; Schroer *et al*, 1998; Vincent *et al*, 2006). Seizures appear in approximately one-third of ASD cases, including comorbid neurodevelopmental disorders such as tuberous sclerosis, Fragile X, Rett, 15q11-13 duplication, 16p11.2 deletion, and Phelan-McDermid syndromes, in which seizures present as a primary symptom (Chao *et al*, 2010; Hagerman *et al*, 2010; Sahin, 2012; Sarasua *et al*, 2014; Shinawi *et al*, 2010). GABAergic spectroscopy and postmortem biomarkers have reported lower GABA in certain brain regions in ASD (Blatt and Fatemi, 2011; Fatemi *et al*, 2002; Gaetz *et al*, 2014; Harada *et al*, 2011; Mori *et al*, 2012; Yip *et al*, 2009).

Mouse models with mutations in GABA receptor subunits and/or reduced GABAergic or parvalbumin positive interneurons display social deficits, repetitive behaviors, and other ASD-relevant behavioral phenotypes (Bissonette *et al*, 2014; Brielmaier *et al*, 2014; DeLorey *et al*, 2008; Karayannis *et al*, 2014; Penagarikano *et al*, 2011; Tripathi *et al*, 2009). Impaired GABA inhibitory transmission has been reported in multiple preclinical models of Fragile X syndrome (Gatto *et al*, 2014; Martin *et al*, 2014; Paluszkiewicz *et al*, 2011).

Benzodiazepines and GABA agonists reversed behavioral and electrophysiological abnormalities in *Fmr1*, *Scn1a*, and BTBR mouse models (Han *et al*, 2014; Han *et al*, 2012; Olmos-Serrano *et al*, 2010; Pobbe *et al*, 2011) and Arbaclofen treatment reversed symptoms in Fragile X mice (Henderson *et al*, 2012). Given the circumscribed but intriguing reversal of some elements of Fragile X and ASD in the first clinical trials of Arbaclofen (Berry-Kravis *et al*, 2012; Erickson *et al*, 2014), we reasoned that extensive preclinical evaluation of R-baclofen in mouse models of autism could be informative.

R-baclofen normalizes multiple aspects of excitatory/inhibitory (E/I) circuit balance in mouse models of E/I dysfunction (Gandal *et al*, 2012). The efficacy of R-baclofen could be the result of its ability to dampen hyperexcitability via both pre- and postsynaptic mechanisms. $GABA_B$ receptors on the presynaptic neuron inhibit GABA release presynaptically and activate postsynaptic, inward-rectifying potassium channels that cause neuronal hyperpolarization. R-baclofen may be beneficial in BTBR because of the reduced frequency of inhibitory synaptic events, reduced inhibitory neurotransmission, and increased excitatory neurotransmission reported for BTBR (Han *et al*, 2014).

Here we report a strongly significant reduction in stereotyped and repetitive behaviors in two unrelated inbred strain mouse models of autism. High self-grooming and high marble burying in BTBR mice and high vertical jumping in C58 mice were normalized by doses of 1 and 3 mg/kg R-baclofen. Furthermore, these low doses of R-baclofen reversed sociability deficits in BTBR, an inbred strain that displays low social interactions on multiple social assays (Bolivar *et al*, 2007; Defensor *et al*, 2011; Lipina and Roder, 2013; McFarlane *et al*, 2008; Pearson *et al*, 2012; Pobbe *et al*, 2011; Silverman *et al*, 2012, 2013a, b). These R-baclofen doses produced no sedative effects on open field locomotion or on number of entries during the three-chambered social approach session. B6, a control inbred strain with normal sociability and low repetitive behaviors, was unaffected by R-baclofen on these assays, indicating lack of deleterious effects in normal controls. Higher doses of the less potent enantiomer S-baclofen were required to reverse abnormalities in BTBR and C58, supporting the use of the R-enantiomer.

It is important to recognize that the replicated rescues of sociability by R-baclofen in BTBR mice did not generalize to male–female reciprocal social interactions. In our initial investigation with a single dose, 1 mg/kg, R-baclofen did not improve or impair the normally high reciprocal social interactions in B6 control mice, confirming no deleterious effects on social interactions. However, this dose of R-baclofen did not reverse the low male–female interactions in BTBR mice. It is possible that the optimal doses of R-baclofen will vary across behavioral assays in mice. Given the sedation detected at the higher dose of R-baclofen in many of our mouse behavior assays, chronic treatment may be useful to provide an opportunity for desensitization to the sedative effects. It may be necessary to conduct comprehensive dose–response curves, for both acute and chronic treatments, across a range of preclinical assays relevant to autism to work out the optimal therapeutic regimen. It is conceivable that similar dosing issues may have affected results in the previous clinical trial with Arbaclofen (Erickson *et al*, 2014), and will require attention in future clinical studies with this compound and other GABAergic agonists.

There are no FDA-approved pharmacologic compounds to improve deficiencies in the core behavioral symptom domains of ASD, deficits in social communication, and restricted, repetitive behaviors. Our novel preclinical results using two mouse models of ASD are strikingly promising. These data support the hypothesis that enhancing inhibitory transmission improves ASD relevant deficits. We discovered that by increasing GABAergic signaling via the $GABA_B$ receptor agonist R-baclofen, lack of sociability of BTBR was reversed on two parameters of sociability and replicated in two independent cohorts. Furthermore, we found dose-dependent reductions in high levels of repetitive self-grooming and marble burying in BTBR and in stereotyped vertical jumping in C58 treated with R-baclofen. Future investigations of chronic R-baclofen treatment, in these and other rodent models, will provide additional insights. Our findings support the hypothesis that enhancing inhibitory synaptic transmission offers a therapeutic strategy for improving the diagnostic symptoms of ASD. $GABA_B$ agonists represent one potential hypothesis-based therapeutic intervention. Although the first clinical trials with Arbaclofen produced significant improvement on only a subset of measures, our preclinical findings suggest that the strategy justifies additional trials with refined outcome measures in a biomarker-stratified patient population.

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The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)