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Autism-like behavioral phenotypes in BTBR T+tf/J mice

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Autism is a behaviorally defined neurodevelopmental disorder of unknown etiology. Mouse models with face validity to the core symptoms offer an experimental approach to test hypotheses about the causes of autism and translational tools to evaluate potential treatments. We discovered that the inbred mouse strain BTBR T+tf/J (BTBR) incorporates multiple behavioral phenotypes relevant to all three diagnostic symptoms of autism. BTBR displayed selectively reduced social approach, low reciprocal social interactions and impaired juvenile play, as compared with C57BL/6J (B6) controls. Impaired social transmission of food preference in BTBR suggests communication deficits. Repetitive behaviors appeared as high levels of self-grooming by juvenile and adult BTBR mice. Comprehensive analyses of procedural abilities confirmed that social recognition and olfactory abilities were normal in BTBR, with no evidence for high anxiety-like traits or motor impairments, supporting an interpretation of highly specific social deficits. Database comparisons between BTBR and B6 on 124 putative autism candidate genes showed several interesting single nucleotide polymorphisms (SNPs) in the BTBR genetic background, including a nonsynonymous coding region polymorphism in *Kmo*. The *Kmo* gene encodes kynurenine 3-hydroxylase, an enzyme-regulating metabolism of kynurenic acid, a glutamate antagonist with neuroprotective actions. Sequencing confirmed this coding SNP in *Kmo,* supporting further investigation into the contribution of this polymorphism to autism-like behavioral phenotypes. Robust and selective social deficits, repetitive self-grooming, genetic stability and commercial availability of the BTBR inbred strain encourage its use as a research tool to search for background genes relevant to the etiology of autism, and to explore therapeutics to treat the core symptoms.

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The diagnosis of autism requires the presence of three defining symptoms: (1) aberrant reciprocal social interactions, (2) qualitative impairments in communication and (3) restricted repetitive and stereotyped patterns of behavior, interests and activities (DSM-IV 2000; Lord *et al.* 2001, 2006; Losh & Piven 2007; Volkmar *et al.* 2004). While the causes of autism spectrum disorders remain unknown, a strong genetic component is evidenced by heritability above $\lambda = 60$ and up to 90% concordance in monozygotic twins, as compared with 4–10% concordance in dizygotic twins, and a 4:1 male:female ratio (Blasi *et al.* 2006; Muhle *et al.* 2004; Polleux & Lauder 2004; Ronald *et al.* 2006; Spence *et al.* 2006; Veenstra-Vanderweele *et al.* 2004). Linkage and association studies have identified many gene candidates, however, none is consistently replicated across cohorts (Blasi *et al.* 2006; Muhle *et al.* 2004; Polleux & Lauder 2004; Spence *et al.* 2006; Veenstra-Vanderweele *et al.* 2004). Large numbers of candidate genes may indicate that alleles mediating the behavioral traits of autism are present in the normal population, but cluster in high concentrations at one extreme of the normal distribution to produce the symptoms of autism (Nadler *et al.* 2006; Ronald *et al.* 2006), and/or may represent various ways to impair the development of essential brain structures and pathways.

Animal models of autism include inbred strains of mice expressing traits relevant to autism (forward genetics) and targeted mutations in candidate genes (reverse genetics) (Bolivar *et al.* 2007; Brodkin *et al.* 2004; Brodkin 2007; Cheh *et al.* 2006; Crawley 2004; Insel & Young 2001; Kuemerle *et al.* 2007; Kwon *et al.* 2006; Levitt 2005; Mineur *et al.* 2006; Moy *et al.* 2004, 2007; Nadler *et al.* 2004; Zoghbi 2005). While investigating priority strains from the International Mouse Phenome Project, we discovered several inbred strains of mice with low levels of social interaction (Bolivar & Flaherty 2003; Bolivar *et al.* 2007; Moy *et al.* 2004, 2007; Nadler *et al.* 2004). The present experiments focus on one of these, BTBR T+tf/J (BTBR), using multiple tasks with putative face validity for each of three diagnostic domains. Procedural abilities were examined to prevent overinterpretations of aberrant

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social phenotypes (Crawley 2004, 2007). To address the strong genetic component of autism, we began a search of single nucleotide polymorphism (SNP) databases to discover polymorphisms between BTBR and B6 in putative autism candidate genes.

Materials and methods

Mice

All procedures were conducted in strict compliance with the NIH guidelines for the Care and Use of Laboratory Animals and approved by the National Institute of Mental Health and Wadsworth Animal Care and Use Committees. Breeding pairs of C57BL/6J (B6) and BTBR inbred strains of mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice at the National Institute of Mental Health (NIMH), Bethesda, MD were bred and maintained in a vivarium at 20 °C and 55% humidity with food and water available *ad libitum*. Within 2 days of birth, most litters were culled to eight pups, with a sex ratio of four males and four females, whenever possible (Ikemoto & Panksepp 1992; Terranova *et al.* 1998). Pups were kept with the dam until juvenile play and open field activity testing were completed, and weaned no later than postnatal day (PND) 25. After weaning, juveniles were housed by sex and strain in standard plastic cages in groups not exceeding four per cage. Consistent with the higher prevalence of autism in human males, male mice were used in all studies.

Experiments at NIMH were conducted with BTBR and B6 raised under reverse lighting conditions, lights on at 2100 h and off at 0900 h, and tested under red lighting during the dark phase of the circadian cycle, when mice are generally engaged in high levels of social interactions (Laviola *et al.* 1994; Panksepp & Lahvis 2006; Terranova *et al.* 1998). Experiments at the Wadsworth Center were conducted with BTBR and B6 bred and housed under a conventional light cycle (lights on 0700–1900 h) and tested during the light phase. After weaning, mice were housed four to five per cage by sex and strain. The different lighting conditions at NIMH and Wadsworth allowed comparison of phenotypes obtained when mice were tested during their active dark phase vs. during their resting light phase.

Behavioral assays

Order of testing

The sequence of testing at NIMH began with scoring of grooming behaviors, conducted from PND 18 to PND 60. Juvenile play testing was carried out on PND 21, open field testing on PND 22 and social approach testing was carried out when the mice were approximately 6 weeks old. Experiments were conducted during the first half of the dark phase when social behaviors are highest (Laviola *et al.* 1994; Panksepp & Beatty 1980; Panksepp & Lahvis 2006; Terranova & Laviola 2005). Test rooms at NIMH were dimly lit at 6–27 lux, with a single 25 W red light bulb in a desk lamp. Test chambers were cleaned with 70% ethanol between test subjects, and with detergent and warm water at the end of each testing day.

Identification coding

Videotapes of adult reciprocal social interactions from the Wadsworth Center and of juvenile play behaviors from NIMH were coded to prevent observer bias. Raters were generally unable to distinguish the fur marking differences between B6 and BTBR from the videotapes. While the slightly different fur color markings of B6 (dark brown) and BTBR (dark brown with a white ventral patch) prevented fully blind rating in real time, the observer sat at a distance from the subject mice and was generally unable to identify the strain while scoring self-grooming, or during real-time scoring of sniffing in the social approach task.

Social approach (NIMH)

Tendency to approach a novel mouse as compared with tendency to approach a novel object was measured in 6-week-old-male mice in a 10-min test session using an automated three-chambered apparatus as previously described (Crawley *et al.* in press; Moy *et al.* 2007; Nadler *et al.* 2004). Eighteen B6 and 14 BTBR subjects were tested. In this task, illustrated in Figure S1 panel A and Videos S1 and S2, the subject mouse freely explores the middle start chamber, the side chamber containing a nonsocial novel wire object and the side chamber containing an unfamiliar stranger mouse. Social approach deficits in BTBR were previously reported (Moy *et al.* 2007) when this task was conducted during the light phase of the circadian cycle, when mice are normally asleep. Therefore, the present experiments were conducted during the dark phase, when mice are generally awake and socially interacting, using BTBR and B6 mice housed on a reverse light cycle. Although previous experiments found similar social approach by subjects when the strangers were of the same or different strains, and of the same or different sex (Moy *et al.* 2004; Nadler *et al.* 2004), the present experiments used strangers of the same strain and sex for internal consistency. Stranger mice were enclosed in a wire cage to ensure that all social approach was initiated by the subject, and to limit interactions to social approach and sniffing, while avoiding complications of fighting and sexual activity. Time spent in each chamber was calculated by the automated software, based on the movements of the subject mouse in breaking and unbreaking a series of photocell beams embedded in the openings between chambers. Time spent sniffing was scored by an observer with two stopwatches. The sociability test was preceded by two 10-min habituation sessions. During the first 10-min session, the subject was in the center chamber with the doors closed, providing the first habituation to the apparatus and establishing the center chamber as the start area. During the second 10-min session, both doors were open, providing the second habituation to the entire apparatus. Lack of innate side preference was confirmed during this 10-min session (Crawley *et al.* 2007). The fourth 10-min session provided a measure of preference for social novelty, in which a second novel stranger was placed in the side chamber previously containing the novel object. The preference for social novelty test was included as a control to confirm olfactory abilities for detection and discrimination of social odors.

Reciprocal social interactions (Wadsworth)

Reciprocal social interactions in freely moving unfamiliar adult mice, illustrated in Figure S1 panel B, were measured in 60 to 70-day-old pairs of male mice of the same strain but different litters. Ten pairs of B6 and 10 pairs of BTBR were tested. Pairs were of the same strain but socially naïve to each other, prior to the social test session. After a 30-min acclimation to the room environment, one male of the pair was placed in a clean cage containing clean bedding for 15 min. The second male was added to the cage and the pair was allowed to freely interact for 20 min. The test session was videotaped, and subsequently scored for behavioral events including sniffing, following, mounting, allogrooming, huddling and wrestling.

Juvenile play (NIMH)

Social interactions at a younger neurodevelopmental time-point were measured in 21±1-day-old pairs of male mice of the same strain but different litters. Fourteen pairs of male B6 mice from 10 litters and 13 pairs of male BTBR from 7 litters were used. Social interaction test sessions were conducted during the first half of the dark cycle in a quiet, dimly lit room illuminated by a single 25 W red light (Laviola *et al.* 1994; Terranova *et al.* 1998). The testing room was kept at 20°C, to match the colony room temperature. One day before testing, the subjects were brought to the testing room in their home cage for a period of pre-exposure to the experimental conditions and procedures (Panksepp & Beatty 1980). Whenever the mice were transported between the colony and the experimental rooms, the cages were enclosed in light-tight boxes to prevent disruption of their circadian cycle. Once in the testing room, the subjects were weighed and marked on the base of the tail with a fine point, metallic marking pen (Sharpie™; Sanford Corporation, Oak Brook, IL, USA). Each mouse was then housed individually in a standard laboratory cage, similar to their home cage but without access to food and water. After an hour of individualized housing, each mouse was placed alone

in the play testing arena for a 10-min habituation period. The play arena was the Noldus PhenoTyper chamber (Noldus, Leesburg, VA, USA). The floor of the arena was covered with a thin, fresh layer of the same type of bedding that was in the home cage (Panksepp & Beatty 1980). After habituation, the test arena was cleaned with 70% ethanol, the floor covered with clean bedding, and the next mouse was habituated. After all mice were habituated, each was replaced in its home cage with cagemates and dam, and returned to the colony room.

The next day, mice were brought to the testing room and again weighed, marked and housed individually. After an hour of single housing, a pair of same sex, nonsibling mice from different litters, matched for similar body weights within 1 g, was placed in the testing arena. Behaviors were video recorded for 30 min. At the end of the 30-min test period, members of the pair were again placed into their individual housing cages and kept there until all members of the litter had been tested. All mice were then returned to their home cages with the mother and littermates.

The Noldus PhenoTyper chamber, illustrated in Figure S1 panel C, was connected to Noldus Observer software run on a Dell Pentium 4 desktop computer. Built into the roof of the PhenoTyper chamber are infrared lights and an infrared-sensitive digital video camera, which recorded the test sessions. Data from this camera were captured with Canopus MediaCruise and stored as MPEG 2 files directly on the computer. Subsequent frame-by-frame analysis of the recorded video files was conducted using Noldus Observer 5.0 software for the 30-min test session. Scores were recorded using the Noldus Observer keypad and event analysis software.

The investigator was blind to the strain of the play pair. A six or eight digit numerical code was created for each play pair consisting of a combination of a subset of the numbers, which identified their litters of origin. This number was used as the label for the computerized video file of the pair's social interactions. After the video file had been scored, the experimenter decoded the file by cross-referencing the file number to the litter numbers, thereby verifying the strain of the members of the pair.

Parameters of juvenile mouse social behaviors were chosen from the established literature (Grant & Mackintosh 1963; Terranova & Laviola 2005; Terranova *et al.* 1993, 1998). Behavioral categories are given below.

Investigative

1 Anogenital sniff: sniffing of the partner's anogenital region.
2 Nose-to-nose sniff: sniffing of the head and snout region of the partner.
3 Body sniff: sniffing anywhere on the body with the exception of the head, snout and tail.
4 Follow: one partner follows the other around the cage without any fast, sudden, or running movements.

Affiliative

1 Social grooming: allogrooming, one mouse grooms the other mouse on any part of the body.
2 Social inactive: close physical contact, while lying or standing still.
3 Other affiliative behavior: close physical contact with the partner while engaged in self-directed behaviors such as grooming.

Play soliciting

1 Push under: pushes underneath the partner's anterior body area (snout or snout and rest of anterior) and rests in that position. May result in allogrooming by the partner.
2 Crawl over: traverses the partner's body by crawling over the back from one side to the other.
3 Crawl under: traverses the partner's body by crawling under from one side to the other.
4 Push past: pushes between the play partner and the cage wall.

Nonsocial behaviors

1 Maintenance: self-grooming, mouse grooms any part of its own body.
2 Exploration: investigates the walls and floor of the chamber.

Social transmission of food preference (Wadsworth)

The social transmission of food preference test, illustrated in Figure S1, panel D (a–c), was employed to assay communication of information obtained through social interactions. Same-strain male cagemates were tested using methods previously described (Wrenn *et al.* 2003). As this assay involved food preference with novel flavored powdered chow, BTBR and B6 male mice were first tested for their innate preference for cinnamon (1% McCormick ground cinnamon; McCormick, Hunt Valley, MD, USA) and cocoa (2% Hershey's cocoa, Hershey, PA, USA) flavored powdered food. There was no difference in innate preference between BTBR (11 pairs) and B6 (11 pairs) for these two novel food flavors ($t = .182$, df $= 20$, $P = 0.8571$).

Having established that the strains did not differ, the social transmission of food preference experiment was conducted in a new cohort of 32 pairs of BTBR and 30 pairs of B6 adult male mice. In one half of these pairs, cocoa was the cued flavor food and cinnamon was the novel flavored food, whereas the other half was given cinnamon as the cued flavor food and cocoa was the novel flavored food. Prior to testing, same-strain subject male mice were housed as pairs for 3 days and acclimated to the unflavored powdered food (Lab Diet 5001; PM1 Feeds, Inc., St Louis, MO, USA) and jar assembly in their home cages. The base of the feeding assembly was a Pyrex crystallizing dish (Corning, NY, USA) 80 mm in diameter and 40 mm in height. A clear glass screw-top bottle (Owens Glass, Owens, IL, USA) 40 mm in diameter and 45 mm in height was glued to the center of the crystallizing dish. The melamine plastic cover of the bottle had a 2-cm diameter round hole drilled in the center. Powdered food was placed inside the bottle such that the mouse reached through the hole in the top to access the food. The crystallizing dish was used to catch food spillage.

At the end of the acclimation period, the mice were separated and the demonstrator was food deprived for 18 h. The demonstrator was then given 2 h access to the cued food. Half of the demonstrators were given powdered food flavored with cinnamon and the other half were given powdered food flavored with cocoa. Only mice that showed consumption of food during this period were included in the rest of the study. Immediately afterward, the demonstrator and observer were allowed to interact freely in the absence of food for 10 min. Twenty-four hours later, after an 18-h food deprivation, the observer mouse was given the choice of cinnamon- or cocoa-flavored food for 2 h. The positions of the two dishes within the test cages were randomized (either placed in front or back of the cage) on the opposite side of the cage from the water bottle, to ensure lack of position bias.

Self-grooming (NIMH)

In addition to the grooming measures scored during the juvenile play session within the Noldus Phenotyper (illustrated in Videos S3 and S4), a separate set of male B6 ($n = 10$) and BTBR ($n = 10$) mice were scored for spontaneous grooming behaviors when placed individually in a clean, empty mouse cage without bedding. Each mouse was given a 10-min habituation period in the empty cage and then rated for 10 min for cumulative time spent grooming all body regions. The investigator sat approximately 2 m from the test cage and recorded cumulative time spent in grooming with a stopwatch. The same mice were tested at 18, 28, 38 and 60 days of age.

Open field locomotion (NIMH and Wadsworth)

General exploratory locomotion in a novel environment was tested in individual male mice placed in a VersaMax Animal Activity Monitoring System (AccuScan Instruments, Columbus, OH, USA) for a 30-min test session at NIMH and for a 15-min test session at Wadsworth. Experiments conducted at NIMH employed 22-day-old mice (B6, $n = 23$;

BTBR, $n = 20$). To compensate for the relatively small size of 22-day-old-male mice, the VersaMax vertical sensor was adjusted to the lowest setting of 7 cm, and the floor of the open field arena was elevated by 1.0 cm so the final height of the vertical sensor was 6.0 cm above the floor of the arena. The testing room was illuminated with a single 25-W red lamp and kept at a similar temperature as the colony room. The animals were transported from the colony room to the testing room inside light-tight boxes, during the dark phase of their light cycle. Testing began 1 h later, using a 30-min session length.

Open field testing conducted at Wadsworth employed adult male mice (B6, $n = 15$; BTBR, $n = 15$). One hour before the start of testing, mice were placed in the testing room to acclimate to the room. Mice were tested during the light portion of the light–dark cycle, and all testing took place in a darkened chamber. Mice were tested individually in a three-step procedure. First, the mouse was taken from the home cage, weighed and placed in a holding cage for 15 min. The subject was then placed in the center of the activity monitor and the computer program was initiated for a 15-min test session.

Elevated zero maze

Anxiety-like behaviors were evaluated in an elevated zero maze using procedures previously described (Cook *et al.* 2002; Crawley 2000), with adaptations described below. Adult male mice, 14 B6 and 14 BTBR, were tested in a Zero Maze Digital Monitoring system (AccuScan Instruments, Inc., Columbus, OH, USA). The maze consists of a black circular platform (5 cm in width) composed of open and closed quadrants surrounded by 28.5 cm high acrylic walls. The platform is raised 28.5 cm from the floor and the diameter of the maze is 40 cm. Eight photocells are located in the closed quadrants to measure movement. The only light source in the room was a 15 W bulb located 2 m away from the maze. Mice were given 1 h to acclimate to the testing room prior to being placed on the maze. At the onset of testing, the mouse was placed in quadrant 1 (one of the two closed quadrants) of the zero maze and allowed to explore the maze for 5 min.

Statistical analysis behavioral data

For the automated social approach task, analysis of variance (ANOVA) compared strain and test condition for time spent in the chamber in the sociability and social novelty tasks. As times spent in each of the three chambers were not independent, the test condition factor compared time spent only in the right vs. left chambers. Center chamber times are shown in the graphs for illustrative purposes. Time spent sniffing the novel object vs. the stranger was similarly analyzed. For the reciprocal social interaction study, time spent engaged in any type of social interaction and time spent sniffing were analyzed by Student's *t*-tests. Juvenile play parameters were analyzed using multivariate analysis of variance (MANOVA), followed by univariate tests. Fisher's protected least significant difference ($P < 0.05$) was used for *post hoc* pairwise comparisons following a significant overall *F*. Innate preference for the two flavored foods and percentage of total amount of food consumed as the cued food were analyzed by Student's *t*-tests. Grooming scores were analyzed by repeated measures ANOVA followed by Bonfferoni/Dunn *post hoc* tests to compare strains and ages. The time–course for open field activity at NIMH was analyzed by repeated measures ANOVA. Additional control measures illustrated in Fig. 5 were analyzed with Student's *t*-tests for strain differences.

SNP analysis

To begin investigating gene differences between BTBR and B6, two SNP databases, http://www.ncbi.nlm.nih.gov/projects/SNP and http://www.jax.org/phenome, were queried for 124 putative autism candidate genes described in Polleux and Lauder (2004). All SNPs differing between BTBR and B6 were categorized as defined by the database into coding or noncoding. Coding SNPs were further classified as synonymous (polymorphism does not result in an amino acid change), nonsynonymous (polymorphism that results in an amino acid change), or unknown (if not yet annotated by the database). Noncoding SNPs were classified as being located in an intron, untranslated region (UTR), or unassigned.

The majority of the SNP differences between BTBR and B6 were located in noncoding sequences. Although polymorphisms located within an intron or the UTR could be important for regulation of a gene, there were too many genes with SNP differences in these categories to narrow the selection to optimal candidates. Of the 24 genes harboring polymorphisms within coding sequence, only four of these represented nonsynonymous SNPs resulting in an amino acid change. The allele distribution patterns for nonsynonymous coding SNPs were investigated among inbred strains, looking for polymorphisms specific for BTBR as the most interesting candidates.

Sequencing was carried out to confirm SNP differences of high interest detected between BTBR and B6, using DNA from two BTBR and two B6 inbred mice. Briefly, genomic DNA was isolated using the Puregene mouse tail kit (Gentra Systems, Inc., Minneapolis, MN, USA) from tail tips of BTBR and B6 mice bred and maintained at the Wadsworth Center. We designed primers to amplify the target regions in BTBR and B6 genomic DNA. Polymerase chain reaction (PCR) products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and purified samples were sent to the Wadsworth Center Molecular Genetics Core Facility for sequencing.

Results

Sociability

The social approach apparatus is illustrated in Figure S1 panel A. B6 displayed high levels of social approach (Fig. 1a) as previously reported (Crawley *et al.* 2007; Moy *et al.* 2004, 2007; Nadler *et al.* 2004). B6 spent significantly more time in the chamber containing the stranger than in the chamber containing the novel object ($F_{1,34} = 36.0$, $P < 0.001$). In contrast to B6, adult male BTBR mice failed to spend more time in the side chamber with a stranger mouse, as compared with time with a nonsocial novel object (Fig. 1a; $F_{1,26} = 1.44$, $P = 0.24$, NS for time spent in the two side chambers). Further, BTBR failed to spend more time sniffing the wire enclosure containing the stranger mouse than sniffing the novel object (Fig. 1b; $F_{1,13} = 3.48$, NS), whereas B6 spent more time sniffing the stranger mouse than the novel object ($F_{1,17} = 22.6$, $P < 0.001$). Figure S1 panel B illustrates the chamber for assaying reciprocal social interactions between freely moving B6 or BTBR pairs from different litters. BTBR spent less time engaged in total interactions and sniffing of each other, as compared with B6 (Fig. 1c; $t = 2.321$, df $= 18$, $P = 0.032$). Similarly, BTBR displayed less time sniffing each other as compared to B6 (Fig. 1d; $t = 2.46$, df $= 18$, $P = 0.024$). Although most B6 mice were observed following each other around the cage, no episodes of following were seen in BTBR mice (Fig. 1e).

Juvenile play

Behaviors scored from videotapes of 30-min test sessions in the Noldus Observer Phenotyper chamber (Figure S1 panel C) showed that pairs of 21-day-old BTBR engaged in significantly less social interaction than B6. Number of bouts of social grooming, in which one member of the pair groomed the other, was significantly less in BTBR than in B6 (Fig. 2a; $F_{1,25} = 13.24$, $P < 0.01$). Total time spent in self-grooming, in which a subject groomed any of his own body regions, was significantly higher in BTBR than B6 (Fig. 2b; $F_{1,25} = 32.79$,

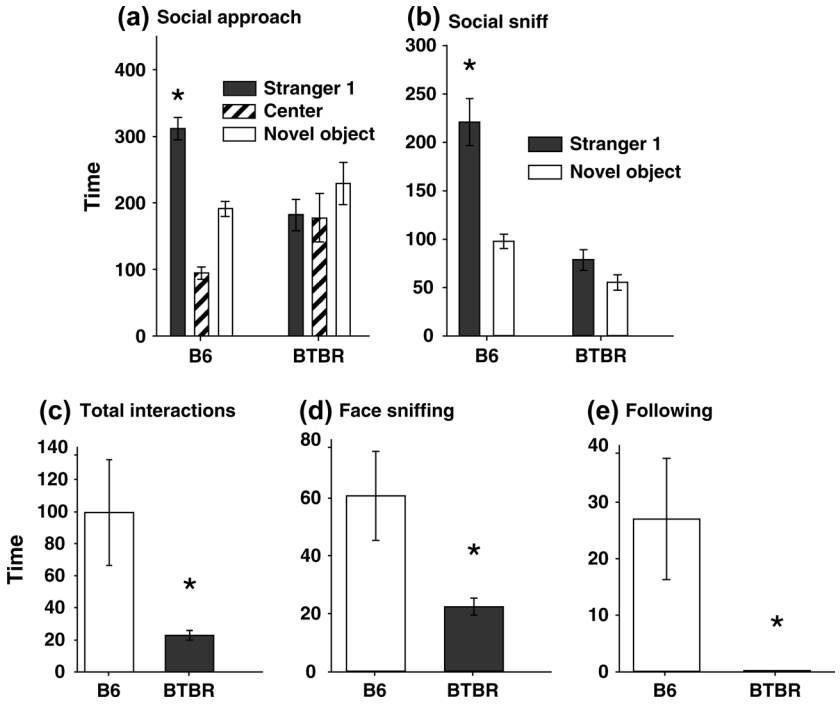

Figure 1: Social interaction in adult male BTBR vs. B6. (a) Social approach in unfamiliar 8-week-old-male mice during a 10-min sociability test session in an automated three-chambered apparatus, conducted during the active nighttime phase under red lighting in mice raised on a reverse circadian cycle. B6 ($n = 18$) spent significantly more time in the side chamber containing the stranger than in the side chamber containing the novel object ($^*P < 0.001$) while BTBR ($n = 14$) showed no significant difference in time spent in the two side chambers. (b) B6 spent more time sniffing the wire enclosure containing the stranger mouse than sniffing the novel object ($^*P < 0.001$), while BTBR did not. (c) Total time engaged in any type of reciprocal social interaction in freely moving adult male mice of the same strain and sex but from different litters during a videotaped 20-mite test session was significantly less for BTBR pairs than B6 pairs ($^*P = 0.032$). (d) Time spent sniffing the face of the other mouse was less in BTBR than B6 ($^*P = 0.024$). (e) Following behavior was never observed in BTBR pairs. In Figs 1–5, all data are expressed as mean ± standard error of the mean. *Significance level. Full statistical results are described in the text.

$P < 0.001$). Nose-to-nose sniffing between members of the pair was significantly less in BTBR (Fig. 2c; $F_{1,25} = 30.84$, $P < 0.0001$), while sniffing the anogenital region was similar between strains (Fig. 2d; $F_{1,25} = 0.461$, $P = 0.50$, NS). Number of times that one member of the pair crawled over or under the other was significantly less in BTBR (Fig. 2e; $F_{1,25} = 9.677$, $P < 0.001$). Number of bouts in which one member of the pair was inactive within the social arena was greater in BTBR (Fig. 2f; $F_{1,25} = 7.433$, $P < 0.05$).

Communication

Figure S1 panels D–F illustrate the social transmission of food preference task. B6 mice ate more of the food familiarized by interacting with their demonstrator cagemate than of a completely unfamiliar food, as compared with BTBR (Fig. 3a; $t = 2.187$, df = 60, $P = 0.033$). BTBR observers sniffed the whiskers and mouth of their demonstrator cagemate less frequently (Fig. 3b; $t = 2.748$, df = 30, $P = 0.01$) and for shorter amounts of time than B6 (Fig. 3c; $t = 2.719$, df = 30, $P = 0.01$).

Repetitive grooming

BTBR displayed an unusual spontaneous repetitive behavior pattern, high levels of repetitive self-grooming. Individual BTBR mice placed in a clean empty standard mouse cage for 10 min displayed considerably more self-grooming than B6 across four developmental ages (Fig. 4; repeated measures ANOVA for strain: $F_{1,18} = 87.9$, $P < .0001$; Bonferroni/Dunn *post hoc* comparison of BTBR vs. B6 $P < 0.05$ at PND 28, $P < 0.01$ at PNDs 18, 38 and 60). Qualitative appearance of self-grooming was similar in adults tested individually in an empty cage (Fig. 4) and in juveniles tested in the Noldus social environment (Fig. 2b).

Procedural controls

Ability to distinguish olfactory social cues was confirmed for both BTBR and B6 in the preference for social novelty test because both strains spent more time with a new stranger than with a familiar mouse (Fig. 5a). Time spent in the side chamber with a new stranger 2 was higher than time spent in the side chamber with the now familiar stranger 1 for both B6 ($F_{1,34} = 29.14$, $P < 0.0001$) and BTBR ($F_{1,26} = 8.86$, $P < 0.01$),

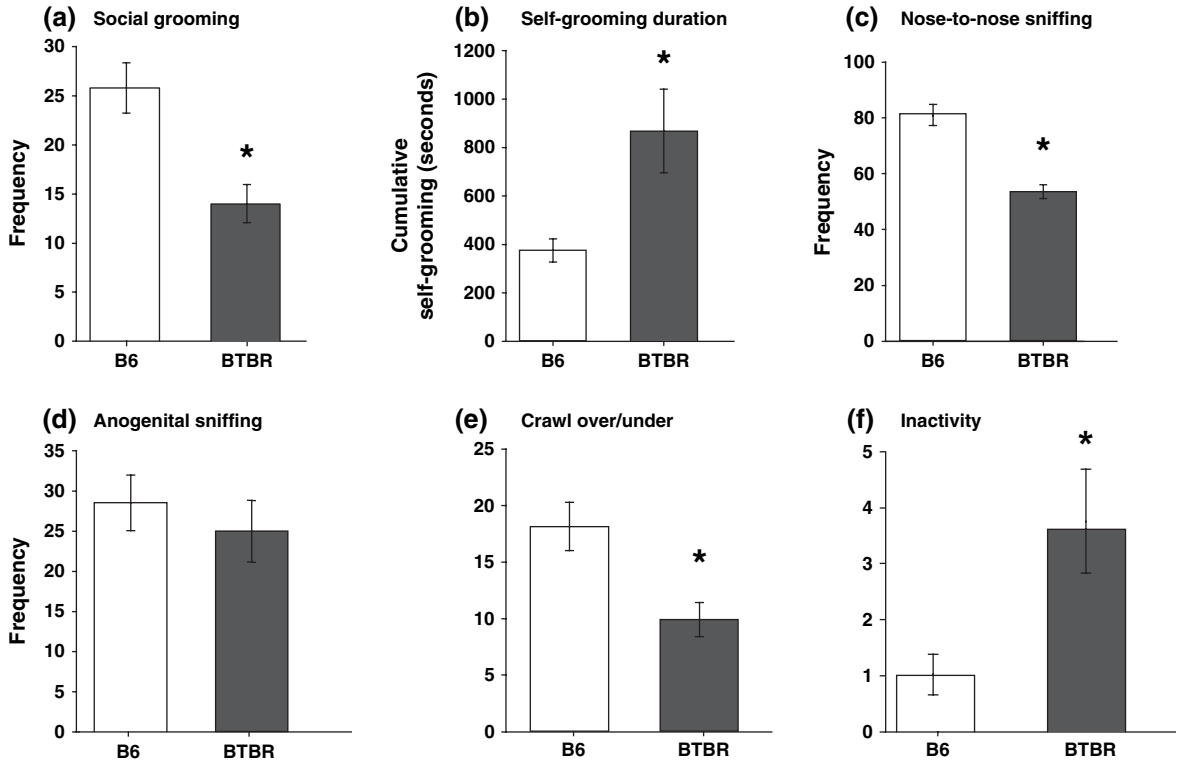

Figure 2: Juvenile play in 21-day-old male B6 and BTBR mice, of the same strain from different litters, during a 30-min videotaped test session. $n = 14$ pairs of B6, 13 pairs of BTBR. (a) Number of bouts of social grooming, in which one member of the pair groomed the other, was significantly less in BTBR than in B6 ($*P < 0.01$). (b) Total time spent in self-grooming, in which a subject groomed any of his own body regions, was significantly higher in BTBR than B6 ($*P < 0.001$). (c) Nose-to-nose sniffing between members of the pair was significantly less in BTBR ($*P < 0.0001$), while (d) sniffing the anogenital region was similar between strains. (e) Number of times that one member of the pair crawled over or under the other was significantly less in BTBR ($*P < 0.001$). (f) Number of bouts in which one member of the pair was inactive within the social arena was greater in BTBR ($*P < 0.05$).

indicating olfactory and other sensory abilities sufficient to discriminate two different stranger mice. General exploratory tendencies were similar between strains, as measured by number of entries into compartments in the social approach task (Fig. 5b; $t = 1.03$, df $= 30$, NS). Neither strain showed anxiety-like scores on the elevated zero maze (Fig. 5c), consistent with the absence of anxiety-like traits previously reported for BTBR and B6 in the elevated plus-maze (Moy *et al.* 2007). Percentage of time spent on the open segments of the elevated zero maze was higher in BTBR than in B6 ($t = 4.387$, df $= 26$, $P = 0.0002$), indicating very low anxiety-like traits in BTBR. Locomotion and exploratory activity in a nonsocial empty novel open field was initially higher in BTBR than B6 in both the Wadsworth (Fig. 5d; $t = 4.55$, df $= 28$, $P < 0.0001$) and NIMH (Fig. 5e; $F_{1,41} = 86.23$, $P < 0.001$) cohorts, and the two strains showed similar activity levels after habituation (Fig. 5e). Further, BTBR previously showed normal scores on measures of general health, home cage activity, startle reflex, visual forepaw placing, open field activity, rotarod performance and the buried food olfactory task (Moy *et al.* 2007).

Single nucleotide polymorphisms

Nonsynonymous coding SNPs resulting in amino acid changes were detected in four genes: *Kmo*, *Slc6a4*, *Smo* and *Pkd1*. For the serotonin transporter gene *Slc6a4*, the unusual polymorphism appeared in B6, not in BTBR. For *Smo* (smoothened, a signaling protein regulated by Sonic hedgehog), and *Pkd1* (polycystic kidney disease 1), the polymorphism appeared in many inbred strains, with neither BTBR nor B6 being the outlier. For *Kmo*, the gene encoding the enzyme kynurenine 3-hydroxylase, BTBR contained the unusual polymorphisms. Table 1 shows the three SNP differences between B6 and BTBR in *Kmo* coding regions resulting in amino acid changes. Both polymorphisms between BTBR and B6 in exon 13 of *Kmo* were subsequently confirmed by sequencing.

Discussion

While no mouse model can fully recapitulate all symptoms of a human neuropsychiatric disorder, BTBR mice display distinctive phenotypic traits with conceptual face validity to

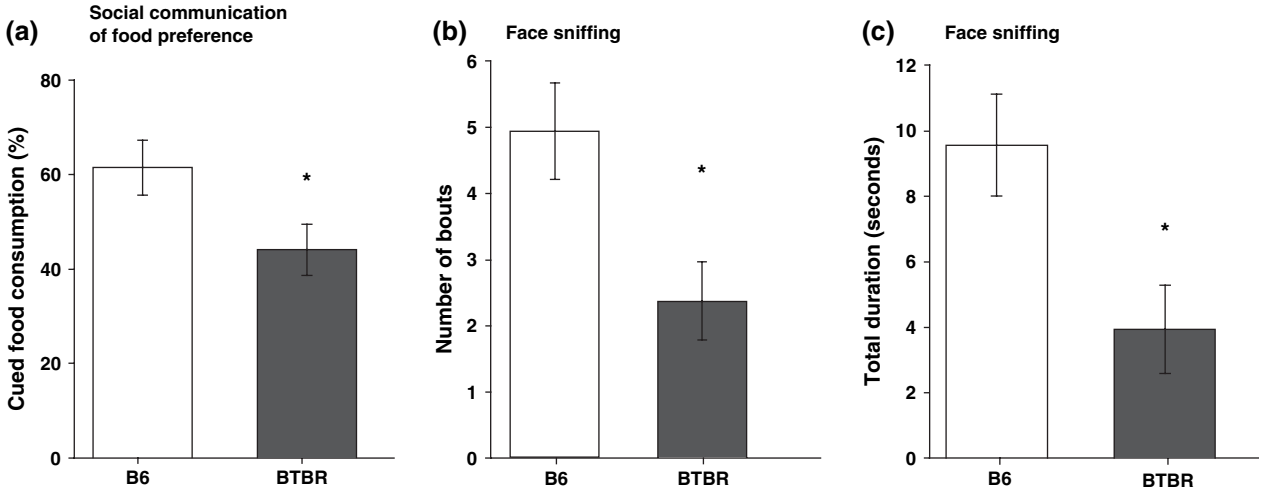

Figure 3: Social transmission of food preference. $n = 30$ B6 pairs and $n = 32$ BTBR pairs of demonstrator + observer. (a) BTBR consumed less of the cued food that the demonstrator had previously eaten than did B6 ($*P = 0.033$). (b) During the 10-min social interaction phase, BTBR observers displayed fewer bouts of face sniffing ($*P = 0.01$), as compared with B6, and (c) spent less time sniffing the whiskers and mouth of their demonstrator cagemates ($*P = 0.01$).

salient components of all three diagnostic symptoms of autism. To fully characterize the initial observation of low social interaction in BTBR (Bolivar & Flaherty 2003; Moy *et al.* 2007), we employed multiple behavioral assays relevant to the three diagnostic criteria. Social approach was quantified with an automated three-chambered social approach task (Moy *et al.* 2007; Nadler *et al.* 2004). Complex reciprocal social interactions (Bolivar & Flaherty 2003; Bolivar *et al.* 2007) were scored in freely moving pairs of mice. As autism is usually detected in early childhood (Lord *et al.* 2006), earlier developmental ages were tested for juvenile play behaviors (Laviola *et al.* 1994; Panksepp & Beatty 1980; Panksepp & Lahvis 2006; Terranova & Laviola 2005; Terranova *et al.* 1993, 1998). Impaired communication in autism (Lord *et al.* 2001, 2006; Losh & Piven 2007; Muhle *et al.* 2004; Volkmar *et al.* 2004) is difficult to model in rodents, where communication is primarily through olfactory cues (Baum & Keverne 2002; Blanchard *et al.* 1991; Wersinger *et al.* 2004) and ultrasonic vocalizations (Branchi *et al.* 2004; Hofer *et al.* 2001). Social transmission of food preference (Ross & Eichenbaum 2006; Wrenn *et al.* 2003) was chosen to model components of social communication in mice. Spontaneous motor stereotypies and repetitive behaviors (Lord *et al.* 2001, 2006; Militerni *et al.* 2002; Symons *et al.* 2005; Volkmar *et al.* 2004) were scored in juveniles and adults.

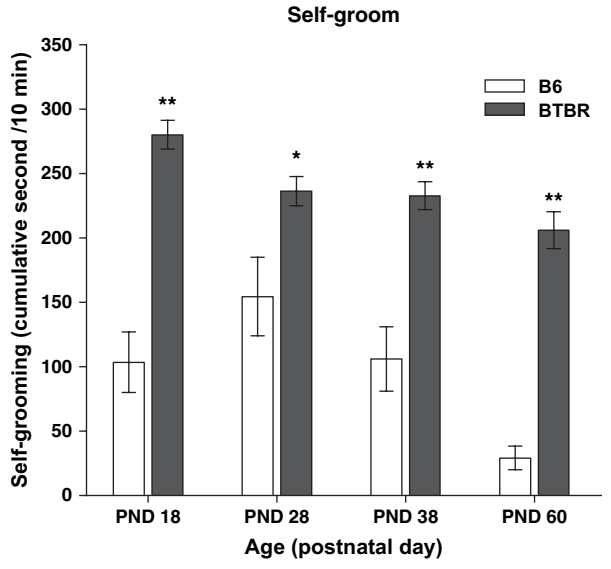

Figure 4: Self-grooming in BTBR. High levels of self-directed grooming were evident in 18, 28, 38 and 60-day-old BTBR male mice placed alone in an empty mouse cage for 10 min, as compared with age-matched male B6 ($*P < 0.05$ at PND 28, $**P < 0.01$ at PNDs 18, 38 and 60). $n = 10$ per strain.

Consistent abnormalities in reciprocal social interactions were discovered in both juvenile and adult male BTBR mice, tested both during their day and night circadian phases. The present experiments with mice housed under reverse circadian conditions and tested in the dark phase, when mice are usually awake and socially active, detected the same social approach deficit in BTBR as previously reported with mice in another laboratory environment, housed under conventional circadian conditions and tested in the light phase (Moy *et al.* 2007). Concordance of findings was evident across the four social tasks, in which BTBR displayed lower scores on social approach to a stranger, reciprocal social interactions, juvenile play and social transmission of food preference. Low social interaction between BTBR observers and cagemate demonstrators during STFP appeared to reduce the amount of information transmitted from the cagemate's olfactory cues about novel foods. It is interesting to speculate that the reduced nose-to-nose sniffing observed in BTBR during both STFP and juvenile

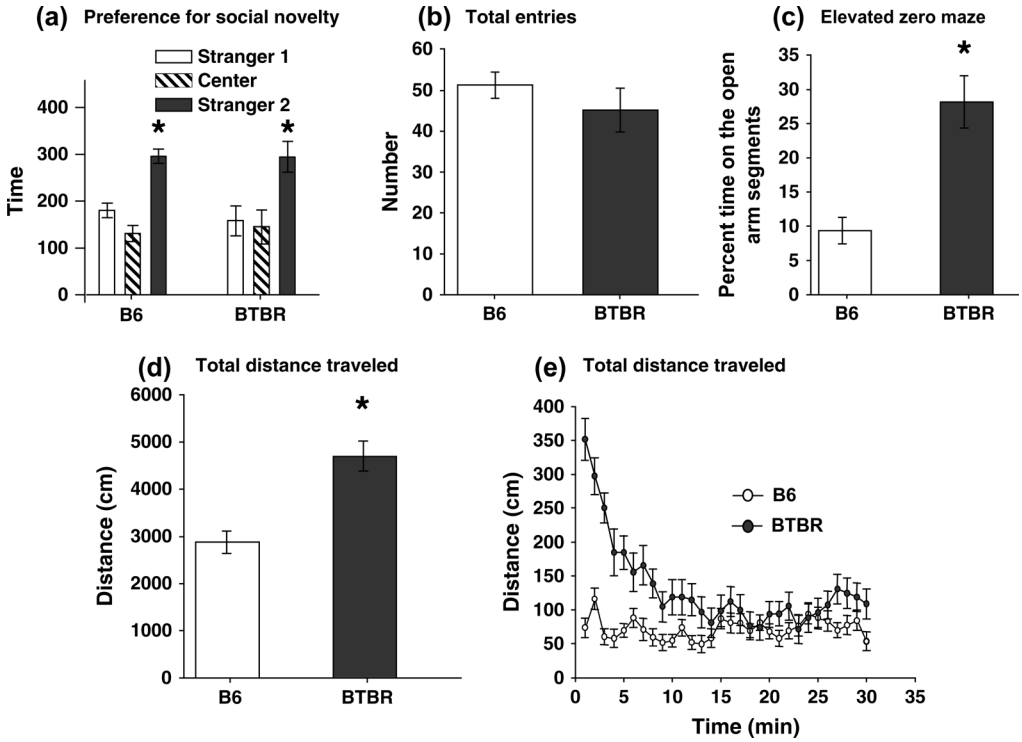

Figure 5: Intact procedural abilities in BTBR. (a) Both strains displayed normal preference for social novelty during the last 10-min session in the three-chambered automated social approach task. Time spent in the side chamber with a new stranger 2 was higher than time spent in the side chamber with the now familiar stranger 1 for both B6 ($*P < 0.0001$) and BTBR ($*P < 0.01$), indicating normal olfactory and other sensory abilities, sufficient to discriminate between two different stranger mice. b) Number of entries into the side chambers was not significantly different between the two strains, indicating similar nonsocial exploratory activity and normal motor functions. (c) Elevated zero maze analysis confirmed low levels of anxiety-related behaviors in both strains, with BTBR spending an even higher percentage of the 5-min session in the open segments of the zero maze than B6 ($*P = 0.0002$). (d) Total distance traveled in a standard Accuscan VersaMax open field was higher in BTBR in a 15-min Accuscan open field test at Wadsworth ($*P < 0.0001$), and (e) initially higher in BTBR ($P < 0.001$), and subsequently not different between BTBR and B6, in a 30-min test at NIMH, supporting the interpretation that reduced social approach, reciprocal social interactions and juvenile play were not caused by low-exploratory tendencies.

play is analogous to the reduced eye contact that is commonly seen in autistic individuals, relevant to communication deficits (DSM-IV 2000; Kanner 1943; Lord *et al.* 2001; Losh & Piven, 2007; Muhle *et al.* 2004; Volkmar *et al.* 2004). Excessive repetitive self-grooming in BTBR juveniles and adults may be conceptually analogous to the repetitive motor stereotypies and self-stimulation common in autism (DSM-IV 2000; Lord *et al.* 2001; Militerni *et al.* 2002; Symons *et al.* 2005; Volkmar *et al.* 2004). Furthermore, our previous inbred strain survey detected normal learning but a selective failure to reverse a spatial habit in the Morris water maze by BTBR (Moy *et al.* 2007), potentially representing a

Table 1: SNP differences between B6 and BTBR in *Kmo* coding regions resulting in amino acid changes. Based on data available for 17 common inbred strains for these SNPs, the B6 allele is most frequently observed. The rare BTBR allele is identified in bold. Potential functional relevance of the amino acid residue change is indicated by the domain type. Rs3254044 and rs32535929 are involved in flavin adenine dinucleotide (FAD) binding. In rs3235930, the amino acid residue is located within the mitochondrial membrane (transmembrane). Conservation of the amino acid between human and mouse may indicate the importance of this residue within the functional protein. Reference SNP ID and SNP information was obtained from NCBI dbSNP build 126 (http://www.ncbi.nlm.nih.gov)

Reference SNP ID	Gene location	Allele	AA residue	Domain type	Human/mouse AA conservation
rs32540044	Exon 9	C/**T**	C250R	FAD binding	No
rs32535929	Exon 13	**A**/G	K374R	FAD binding	No
rs32535930	Exon 13	C/**T**	L386F	Transmembrane	Yes

perseverative trait analogous to the autistic insistence on sameness (DSM-IV 2000; Lord *et al.* 2001; Volkmar *et al.* 2004).

Several other intriguing mouse models of autism have reported impressive social deficits, communication abnormalities or repetitive behaviors (Boylan *et al.* 2007; Brodkin 2007; Brodkin *et al.* 2004; Carter 2007; Cheh *et al.* 2006; Hammock & Young 2006; Kuemerle *et al.* 2007; Kwon *et al.* 2006; Levitt 2005; Lewis *et al.* 2007; Mineur *et al.* 2006; Moretti *et al.* 2005; Shu *et al.* 2005; Spencer *et al.* 2005; Zoghbi 2005). Some of these lines of mice display behavioral traits relevant to one or two of the three diagnostic criteria for autism. BTBR is remarkable for incorporating unusual behavioral traits with putative face validity to all three diagnostic criteria for autism. Several of the other mouse models display impaired locomotion or anxiety-related traits that limit an interpretation of specific social abnormalities. In BTBR, the present control measures and those previously reported (Moy *et al.* 2007) confirmed normal scores on learning, olfaction, social recognition, exploratory activity toward the high range and low anxiety-like traits. In our initial strain distribution, BTBR was the only strain to show reduced social approach in the absence of hypoactivity in the open field and open arm aversion in the elevated plus-maze (Moy *et al.* 2007). The absence of anxiety-related and motor confounds supports the interpretation that BTBR displays highly selective behavioral abnormalities that model the defining symptoms of autism. It will be interesting to evaluate additional BTBR behaviors with face validity to associated symptoms of autism, which include mental retardation, seizures, anxiety, sleep disruption, idiosyncratic hypersensitivity to sensory stimuli, gastrointestinal disturbances, larger head circumference and brain volume, and impaired attentional disengagement (DSM-IV 2000; Lord *et al.* 2001; Muhle *et al.* 2004; Piven *et al.* 2007; Volkmar *et al.* 2004). Assays at even earlier ages may show additional parallels to the neurodevelopmental aspects of autism. Comparisons of male vs. female BTBR will be useful to address the 4:1 prevalence of autism in boys vs. girls.

BTBR is a minimally characterized inbred strain, originally developed in 1956 from the tufted mutation T bred into a 129 strain (described in http://www.informatics.jax.org/external/festing/mouse/docs/BTBRTF.shtml) and occasionally used as a background strain in diabetes and phenylketonuria research (Clee *et al.* 2005; Ranheim *et al.* 1997; Shedlovsky *et al.* 1993). BTBR has been included in several inbred mouse strain distributions for traits including rotatod deficits during ethanol intoxication, water maze performance, four-arm water maze escape and antidepressant-like response to citalopram in the tail suspension test (Crowley *et al.* 2005; Rustay *et al.* 2003a,b; Wahlsten *et al.* 2005). The first neuroanatomical investigation of BTBR showed a severely reduced hippocampal commissure and absent corpus callosum (Wahlsten *et al.* 2003). Corpus callosum abnormalities have been reported in some autistic individuals (Alexander *et al.* 2007; Barnea-Goraly *et al.* 2004; Egaas *et al.* 1995; Piven *et al.* 1997). Genetic analysis of the X chromosome contrasting BTBR with BALB/cByJ showed two quantitative trait loci that may be responsible for the callosal abnormalities in BTBR (Kusek *et al.* 2007).

Autism spectrum disorders are currently diagnosed by behavioral abnormalities, while the underlying etiologies including genetic contributions remain elusive. It is increasingly recognized that autism will not be defined by a single gene mutation, but by complex interactions between multiple genes, influenced by neurodevelopmental and environmental factors (Blasi *et al.* 2006; Dong & Greenough 2004; Muhle *et al.* 2004; Polleux & Lauder 2004; Ronald *et al.* 2006; Spence *et al.* 2006; Veenstra-Vanderweele *et al.* 2004). By analogy, we do not expect a single gene mutation to underlie autistic-like phenotypes in BTBR. However, genetic differences such as coding polymorphisms in genes associated with autism warrant further investigation. Our initial SNP database analysis for 124 putative candidate genes identified from the autism literature (Polleux & Lauder 2004) sought to identify genetic differences between BTBR and B6 that might explain their social behavior differences. Querying current databases for SNPs between BTBR and other inbred strains yielded one promising lead, *Kmo*, a gene encoding a protein that affects excitatory neurotransmission (Sapko *et al.* 2006; Yu *et al.* 2004). *Kmo* produces the kynurenine 3-hydroxylase protein, an enzyme that is indirectly involved in the regulation of kynurenic acid synthesis. Kynurenate, a glutamate and nicotinic receptor antagonist, may play a role in neuroprotection, dendritic spine formation and dopamine release (Alkondon *et al.* 2004; Hilmas *et al.* 2001; Sapko *et al.* 2006; Wu *et al.* 2007; Yu *et al.* 2004). Unusual levels of kynurenic acid have been implicated in other neuropsychiatric diseases including schizophrenia and Huntington's disease (Sapko *et al.* 2006; Schwarcz *et al.* 2001). The *Kmo* exon 13 coding region polymorphisms identified in our SNP database search were sequenced and confirmed. The three intriguing SNPs in *Kmo* were found only in BTBR compared with documented SNP data from 12 other inbred strains. The contribution of these polymorphisms to the behavioral abnormalities observed in BTBR is currently under investigation.

Three additional genes yielded positives in our BTBR SNP analyses. The coding polymorphism in the serotonin transporter, *Slc6a4*, is particularly interesting because it has been linked to several neuropsychiatric diseases including autism (Devlin *et al.* 2005). However, in our B6 vs. BTBR comparison, the unusual *Slc6a4* SNP was detected in B6, not in BTBR, making it less likely that this polymorphism contributes to the behavioral abnormalities present in BTBR. The other two SNPs were for *Smo*, coding for smoothened, a signaling protein regulated by Sonic hedgehog, and *Pkd1*, coding for the polycystic kidney disease protein. However, the polymorphisms for *Smo* and *Pkd1* were distributed evenly among many inbred strains, with neither BTBR nor B6 being the outlier.

It is interesting to note that the allelic distribution of these SNPs, wherein some alleles present in BTBR are shared with other inbred strains but not with B6, is consistent with the notion that genetic differences mediating the behavioral traits of autism may be present in the normal population, but the full autism syndrome occurs only when high numbers of the relevant mutations are clustered in one individual, analogous to alleles clustered in one inbred mouse strain (Nadler *et al.* 2006; Ronald *et al.* 2006). Our first pass at SNP database

mining for BTBR alleles also identified hundreds of noncoding SNP differences within the introns and UTRs of these 124 candidate genes (data not shown), that may alter gene regulation or expression, whose contribution to autistic-like phenotypes will be even more difficult to interpret. New approaches are needed to explicate the contributions of these coding and noncoding polymorphisms to the observed behavioral abnormalities in BTBR.

A fundamental question in the autism field is whether the same or different genetic and neurobiological mechanisms underlie each of the three diagnostic symptoms (Hurley *et al.* in press; Ronald *et al.* 2006; Sung *et al.* 2005). A new approach is genetic analysis of the broader autism phenotype, to search for genes underlying each symptom domain in nuclear family members who do not meet all three diagnostic criteria, but represent a continuum of severity of impairments in one or more domains (Hurley *et al.* in press; Losh & Piven 2007; Sung *et al.* 2005). Inbred strains closely related to BTBR, such as 129 substrains and LP/J (Petkov *et al.* 2004), offer research tools analogous to the broader autism phenotype. The forward genetics approach, highlighted by our discoveries in the BTBR inbred strain, presents a similar research tool for dissecting out differential genetics and neurobiology of sociability, communication and repetitive behaviors in mice, to show common vs. divergent underlying mechanisms (Crawley 2004; Moy *et al.* 2007), Furthermore, the relatively rapid SNP strategy, although still in its infancy, appears promising for dissecting genetic polymorphisms related to complex behavioral traits in inbred strains of mice (Chesler *et al.* 2005). Discovery of mouse genes responsible for deficits in a subset of autism-related traits, including social interaction, communication, perseveration and associated symptoms, will suggest new candidate genes to search for polymorphisms in clinical databases of autistic genomes.

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Supplementary material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1601-183X.2007.00330.x. Please note that the file sizes are large for some of these files.

Figure S1: Illustrations of behavioral tasks.

Video S1: Typical sociability in the automated social approach apparatus.

Video S2: Subject mouse sniffs a stranger mouse in the automated three-chambered social approach task.

Videos S3 and S4: Repetitive self-grooming in BTBR T+ tf/J in the Noldus Observer Phenotyper arena.

Appendix S1 Legends etc.

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