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Somatosensorimotor and odor modification, along with serotonergic processes underlying the social deficits in BTBR T + Itpr3^{tf}/J and BALB/cJ mouse models of autism

Hiroyuki Arakawa, PhD^{*,1,2}

¹Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, MD, USA

²Rodent Behavioral Core Department of Research Administration, Case Western Reserve University School of Medicine, Cleveland, OH, USA

Abstract

Autism is a complex spectrum of disorders characterized by core behavioral deficits in social communicative behavior, which are also required for comprehensive analysis of preclinical mouse models. As animal models of the core behavioral deficits in autism, two inbred mouse strains, BTBR T+ Itpr3^{tf}/J (BTBR) and BALB/cJ (BALB), were compared with the standard social strain, C57BL/6J (B6), regarding a variety of behavioral factors underlying social communicative interactions, including olfactory and tactile sensory processes, social recognition abilities and behavioral expression strategies. Although both female BTBR and BALB mice can express social recognition and approach behavior depending on the stimuli they encounter, the available sensory modalities, along with modulation of the serotonergic system, differ between the two strains. BALB mice have deficits in using volatile olfactory cues and tactile information in a social context; they fail to exhibit a social approach to volatile cues and seek nonvolatile cues by exhibiting substantial sniff/contact behavior when allowed direct contact with social opponents. Systemic injection of the serotonin (5-HT_{1A}) agonist buspirone has little effect on these social deficits, suggesting a congenitally degraded serotonergic system in BALB mice. In contrast, BTBR mice exhibit impaired body coordination and social motivation-modified olfactory signals, which are relevant to a reduced social approach. A systemic injection of the 5-HT_{1A} agonist restored these social deficits in BTBR mice, indicating that a downregulated serotonergic system is involved in the social deficits exhibited by BTBR mice.

Keywords

Social approach; Odor signal; Tactile function; Serotonin; Autism model

^{*}Corresponding author: Hiroyuki Arakawa, PhD, Department of Anatomy and Neurobiology, University of Maryland School of Medicine, 20 Penn St. HSFII/S251, Baltimore, Maryland, 21201, USA, harakawa@som.umaryland.edu, Phone: 1-410-706-2620.

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Introduction

Neurodevelopmental disorders such as autism spectrum disorder (ASD), are characterized by social behavioral deficits (Kennedy and Adolphs, 2012), including persistent deficits in effective social and communicative interactions and the maintenance of social relationships (Kogan et al., 2009). Laboratory mice are often used as models to investigate the neural mechanisms underlying human disorders, and, thus, various assays and measurements have been proposed for mouse models to elucidate a primary behavioral phenotype of ASD (Ricceri et al., 2007; Wöhr, 2014; Kazdoba et al., 2016). Mice are a social species and, as such, engage in a wide array of social interactions and a social structure that mimics fundamental components of human social behavior (Kazdoba et al., 2016; Arakawa & Iguchi, 2018). One requirement for working successfully with preclinical mouse models is the necessity to account for species-specific/typical differences in the construct of interest. Laboratory mice are nocturnal; thus, they primarily use olfaction along with tactile sensations to determine the expression of adaptive behaviors in social settings (Eisenberg and Kleiman, 1972). Thus, it is important to understand the species-typical features of sensory modalities that underly the performance of social behavior.

ASD-related behavioral deficits are measured through interactions between two mice. The sociability test, also known as the three-chamber social preference test, is widely used to evaluate various aspects of social behavior relevant to ASD symptoms such as social approach and preference, as well as social novelty discrimination (Nadler et al., 2004; Silverman et al., 2010a). In this test, the behavioral response of the test mouse is recorded with respect to a (novel) conspecific (i.e., “stimulus” mouse) placed in an inverted, wire-mesh or grid cup on one of the three chambers. Social approach is measured as the increased ratio of investigation time of the cup containing the stimulus mouse over an empty cup. The holding cup of the stimulus mouse permits only limited contact between the two mice. The mesh or the wire bars of the holding cup impedes whisker tactile exploration and contact (specifically facial and anogenital region)-mediated olfaction via nonvolatile odors (Arakawa and Iguchi, 2018); thus, mice are capable of using only volatile olfactory cues in this limited social setting.

In direct social interaction, mice seek further information through physical contact via typically whisking at and sniffing the facial and anogenital areas of stimulus mice (Arakawa et al., 2011). For example, BALB/cJ (BALB) inbred mouse strains are characterized by low sociability, as expressed by a lower amount of social approach compared with the social standard strain, C57BL/6 (B6) mice in the social preference chamber setting (Brodin et al., 2004; Sankoorikal et al., 2006). However, when allowed direct contact with stimulus mice (i.e., male versus male interactions), BALB mice engage in a substantial amount of social contact, especially the anogenital regions, and discrete responses to the social features of opponents (Arakawa, 2018). Therefore, it is imperative to evaluate the details of the characteristics of social behavior/interactions in mice that primarily use olfactory and tactile cues to assess social features.

Several studies examining social and sensory functioning have implicated a specific neural pathway in the regulation of social approach/investigation, including the serotonergic (5-HT)

system (Gould et al., 2011; Beis et al., 2015). Essential olfactory processing does not require the 5-HT system (Carlson et al., 2016); however, social odor preference (e.g., approach) does require this system, as demonstrated using neuronal 5-HT synthesis knockout mice (Tph2^{-/-}) (Kane et al., 2012; Zhang et al., 2013). Accordingly, multiple lines of evidence support a linkage between social approach and modulation of serotonergic 5-HT_{1A} receptors (Thompson et al., 2007; Akimova et al., 2009). A systemic injection of 5-HT_{1A} agonist produces increased sniffing in response to volatile social odor and approach toward unfamiliar conspecifics (Arakawa, 2017; Gould et al., 2011), and robustly decreases the flight response in the approached animals (Arakawa, 2017). Of particular interest is that BALB mice, which genetically possess a low level of 5-HT synthesis (>70% reduction) along with higher anxiety compared with the standard B6 strain (Bach et al., 2011), fail to exhibit social approach/preference when allowed to access only volatile odorants (Sankoorikal et al., 2006; Brodtkin, 2007), while maintaining intact social recognition and general olfactory abilities (Arakawa, 2017). Male BALB mice also fail to exhibit social preference to a stimulus mouse that receives a 5-HT_{1A} agonist, which enhances social preference in confronted B6 mice (Arakawa, 2017).

In the present study, we will further investigate multisensory social behavioral strategies in BALB strain mice and in another ASD mouse model, BTBR T+ Itpr3^{tf}/J (BTBR) (McFarlane et al., 2008; Meyza and Blanchard, 2017) to assess and compare mechanistic differences. BTBR mice exhibit a reduced social approach to a juvenile stimulus mouse (Yang et al., 2009; McFarlane et al., 2008; Jones-Davis et al., 2013) and to an adult stimulus mouse (Bolivar et al., 2007; Moy et al., 2007; Defensor et al., 2011) in the social preference test setting. BTBR mice typically exhibit a reduced motive to approach social opponents in several social situations in which mice are permitted direct contact (Pobbe et al., 2010; Pearson et al., 2012; Martin et al., 2014). We hypothesize that the availability of sensory inputs (olfactory versus tactile), along with modulation of the 5-HT system, is a key factor in regulating the deficit of social approach behaviors in these two strains, and, thus, we will find a mechanistic difference in sensory processes and social strategies linked to the 5-HT system between these strains. The outcome(s) of the present comparison will critically inform the mechanistic processes of social approach behaviors expressed in the social preference chamber, along with the central mechanism for species-typical social behaviors that we need to appropriately evaluate for further studies in preclinical mouse models of ASD.

Materials and methods

Animals.

We performed all animal procedures according to the regulations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with approval by the Case Western Reserve University School of Medicine Institutional Animal Care and Use Committee and the University of Maryland School of Medicine Institutional Animal Care and Use Committee. We obtained C57BL/6J (B6) mice (Stock No: 000664), BALB/cJ (BALB) mice (Stock No: 000651), and BTBR T+ Itpr3^{tf}/J (BTBR) mice (Stock No: 002282) from the Jackson Laboratories (Bar Harbor, ME, USA) as breeders, and maintained and bred

them in the colony room of the facility in the University of Maryland School of Medicine. The colony room was temperature-controlled at 22 °C, with a humidity of approximately 50% under a 12-h light–dark cycle (lights on at 6:00 a.m.). Mice were maintained in standard shoe-box cages (32 × 20 × 16.5 height cm) with water and food provided ad libitum. All mice were housed in groups of three to four of same sex siblings. Behavioral tests were performed when subject mice (all females) reached an age of at least 12 weeks. Juvenile BTBR mice as a stimulus mouse for the odor preference test were provided at an age of approximately 25–32 days. All test trials were conducted during the light phase of the light/dark cycle under dimly lit conditions of the experimental room.

Immunohistochemistry

To visualize the barrel cortex, adult mice of all three strains (B6, BALB, and BTBR, each n=5) were perfused with 4% paraformaldehyde and the dissected brains were immersed in the same fixative for 2 – 4 h while cryoprotected (30% sucrose in 0.1 M phosphate buffer saline (PBS); pH 7.4). One hemisphere was separated from the brain, flattened between glass slides (1 mm space) overnight, and tangentially sectioned at a thickness of 60 µm with a vibratome (Leica 1000S). The other hemisphere was serially cut in the coronal plane into 60-µm-thick frozen sections. Alternate series of sections were used for cytochrome oxidase (CO) staining and double-staining (Vglut2 and NeuN) of immune-histochemistry.

For CO histochemistry, we incubated flattened cortex sections in cytochrome C solution consisting of PBS with diaminobenzidine and 10% sucrose until the sections turned gold brown in color. Then, we rinsed the sections in PBS three times, mounted them on glass slides, and cover-slipped them. For immunohistochemistry, free-floating sections were processed; the sections were treated with normal donkey serum (10%) and 0.5% Triton X-100 in PBS for 2 h. After several rinses, we incubated the sections in two antibody solutions at 4°C overnight. The primary antibodies were guinea pig polyclonal VGluT-2 antibody (1:4000; Millipore Bioscience) and mouse polyclonal NeuN antibody (1:1000; Millipore Bioscience)(Arakawa et al., 2014). After the primary antibodies were washed three times, we applied fluorescent secondary antibodies (CY3-conjugated donkey anti-guinea pig, 1:500, and CY2-conjugated donkey anti-mouse, 1:500, Jackson ImmunoResearch) at room temperature for 2 h. The sections were agitated on a rotator between each incubation and rinse step. Following this incubation, the sections were washed three times and mounted on glass slides, dried, and cover-slipped.

We examined the sections under epifluorescence and photographed regions of interests, using filters of different wavelengths appropriate for the fluorescent tag of the secondary antibodies.

Drugs

The serotonin agonist buspirone (a serotonin 5-HT_{1A} receptor partial agonist; Sigma-Aldrich) was dissolved in sterile saline (0.9%, pH 7.4) at a concentration of 3 µg/ml. An intraperitoneal injection of buspirone (3 mg/kg) was administered to each subject mouse (in the social preference test and social interaction test) or stimulus mouse (in odor preference test) 30 min before the test sessions.

Behavioral test experimental design:

We conducted a series of behavioral tests to assess the details of the somatosensory and recognition abilities relevant to mouse-typical social behaviors, and social interaction processes between females and to investigate the anti-anxiety (buspirone) effect on these processes in female B6, BALB, and BTBR mice. Our test battery consisted of a thorough set of somatosensory tasks including the Von Frey touch test (tactile sense), adhesive removal test (tactile sense), gap-crossing test (whisker function), bridge walking test (whisker function), and rotarod test (body coordination) (n=12 for each strain); recognition tasks including the object recognition test (recognition/approach process) and social recognition test (odor-based recognition of social and strain differences) (n=12–14 for each strain); social behavior tasks including the social preference test (odor-based social preference) (n=12 per group of vehicle and buspirone injection in each strain) and social interaction test (full contact interaction) (n=13 for B6 vehicle, n=12 for BALB and BTBR vehicle and buspirone injection); and social odor preference test (n=12 B6 females for each stimulus condition). Testing was performed in tandem with small groups of mice consisting of three cohorts of animals at different times.

Somatosensory tasks

Tactile touch sense (von Frey touch test): The von Frey test is used for the assessment of the tactile touch sense. To assess mechanical touch sensitivity, calibrated von Frey monofilaments (Stoetling Co., IL, USA) were used to measure the paw withdrawal threshold and sensitivity. Mice were allowed to acclimate for 7 min in an inverted dark-screened Plexiglas chamber (10 cm cube) with a wire mesh grid bottom (1 cm spacing), allowing access to the plantar surface of the hind paw. When all four paws were touching the wire mesh, filaments (ranging from 0.07–8.0 g) were applied to the plantar surface of each hind paw for 1–2 s with an inter-stimulus interval of at least 10 s, while being careful to avoid the toes, heels, and pads. Sudden paw withdrawal, sudden flinching, or sudden paw licking were considered withdrawal responses. The paw withdrawal threshold was calculated using the up-down method with 20 trials. Beginning in the middle of the filament series (1.4 g), ascending (if no response was observed a stronger stimulus was applied) or descending (if a response was observed) stimuli were applied. The resulting pattern of positive and negative responses was calculated using the 50% response threshold formulated from the mean of the logarithmic tabular values [$\log_{10}(\text{value g} \times 10000)$] for the pattern of positive and negative responses (Bradman et al., 2015). The responsivity of withdrawal was determined using a standard monofilament (1.0 g; 4.08 mN) applied 10 times to the plantar hind paw, with an inter-stimulus interval of at least 15 s. These data were then expressed as a response ratio to a 1.0 g stimulus (Detloff et al., 2010).

Adhesive removal test: We selected the adhesive removal test (Albertsmeier et al., 2007; Bouet et al., 2009) to assess dorsal column-lemniscal sensitivity. We placed an adhesive-backed label (0.5-cm square) on the palmar side of the hind paw and recorded the latency of the first reaction to the stimulus (sniffing, paw lifting, biting, and removal of the label). We performed the test in a clean home cage setting.

Gap crossing test: To test whisker tactile function, the gap crossing test was performed. The test consists of a series of trials that require the mouse to cross a gap between two glass cups with variable distances. We placed the subjects in the glass cup (10-cm diameter) that was elevated (20-cm height) and connected to another same-sized, dark-screened glass cup (face-to-face). The gap distance between the glass cups varied from 0 to 8 cm by 1-cm increments. We measured the gap distance (in centimeters) that the mice were able to cross to get to the dark-screened glass cup with a 5-min cut-off period. We performed the trials at the beginning under dim lighting and then switched to infrared lighting (0 lux) so that the mice could see the glass cups initially and then relied on tactile rather than visual cues to cross the gap.

Bridge walking test: To test whisker somatosensory function, the bridge walking test was conducted in two trials (training and test) for each mouse (Arakawa et al., 2014). Each mouse was placed on the edge of a 30-cm runway (4cm width and 15-cm height) connected via a gap (2 cm below) to a platform. Each mouse was required to cross the bridge and gap to reach the safe platform under infrared lighting (0 lux) conditions. The latency to reach the platform was recorded in each of the two trials. The latency for the first trial represented mouse anxiety and exploratory behaviors along with whisker tactile abilities, but those for the second trial were purely representative of whisker tactile ability.

Rotarod test: The rotarod test was performed to assess body coordination abilities, which would include whisker and tactile senses and somatosensory coordination (Deacon, 2013). For the constant speed rotarod task using a rotarod (3 cm diameter, Pan Lab), mice were given 2 trials (upto 60 s) in the same day, with the rotarod rotating at 0 rotations per minute (rpm) in the first habituation trial and 4 rpm in the second trial. The habituation data were excluded from the results section. For the accelerating rotarod task accelerations from 4 to 40 rpm in 300 s. with a speed increase of 1 rpm per 8.5 s for 3 trials (20-min ITI) were administered during the same day. The latency for the mice to fall from the rotarod was measured, and the average of the latencies was taken as a representative value for each mouse.

Habituation/dyshabituation test to an object and stimulus mouse—The test was conducted in a standard shoe-box cage with clean corn cob bedding, the top of which was covered with clear Plexiglas to videotape the behavior of the mice from above. In the object session, each subject mouse was placed in the test cage for 2 min for acclimation. Next, a small object (wooden Easter egg, wooden cube, or flowerpot) was placed at the wall side of the cage (Fig. 3A), and the mouse was allowed to investigate it for 3 min. The order of the objects was counterbalanced for each animal. At the end of the 3-min trial, the object was removed, and the mouse was kept in the test cage for 3 min as an inter-trial interval. The trials were repeated four times with the same object, and in the fifth discrimination trial, a novel object was presented.

In the social session that was conducted 3 days after the object session, each subject mouse was placed in the test cage, in which an empty wire-mesh bin (6-cm diameter, 7.5-cm height, 3-mm square grids) was placed at the wall side of the cage for a 2-min acclimation (Fig. 3E). Next, a wire-mesh bin containing a stimulus mouse of either adult familiar cagemate (same

strain) or adult stranger (different strain of either B6, BALB, or BTBR) and the subject mouse was allowed to investigate the mouse bin for 2 min, in which direct physical contact between animals was prevented but olfactory and auditory cues could be received. The trial was conducted four times with a 5-min inter-trial interval. In the fifth discrimination trial, a novel mouse of either adult familiar (e.g., cagemate → other cagemate) or adult stranger (e.g., BALB → BTBR) was presented. To evaluate the investigation, the duration that the subject mouse spent in proximity with sniffing of the object or bin (<3-cm distance of the snout) was measured. The interval between sessions (stranger vs. familiar) was 1 week.

Social preference test—The apparatus for the social preference test has been described elsewhere (Arakawa, 2017). The chamber consists of an open-topped box made of acrylic (58 × 24 × 24 cm) and divided into three chambers (2 compartments, 24-cm cube, and an inter-connected box, 10 × 24 × 2 cm) with two clear acrylic walls. Dividing walls had retractable doorways allowing access to each chamber. Two inverted grid bins (7.5-cm diameter, 1-cm grids space) were placed by the wall of each compartment.

Following a 15-min acclimation to the experiment room, the subject mice received an ip injection of vehicle or buspirone. Thirty minutes later, the mice were placed in the center box, and then the doorways to the side chambers were opened to allow the subject mice to freely explore for 8 min. During the habituation phase (empty), each of the two side chambers contained an inverted empty bin. Following the empty phases, an unfamiliar mouse (either B6, BALB, or BTBR, but not cagemates) was enclosed in one bin in a side chamber. The order and location of the stimulus mouse were counterbalanced. After these social exposure sessions, the test subjects were returned to their home cages, and the apparatus was cleaned with 15% ethanol and dried with a paper towel.

The behaviors of the subject mice were video-recorded and analyzed in terms of exploratory behaviors when orienting to the grid bins using video tracking software (ANY-maze, Stoetling Co.). The duration that a subject mouse spent in proximity to each bin was measured. Proximity was defined when the body center of the subject mice (view from above) was within reach of the wire-mesh bins (less than 4-cm distance). This measure reflects contact with the stimulus mice via a wire mesh as well as exposure to social cues. The time during which a subject mouse climbed on top of the bins was excluded from the analysis. Preference was calculated by a 8-min test duration as the percentage of time spent in proximity to a stimulus mouse compared with the total time spent in proximity to empty and stimulus mouse bins.

Social interaction test—The social interaction session was conducted in the same setting as the previous social recognition session (i.e., a clean home cage setting). Following a 15-min acclimation to the experiment room, the subject mice were injected with a vehicle or buspirone solution. After 30 min remained in their home cages, each subject mouse was placed in the test cage (a regular shoe-box cage with bedding) for a 3-min acclimation. Next, a stimulus mouse of either strain (B6, BALB, or BTBR) was introduced in the test cage for 3 min. Both the subject and stimulus mice were allowed to directly contact each other. Following a 3-min interaction, the stimulus mouse was moved back to its home cage and the subject mouse remained in the test cage. The trial was continued 3 times with 3-min

intertrial intervals. The order of the stimulus mice exposed was counterbalanced for each animal.

Social interactions between mice were videorecorded and later analyzed. Evaluated behaviors included sniff, duration of time that an animal's nose was oriented and in proximity to the opponents' body (mainly facial, side, and anogenital area of the opponent, within a 2-cm distance); social contacts, the duration an animal laid in direct contact with another animal for more than 1 s; sniff by the stimulus mouse, the duration of time that a stimulus mouse spent time for sniffing of the subject mice; and evasion, defined as the percentage of events of quick locomotion away from an approaching animal during sniffing/approaching by stimulus mouse.

Odor preference test—Following a 30-min acclimation to the experiment room, the subject mice (all female B6) were exposed to the social preference chamber containing two wire-mesh bins placed by a wall of each compartment in the social preference test. In this test, the initial session consisted of two empty bins as a habituation (Empty), and the following two sessions included one of the wire-mesh bins with a stimulus mouse of either strain (B6, BALB, adult BTBR, or juvenile BTBR). These stimulus mice were given an injection of either vehicle or buspirone 30 min before the test sessions. Each stimulus mouse was exposed to up to two subjects during the sessions. After the 3 exposure sessions, the test subjects were returned to their home cages, and the apparatus was cleaned with 15% ethanol, dried using a towel, and blown to remove air/odor with a fan.

The behaviors of the subject mice were video-recorded and analyzed in terms of exploratory behaviors when orienting to the wire-mesh bins using ANY-maze software, as performed in the social preference test. Behaviors to be evaluated included the duration of time that a subject mouse spent in proximity to each bin and the distance the subject mouse traveled during the sessions. Time during which a subject mouse climbed on top of the bins was excluded from the analysis. Preference was calculated as the percentage of time spent in proximity of the social bin over the empty bin.

Statistical analysis

All data are expressed as the mean $\pm$ the standard error of the mean (SEM). We used one-way analysis of variance (ANOVA) for the statistical analysis of measurements of somatosensory tasks, with between-subject factors of strain (B6, BALB, or BTBR), including the von Frey test, adhesive removal test, gap-crossing test, bridge walking test, and rotarod test. We used one-way ANOVA for the measurement of object recognition, with within-subject factors of trials (T1 to T5). We used two-way ANOVA for statistical analysis of the measurements of social recognition tasks, with within-subject factors of trials (T1 to T5) and stimulus mice (different vs. same strain). For the social preference test and odor preference test, we tested equal variances by the F-test, followed by the Student's t-test for the values between empty vs. Social. The values of the preference ratio were analyzed by two-way ANOVA with a within-subject factor of the stimulus mice and a between-subject factor of drug (vehicle vs. buspirone). For the social interaction test, we first used a two-way ANOVA for measurements of vehicle-injected mouse conditions, with a within-subject

factor of the stimulus mice (B6, BALB, or BTBR) and a between-subject factor of the subject mice (B6, BALB, or BTBR). Second, we used one-way ANOVA on the measurements of vehicle-injected mice confronted with a same strain of stimulus mouse, with a between-subject factor of pairs (B6-B6, BALB-BALB, or BTBR-BTBR). Third, we used two-way ANOVA for the measurement of social interactions for each strain of subject mice, with a within-subject factor of the stimulus mice (B6, BALB, or BTBR) and a between-subject factor of the drug (vehicle vs. buspirone). We used Bonferroni correction for post hoc comparisons when necessary. We tested equal variances by the F test and found equal variances and normality for all data distributions. For all tests, differences were accepted as significant at $p < 0.05$. All statistical analyses in the present experiments were performed using R statistical software version 3.3.3.

Results

Tactile sensory tests

Sensory information from whiskers are clustered into whisker-related bundles (Erzurumlu & Kind, 2001; Petersen, 2007). We used CO histochemistry and immunohistochemistry for glutamate transporters (VGluT2; thalamocortical marker) along with neuron-specific nuclear protein (NeuN; neuronal marker), to assess the formation and morphological structures of whisker-related patterns in the barrel cortex of these three strains of mice (Fig. 1). CO histochemistry of the cortical barrel depicts clear barrel field patterning for each representative whisker cluster in B6 and BALB mice, but weak expression of the boundaries of CO-stained patches in BTBR mice. To simultaneously examine the distribution of cortical neurons in the barrel, double labeling with VGluT2 and NeuN was conducted with tangential and coronal sections. The localization of vesicular glutamate transporters can represent the interior septa structures of the barrel cortex (Liquz-Lecznar & Skangiel-Kramska, 2007). In the interior barrel septa, the neurons surround the barrel structure of the septa, forming an organized pattern (Ballester-Rosado et al., 2010). Therefore, double immunohistochemistry staining of Vglut2 and NeuN confirmed the structured barrel formation and patterning in the barrel cortex of B6 mice; each whisker-related VGluT2-positive patch was matched with one patch of NeuN-positive neurons, with the majority of neurons lining up at the boundary. In contrast, diffuse VGluT2-positive patches were observed in BALB and BTBR mouse cortices, particularly for the septa clusters related to whiskers. Almost no whisker-related organization in the BTBR cortex was revealed by NeuN staining. These representative images suggested that cortical glutamatergic neurons that form specific patterning in the barrel of B6 mice were disrupted in the BALB cortex and disorganized in the BTBR cortex, indicating a disturbance in coordinated development of synaptic components of thalamocortical connections.

Given the comparison of morphological representations in the barrel cortex for these three strains, we first monitored the three strains of female mice by applying commonly used behavioral tests relevant to whisker-tactile sense and body coordination. A whisker-independent tactile touch sense was assessed by the von Frey hair touch sense test (Fig. 2A,B). The thresholds for withdrawal responses of mice to filament stimulations applied to the plantar surface of each hind paw indicated that BALB mice displayed significantly

greater thresholds than control B6 mice ($p < 0.0001$), while BTBR mice exhibited lower thresholds than B6 mice ($p < 0.0001$), $F_{(2,33)}=37.344$, $p < 0.0001$. The impairment of the tactile sense in BALB mice was confirmed by the responsivity of von Frey touch stimulation, in which BALB mice exhibited lower responsivity than either B6 mice ($p = 0.01$) or BTBR mice ($p < 0.0001$), $F_{(2,33)}=9.19$, $p = 0.0007$. We further assessed the tactile sense of these mice by the sticky tape test (Fig. 2C), and confirmed that BALB mice exhibited an impaired tactile sense compared with either B6 ($p = 0.0094$) or BTBR mice ($p = 0.0053$), $F_{(2,33)}=6.7713$, $p < 0.0034$.

We assessed the whisker-barrel function by the gap-crossing test (Fig. 2D). Mice utilize the whisker tactile sense to assess the gap. BALB mice crossed a shorter (3.67 ± 0.40 cm) gap distance than either B6 mice (4.92 ± 0.22 cm) ($p = 0.0218$) or BTBR mice (5.08 ± 0.34 cm) ($p = 0.0186$), $F_{(2,33)}=5.1057$, $p = 0.0117$. A similar impairment of whisker sense in BALB mice was found in the bridge-walking test (Arakawa et al., 2014). Mice must use the whisker tactile sense to cross the narrow, elevated bridge in the darkness (Fig. 2E). BALB mice required more time to cross the bridge than either B6 ($p = 0.0219$) or BTBR ($p = 0.0135$), $F_{(2,33)}=4.7003$, $p = 0.016$. These whisker sense tests clearly indicated an impairment of whisker function in BALB mice. Furthermore, we tested motor coordination abilities in these mice, which are relevant to sensorimotor function (Fig. 2F). The average scores in three trials of the rotarod performance test revealed significant impairment in both BALB mice ($p = 0.0044$) and BTBR mice ($p < 0.0001$) compared with B6 mice, $F_{(2,33)}=19.487$, $p < 0.0001$. The scores for BTBR mice were worse than those for BALB mice ($p = 0.0047$).

Social recognition tasks

We investigated the recognition abilities of these mice using the habituation/dyshabituation paradigm (Arakawa, 2018), including a novel object and social investigation with an adult stimulus mouse of a different or the same strain.

In the object recognition test shown in Fig. 3A, when confronted with a small object in a shoe-box test cage, all B6, BALB, and BTBR mice exhibited similar profiles of performance to detect and differentiate objects. In the first trial, all mouse strains spent more time investigating the object, and the investigation time decreased over trials with habituation; B6 [$F_{(4,44)} = 5.1296$, $p = 0.0018$](Fig. 3B), BALB [$F_{(4,44)} = 6.9486$, $p = 0.0002$](Fig. 3C), or BTBR [$F_{(4,44)} = 17.4669$, $p = 0.0001$](Fig. 3D). In the fifth trial, all mouse strains exhibited a significant increase in time spent sniffing a novel object as a dyshabituation; 4th trial vs. 5th trial: B6 ($p = 0.0387$), BALB ($p = 0.0269$), and BTBR ($p = 0.0009$).

In the odor recognition test, the subject mice were placed in a clean shoe-box cage with bedding and confronted with a wire-mesh bin containing a stimulus mouse (Fig. 3E). In this case, the subject mice could smell and see a stimulus mouse via a wire-mesh but were incapable of engaging in full physical contact. Fig. 3F shows the mean time B6 mice spent investigating the bin containing the same (B6) or a different (BALB or BTBR) strain of stimulus mouse that was initially an unfamiliar female for the first four trials and then containing a novel (e.g., same strain: B6→B6, different strain: BALB→BTBR) female in trial 5. In the first trial, B6 mice spent more time investigating a different strain of stimulus mouse than those same strains ($p = 0.0018$). In the fifth trial, B6 mice exhibited an extended

time investigating a novel, different strain of stimulus mouse compared with a novel, same strain of stimulus mouse ($p = 0.0219$). B6 mice showed a greater amount of investigation of a different strain of stimulus mouse in the first trial, which decreased over trials (trial 1 vs. 4; $p = 0.0327$). Subsequent exposure to a novel, different strain of stimulus mouse restored the investigation time in the fifth trial (dishabituation, $p = 0.0102$). However, B6 mice showed a similar amount of investigation of the same strain of stimulus mouse over trials through trials 1 to 5, $F_{(4,104)} = 0.3737$, $p = 0.8269$ [strain, $F_{(1,26)} = 1.2587$, $p = 0.2722$, trials, $F_{(4,104)} = 2.6915$, $p = 0.0351$, and strain x trials, $F_{(4,108)} = 3.661$, $p = 0.0078$).

Fig. 3G depicts the mean time of BALB mice spent investigating the bin containing either the same (BALB) or different (B6 or BTBR) strain of stimulus mouse that was initially an unfamiliar female for the first four trials and then containing a novel (as previously) female in trial 5. BALB mice displayed a reduced time sniffing the opponent compared with the different strain ($p = 0.0013$), but a longer time sniffing the opponent compared with the different strain ($p = 0.0245$). When confronted with the same strain of opponents, there were no differences in the investigation time throughout the trials. In contrast, BALB mice displayed more sniffing in the first trial compared with later trials; vs. trial 2 ($p = 0.0194$), vs. trial 3 ($p = 0.0258$), vs. trial 4 ($p = 0.0013$), or vs. trial 5 ($p = 0.0969$), and subsequent exposure to a novel, different strain of the stimulus mouse increased the investigation time in the fifth trial (dishabituation, $p = 0.0007$) compared with the fourth trial [strain, $F_{(1,22)} = 0.0609$, $p = 0.8074$, trials, $F_{(4,88)} = 7.6031$, $p < 0.0001$, and strain x trials, $F_{(4,88)} = 7.2999$, $p < 0.0001$).

Fig. 3H displays the mean time BTBR mice spent investigating the bin containing either the same (BTBR) or a different (B6 or BALB) strain of stimulus mouse, in which the same stimulus mouse was exposed over 4 trials and then a novel (as previously), stimulus mouse was exposed in the fifth trial. In the first ($p = 0.0006$) and fifth trial ($p = 0.0004$), BTBR mice spent more time investigating the bin containing a different strain of stimulus mouse than those containing the same strain of stimulus mouse. When confronted with the same strain of stimulus mice, BTBR mice exhibited more time investigating, which decreased over the trials [1st trial vs. 2nd: $p = 0.3994$], 1st trial vs. 3rd trial: $p = 0.0296$, 1st trial vs. 4th trial: $p = 0.026$, and 1st trial vs. 5th trial: $p = 0.1259$]. The BTBR mice displayed a similar amount of investigation of a novel stimulus mouse vs. trial 4 and trial 5) ($p = 0.5425$). Conversely, when confronted with the different strain of stimulus mice, BTBR mice showed a rapid reduction of investigation over the trials (1st trial vs. 2nd: $p = 0.0004$, 1st trial vs. 3rd trial: $p < 0.0001$, 1st trial vs. 4th trial: $p < 0.0001$, and 1st trial vs. 5th trial: $p = 0.5425$); the investigation time was restored in the fifth trial (vs. 4th trial: $p < 0.0001$) [strain, $F_{(1,22)} = 7.1195$, $p = 0.014$, trials, $F_{(4,88)} = 28.654$, $p < 0.0001$, and strain x trials, $F_{(4,88)} = 11.014$, $p < 0.0001$].

Social preference test

The social approach behavior of these three strains of mice was tested by the three-chamber social preference test (Crawley, 2004)(Fig. 4A). In this test, mice were exposed to an empty bin and a grid bin containing a stimulus mouse for 8 min, and then all the mice spent substantial time investigating in proximity to the two bins. Supplemental Fig. 1A–F displays

the duration of sniffing investigation of either a wire-mesh bin containing a stimulus mouse (B6, BALB, or BTBR) or an empty bin (empty). When the subject B6 mice were introduced into the test chamber (Fig. 4A), B6 mice investigated both two bins (empty vs. social (i.e., empty)) equally during the test, $t_{11} = 0.6819$, $p = 0.2549$. The B6 mice then spent an increased amount of time investigating the mouse bin (social) in response to the B6 stimulus mouse, $t_{11} = 3.624$, $p = 0.002$, and BALB stimulus mouse, $t_{11} = 3.4334$, $p = 0.0028$, but not the BTBR mouse, $t_{11} = 0.0053$, $p = 0.4979$. When the subject B6 mice were injected with buspirone 30 min before the session (Supplemental Fig. 1D), the B6 mice investigated two empty bins equally during the initial test, $t_{11} = 0.0952$, $p = 0.4629$. Then, B6 mice that received a buspirone injection spent an increased amount of time investigating the mouse bin (social) in response to the B6 stimulus, $t_{11} = 11.056$, $p < 0.0001$, and BALB stimulus mouse, $t_{11} = 6.7302$, $p < 0.0001$, but not the BTBR mouse, $t_{11} = 0.8149$, $p = 0.2162$.

To compare the effect of buspirone injection, the scores of the B6 mice were calculated as the percentage of preference toward a wire-mesh bin containing a stimulus mouse over an empty bin (Fig. 4B). B6 mice that received either vehicle or buspirone injection showed a 50% (chance level) preference when exposed to two empty bins, while these B6 mice exhibited an increased preference for the B6 mouse bin ($p < 0.0001$) and BALB mouse bin ($p < 0.0001$), but not for the BTBR mouse bin ($p = 0.5456$). B6 mice that received buspirone showed an increased preference than B6 mice that received vehicle, $F_{(1,22)} = 4.8997$, $p = 0.0375$, stimulus effect: $F_{(3,66)} = 20.7212$, $p < 0.0001$, and treatment x stimulus: $F_{(3,66)} = 0.834$, $p = 0.48$.

Subject BALB mice exhibited a completely different profile of social approach behavior than B6 mice (Fig. 4C). As an initial trial, BALB mice investigated both bins (empty vs. social) equally, $t_{11} = 1.057$, $p = 0.1566$, and the BALB mice maintained these equal investigations toward either the B6 stimulus mouse, $t_{11} = 0.3535$, $p = 0.3652$, BALB stimulus mouse, $t_{11} = 0.483$, $p = 0.3193$, or BTBR stimulus mouse, $t_{11} = 0.3828$, $p = 0.3546$ (Supplemental Fig. 1B). These tendencies were maintained when BALB mice were injected with buspirone 30 min before the session (Supplemental Fig. 1E); thus, there were no differences in investigation time between empty and social when exposed to empty, $t_{11} = 1.5046$, $p = 0.0803$, B6 stimulus mouse, $t_{11} = 0.3186$, $p = 0.378$, BALB stimulus mouse, $t_{11} = 1.3581$, $p = 0.1008$, or BTBR stimulus mouse conditions, $t_{11} = 1.1378$, $p = 0.1397$. Therefore, buspirone injection had no effects based on the calculation of the percentage of preference toward the social bin over the empty bin [treatment, $F_{(1,22)} = 0.1843$, $p = 0.6719$, stimulus effect: $F_{(3,66)} = 0.4321$, $p = 0.7307$, and treatment x stimulus: $F_{(3,66)} = 0.1097$, $p = 0.9541$] (Fig. 4C).

Supplemental Fig. 1C,F displays the duration of sniffing investigation expressed by BTBR mice toward either an empty wire-mesh bin or a social (B6, BALB, or BTBR) bin. BTBR mice investigated both bins (empty vs. social (i.e., empty)) equally during the initial empty trial, $t_{11} = 0.1703$, $p = 0.4339$. Then, BTBR mice maintained equal investigation periods toward a bin containing the B6 stimulus mouse, $t_{11} = 0.4558$, $p = 0.3287$, or BALB stimulus mouse, $t_{11} = 0.1212$, $p = 0.4529$, and a decreased time investigating a bin containing a BTBR mouse, $t_{11} = 2.3411$, $p = 0.0195$. When the subject BTBR mice received a buspirone injection 30 min before the session (Supplemental Fig. 1F), the BTBR mice investigated the

two empty bins equally during the initial test, $t_{11} = 0.063$, $p = 0.4755$. Then the buspirone-injected BTBR mice spent an increased time investigating the mouse bin (social) in response to the B6 stimulus mouse, $t_{11} = 3.017$, $p = 0.0059$, and BALB stimulus mouse, $t_{11} = 2.4958$, $p = 0.0149$, but not the BTBR mouse, $t_{11} = 1.1191$, $p = 0.1435$.

To assess the effect of buspirone injection, the preference scores of BTBR mice were calculated (Fig. 4D). BTBR mice that received either vehicle or buspirone injection showed a 50% (chance level) preference when exposed to two empty bins, while either vehicle-injected or buspirone-injected BTBR mice exhibited an increased preference for the B6 mouse bin ($p = 0.047$) and BALB mouse bin ($p = 0.047$), but not the BTBR mouse bin ($p = 0.2068$). BTBR mice that received a buspirone injection showed a significantly increased preference compared with those that received a vehicle injection, $F_{(1,22)} = 6.2919$, $p = 0.02$, stimulus effect: $F_{(3,66)} = 5.3451$, $p = 0.0023$, and treatment x stimulus: $F_{(3,66)} = 0.7854$, $p = 0.5063$.

Social interaction test

In the social interaction test, subject mice could directly contact and interact with stimulus mice in familiar shoe-box cages over 3 trials consisting of 3-min sessions. The subject mice displayed a variety of social behaviors and interactions with a stimulus mouse, and thus, the data were analyzed based on the investigation and physical contacts of subject mice oriented to the stimulus mice and the subsequent responses of the subject mice during the interactions (Figs. 5 and 6).

First, the social interaction data were analyzed only in vehicle-injected mice, with factorial interactions of the subject mice (B6, BALB, or BTBR) and the stimulus mice (B6, BALB, or BTBR)(Fig. 5). Supplemental table 1 summarizes all the statistical analyses of the behavioral measurements, including sniff, social contact, sniff by stimulus mice, and evasion. Briefly, the subject mice exhibited sniff varied depending on the strain of the stimulus mice; they showed an increased sniff to a BALB stimulus mouse, but a decreased response to a BTBR stimulus mouse. The duration of social contacts observed during the social interaction tests also varied depending on the strains of the stimulus mice. The subject mice of either strain showed increased social contacts when confronted with the stimulus mouse of the BALB strain. Accordingly, the BALB strain stimulus mice exhibited a greater amount of sniff to the subject mice than those of either the B6 or BTBR strain. The subject mice of either strain consistently expressed a higher evasion to approaches of BTBR stimulus mice than those of BALB stimulus mice.

Second, the effects of buspirone injection on the social interactions of mice were analyzed for each mouse subject strain (B6, BALB, or BTBR). Supplemental table 2 shows a summary of the statistical results for these scores of social interactions. Buspirone-injected mice tended to show decreased amounts of sniff time compared with vehicle-injected mice, when BALB subject mice were confronted with BALB stimulus mice or BTBR subject mice were confronted with either B6 or BTBR stimulus mice. An increased sniff following buspirone injection was found only when B6 subject mice were confronted with BTBR stimulus mice. In contrast, the buspirone injection was discretely effective in the following behavioral measurements. Both B6 and BTBR subject mice that received a buspirone

injection showed enhanced contacts when they were exposed to either B6 or BTBR stimulus mice, but not to BALB stimulus mice. BALB subject mice showed little impact of buspirone injection on contact behavior. In a similar fashion, the stimulus mice of either strain showed a substantially increased sniff to buspirone-injected subject mice of either B6 or BTBR strain, but not to those of the BALB strain. Finally, the buspirone-injected subject mice of all strains consistently exhibited a reduced evasion to the approaches of either stimulus mouse compared with the vehicle-injected subject mice.

Odor preference test

We assessed the odor characteristics of stimulus mice that received a buspirone injection by the odor preference test paradigm. B6 mice as a subject were placed in the 3-chamber apparatus as shown in the social preference test and allowed to investigate a wire-mesh bin containing a stimulus mouse that was given either vehicle or buspirone injection (Fig. 7A).

When confronted with a B6 stimulus mouse (Fig. 7B), B6 subject mice exhibited a similar investigation of the two empty bins, $t_{11} = 0.5839$, $p = 0.571$, and discrete investigation of the vehicle-injected B6 stimulus mouse, $t_{11} = 4.95$, $p = 0.0004$, and buspirone-injected B6, $t_{11} = 23.614$, $p < 0.0001$ (Supplemental Fig. 2). The odor preference of B6 mice revealed that the social bin containing vehicle-injected B6 was preferred to empty bins ($p = 0.0003$), and the social bin containing buspirone-injected B6 was preferable to those containing vehicle-injected B6 ($p = 0.038$), $F_{(2,22)} = 43.1685$, $p < 0.0001$ (Fig. 7B).

When confronted with a BALB stimulus mouse via a wire-mesh bin (Fig. 7C), B6 subject mice displayed similar investigation time of the two empty bins, $t_{11} = 0.0811$, $p = 0.9369$, and a longer investigation time of a social bin than an empty bin, containing either vehicle-injected BALB stimulus mice, $t_{11} = 5.7848$, $p = 0.0001$, or buspirone-injected BALB stimulus mice, $t_{11} = 9.7429$, $p < 0.0001$. B6 subject mice preferred to investigate the social bin containing either vehicle-injected BALB ($p = 0.0051$) or buspirone-injected BALB ($p < 0.0001$) than the empty bin, $F_{(2,22)} = 15.9558$, $p = 0.0001$ (Fig. 7C). There was no difference in preference between buspirone- and vehicle-injected mouse bins ($p = 0.7939$).

When exposed to social bins containing adult BTBR stimulus mice (Fig. 7D), B6 subject mice exhibited similar investigation times of the two empty bins, $t_{11} = 0.6736$, $p = 0.5144$, and of a social bin containing a vehicle-injected adult BTBR vs. an empty bin, $t_{11} = 0.0927$, $p = 0.9278$. B6 subject mice showed a longer investigation time of a social bin containing a buspirone-injected adult BTBR over an empty bin, $t_{11} = 4.1607$, $p = 0.0016$. The preference scores confirmed these tendencies (Fig. 7D); there was no difference between the empty bin preference and vehicle-injected BTBR preference ($p = 0.4924$), but B6 subject mice preferred investigating a social bin containing a buspirone-injected adult BTBR mouse than either an empty ($p = 0.0139$) or vehicle-injected BTBR ($p = 0.0317$), $F_{(2,22)} = 7.4377$, $p = 0.0034$.

Finally, the exposures of social bins containing juvenile BTBR stimulus mice that received a vehicle or buspirone injection revealed quite distinguishable responses from those toward adult BTBR stimulus mice in B6 subject mice (Fig. 7E). As a control, B6 subject mice showed similar investigation times of the two empty bins, $t_{11} = 0.2673$, $p = 0.7942$. Then,

B6 subject mice exhibited a longer investigation time of a social bin containing a vehicle-injected juvenile BTBR stimulus mouse over an empty bin, $t_{11} = 5.0194$, $p = 0.0004$; however, B6 subject mice did not exhibit any differences in investigation between a social bin containing a juvenile BALB mouse with buspirone and those with a juvenile BALB mouse with vehicle treatment, $t_{11} = 1.8476$, $p = 0.0917$. The preference ratio calculated in response to B6 subject mice confronted with a juvenile BTBR illustrated that B6 subject mice preferred the odor from a juvenile mouse with a vehicle injection ($p = 0.0004$), but not those with a buspirone injection ($p = 0.1725$), $F_{(2,22)} = 7.0448$, $p = 0.0043$ (Fig. 7E).

Discussion

Analyses of sequential communicative behaviors in mice along with olfactory and tactile sensory abilities that corporately regulate social behaviors have illustrated a more profound, complicated process between subject and stimulus mice (Hurst & Beynon, 2004; Arakawa & Iguchi, 2018). BALB mice have impaired sensorimotor processes including whisker (gap crossing) and non-whisker (Von Frey) tactile hyposensitivity and body coordination (Rotarod), which are accompanied by modification of the cortical barrel structure. When social contacts are limited (social preference test), BALB mice consistently fail to express social preference, indicating a poor responsivity to volatile social cues. As compensation, BALB mice use nonvolatile cues to assess the social features of opponents, resulting in heightened sniff and contact when allowed full contact with them. These behavioral strategies in BALB mice were not altered by 5-HT1A agonist injection, which indicates that a degraded 5-HT system, including its receptors, is involved in the social behavioral characteristics of BALB mice. In contrast, BTBR mice may have a modulated tactile sense and body coordination deficit, along with a fussy cortical barrel structure. BTBR mice reduced social preference and sniff/contact, regardless of physical contact availability; however, they have intact olfactory abilities. In particular, BTBR mice demonstrate impaired elimination of social olfactory signals that stimulate approach behavior in odor recipients following maturation, which is linked to the social impairment of this strain demonstrated in the social preference and interaction test paradigms. A 5-HT1A agonist could restore these modifications of social preference, contact, and olfactory signaling in BTBR mice, suggesting that downregulation or modulation of the 5-HT system partially underlies the social deficits in these mice.

First, we examined the cortical barrel pattern and structure in the standard B6 strain and the ASD-like BALB and BTBR strains. B6 mice exhibited regularly structured barrel formation and a patterned neuronal structure, while BALB mice exhibited patterned barrel formation but with an incomplete structure (e.g., congested neurons on the boundary of barrel septa), and BTBR mice had mostly lost the patterned array of neural structures in the barrel cortex. The BALB strain is an albino and has reduced visual performance compared with the B6 strain (Yeritsyan et al., 2012) due to a deficit in cortical representation (Heimel et al., 2007). A gross deficiency in the corpus callosum (Wahlsten, 1989; Morin et al., 1994; Fairless et al., 2008) and difference in cortical volume compared with the substrain (BALB/cByJ)(van Heukelum et al., 2019) have been reported in the BALB/cJ strain, which would be responsible for the modified pattern of barrel structures in BALB strains. Moreover, BTBR mice are characterized by alterations in brain development and morphology, including an

absent corpus callosum (Wahlsten et al., 2003), along with callosal abnormalities (Kusek et al., 2007; MacPherson et al., 2008) and altered functional connectivity networks (Dodero et al., 2013; Ellegood et al., 2013, 2015; Miller et al., 2013). The neural cratered structure of the barrel field is developmentally constructed throughout the neonatal period (Iwasato & Erzurumlu, 2018), and the interconnection between contralateral barrel cortices via the corpus callosum plays a key role in forming the pattern of the barrel structure (Ballester-Rosado et al., 2010; Erzurumlu and Gaspar, 2012). Therefore, the deficiency in neural connectivity in the corpus callosum would be relevant to the disturbance of the pattern of the barrel structure commonly observed in these particular BALB and BTBR strains.

Whiskers are essential for several somatosensory tasks, such as gap/cliff distance assessment(s), shape and texture discrimination, and coordinated body movements (O'Connor et al., 2010; Deschenes et al., 2012). We found a significant impairment of whisker function (cf. Gap crossing) and the whisker-independent tactile sense (cf. Von Frey test) in BALB mice, but no impaired whisker function and a complementary sensitive whisker-independent tactile sense in BTBR mice. Accordingly, BTBR mice were less likely to use their whiskers, particularly while searching gaps, and tended to jump across the gap (personal observation) with slipping of the foot (B6, total slips, 4 per 63 total crossings; BALB, total slips, 2 per 44 total crossings; and BTBR, total slips, 27 per 62 total crossings). Furthermore, both BALB and BTBR mice exhibited substantially impaired performance in the rotarod test compared with B6 mice, suggesting that coordinated body abilities that are partially associated with whisker-related body coordination and more relevant to somatic sensorimotor function (Schaar et al., 2010) were impaired in these mice.

Adequate social interaction between mice requires cognitive abilities in recognizing individual characteristics (social recognition) and expressing appropriate behavioral responses to the conspecifics (investigatory strategy) (Ellegood & Crawley, 2015). The habituation/dyshabituation paradigm, developed to assess object and social recognition abilities (Thor et al., 1982), demonstrated that all three mouse strains exhibited normal object and social recognition. In this situation, in which the subject mice could not directly contact the stimulus mice, all B6, BALB, and BTBR mice were able to distinguish differences in social individuals of the different strain; however, they were unable to distinguish individual cage mates of the same strain via volatile odor cues. Accordingly, individual mice within the same inbred strain possess genetically identical volatile odor cues (Nevison et al., 2001; Hurst & Beynon, 2004). These data illustrate intact recognition ability along with the detection of social olfactory cues in these ASD model, BALB, and BTBR mice.

The social preference test consists of exposing a subject mouse to wired or grid holding cups containing a stimulus mouse, which prevents direct contact between the animals (Arakawa & Iguchi, 2018). In this situation, the subject mouse is able to use olfactory cues along with auditory cues emitted by the stimulus mouse, but limited to access tactile cues, and the stimulus mouse is restricted from moving and posturing, limiting the natural interaction between the two animals (Fairless et al., 2013). This controlled situation in the social preference test is similar to those in the social recognition test, in which all three strains of mice demonstrated an intact ability to recognize strain differences via olfactory cues. Thus,

we can evaluate purely the preference behavior/motive of these strains toward the social sources in the social preference test. While B6 subject mice consistently exhibited social preference to the B6 and BALB stimulus mice, both BALB and BTBR subject mice failed to exhibit social preference to stimulus mice of either strain, which is consistent with previous findings (Gould et al., 2011; Yang et al., 2012; Scattoni et al., 2013; Arakawa, 2017). Interestingly, B6 mice failed to exhibit social preference to BTBR stimulus mice, and BTBR mice exhibited avoidance to exposure to the same strain BTBR stimulus mice. These findings indicate that BTBR (stimulus) mice have olfactory characteristics that inhibit approach behavior in odor recipients, and even induce avoidance in the same strain (BTBR) of odor recipient mice. BTBR mice also demonstrate impaired social signaling in the auditory context, such as the ultrasound vocalization (Wöhr et al., 2011, 2014; Scattoni et al., 2013), and the olfactory context, such as scent marking (Wöhr et al., 2011), and the social transmission of food preferences (McFarlane et al., 2008). Similarly, BTBR mice display motor-related modifications, such as perseverative self-grooming (McFarlane et al., 2008; Pearson et al., 2011; Amodeo et al., 2012) or altered exploratory behavior (Moy et al., 2008; Pearson et al., 2011), along with cognitive deficits (Moy et al., 2007; McTighe et al., 2013), indicating that a broad range of morphological and cognitive changes are involved in BTBR behaviors.

It has been reported that the 5-HT system is involved in the expression of social preference in B6 and BTBR mice (Arakawa, 2017; Gould et al., 2011). Systemic administration of buspirone, a 5-HT_{1A} receptor partial agonist, in B6 and BTBR mice enhanced the social preference to either strain of the stimulus mouse. Conversely, buspirone injection had little impact on social preference in BALB mice. BALB mice genetically exhibit a low level of 5-HT synthesis (>70% reduction) (Bach et al., 2011), along with serotonergic manipulation hyposensitivity (Arakawa, 2017) and, thus, a congenitally degraded 5-HT system. BTBR mice have been reported to exhibit altered serotonin transporter and 5-HT_{1A} receptor function (Gould et al., 2011). The density of serotonin axons is reduced and increases in the compensatory number of serotonin neurons and responsivity are observed in BTBR mice (Guo & Commons, 2017). Therefore, 5-HT agonists, such as buspirone or fluoxetine, have little effect on social preference in BALB mice, but enhance social preference in BTBR mice (Gould et al., 2011; Chadman, 2011; Cai et al., 2019), suggesting that differences in 5-HT processing could be a key mechanism for discriminating these two ASD-like mouse model strains.

In the social interaction test, the subject mice were permitted full contact with the stimulus mice, and they engaged in naturalistic interactions with one another in a familiar home cage environment. The strain differences in social interactive behaviors indicated that BALB subject mice tended to exhibit more sniff and contact with the stimulus mice compared with either B6 or BTBR mice, while BTBR mice were likely to exhibit a reduced sniff and higher evasion to being approached by the stimulus mice than the other strains. However, these behavioral trends depended highly on the strain of the opponents (i.e., stimulus mice) and, thus, the types of social pairing. For example, BALB stimulus mice tended to receive substantial amounts of sniff and contact from the subject mice, and BTBR stimulus mice tended to receive reduced sniff and contact from subject mice. In this context, female and male BALB mice (Arakawa, 2017), failed to exhibit social preference when allowed to

access only volatile social cues, as shown in the social preference test, while maintaining intact (or robust) sniff/contact with the stimulus mice when allowed full contact (e.g., Fairless et al., 2013; Arakawa, 2018). Although social behaviors are regulated by multiple mechanisms (Arakawa & Iguchi, 2018), the availability of tactile sensory cues (i.e., via direct contact) primarily mediate social strategies in BALB mice.

Buspirone has anti-anxiety effects and enhances social investigation in mouse models (Gao & Cutler, 1993; Loane & Politis, 2012). An acute injection of buspirone induced ambiguous inhibitory effects on sniff behaviors in the subject (i.e., buspirone-injected) mice, while it increased social contacts in B6 or BTBR mice, but not in BALB mice. Furthermore, buspirone robustly suppressed the evasion of subject mice to the approaches of the stimulus mice. These strain-independent effects of buspirone imply that 5-HT_{1A} receptor activation is relevant to the blunted responsivity of subject mice in situations that allow direct social contact with one another. Moreover, buspirone injection of subject B6 or BTBR mice—but not BALB mice—induced a profound increase in sniff by the stimulus mouse of all three strains. These data were consistent with those in the odor preference tests, clearly indicating that buspirone injection in B6 or BTBR mice, but not BALB mice, changed the olfactory cues emitted from buspirone-injected mice and, thus, the mice that detected these olfactory cues increased sniff behaviors toward the buspirone-injected B6 or BTBR mice. As such, we argue that an enhancement of social interactions by buspirone is systemically attributable to a decreased evasion and elimination of the social (olfactory) signal by the buspirone-injected mouse, which enhances sniff by odor recipients of B6 and BTBR mice. BALB mice may have a deficit of the processing of 5-HT-relevant social olfactory signals, but intact 5-HT-induced suppression of the evasion.

In addition, the results of the odor preference tests indicated that (1) the release of olfactory cues that activate sniff in odor recipients is regulated by a 5-HT mechanism in both B6 and BTBR strains; (2) this change in olfactory cues by 5-HT modulation is not observed in the BALB strain and juvenile BTBR strain; and (3) the odor properties in adult BTBR mice that inhibit approach in odor recipients emerge after the juvenile stage. Systemic buspirone injection mainly activates 5-HT_{1A} receptors in the forebrain (Garcia-Garcia et al., 2014). The 5-HT_{1A} receptor-positive neurons activate the release of olfactory signals that have amicable properties in both male and female B6 and BTBR mice (e.g., Arakawa, 2017). The 5-HT_{1A} receptor system is known to be altered developmentally (Donaldson et al., 2014); an acute injection of 5-HT_{1A} agonist decreases the social approach in juvenile mice (Payet et al., 2018), but increases it in adult mice (Loane & Politis, 2012). Moreover, developmental blockade of 5-HT_{1A} receptors during the neonatal or juvenile period results in a residual increase in the anxiety phenotype, in contrast to a similar antagonism during adulthood (Lo Iacono & Gross, 2008; Vinkers et al., 2010). In this fashion, the functions of 5-HT_{1A} autoreceptors are developmentally modulated (Donaldson et al., 2014), which would be relevant to the weak, if not absent, effect of the 5-HT_{1A} agonist on social odor properties in juvenile BTBR mice. It is noteworthy that both male and female BTBR mice typically express a developmental transition in fur/coat appearance during the pubertal stage (personal observation), during which fur/hair loss occurs, accompanied by the secretion of an oily fluid that covers the coat. These phenotypes may be relevant to a pubertal change in olfactory properties in BTBR mice.

BALB mice are known to be highly anxious in a variety of anxiety test paradigms (Belzung & Griebel, 2001; Bouwknecht & Paylor, 2002) and highly aggressive compared with other inbred strains (Southwick & Clark, 1968; Mondragon et al., 1987). The present study used female mouse subjects, which could exclude these behavioral features of BALB mice, especially higher aggression, from the present test situations. Additionally, the impact of the estrous cycle on social behavior between female mice would be negligible given that the present study assessed same-sex (female to female) investigation and interaction (e.g., sexual behavior in females, Kim et al., 2016). Considering clinical evidence that ASD is a heterogeneous neurodevelopmental disorder, the male:female ratio ranges from 3 to 4:1 (Yeargin-Allsopp et al., 2003; Kogan et al., 2009), relatively few studies have used female mice as subjects, and the results of these female BTBR data are not consistent (e.g., no sex difference (Silverman et al., 2010b; Rein et al., 2019), females exhibited social preference (Defensor et al., 2011), females avoid novel objects compared with social stimuli (Amodeo et al., 2019), and no preference in choice test between BTBR versus B6 stimulus mouse (Ryan et al., 2019). This may be attributable to differences in the stimuli used and its exposure. In the present test, mice were exposed to a choice test between empty bin versus social bin, and the social stimuli were exposed separately (e.g., 1. B6 → 2. BALB → 3. BTBR) in a counterbalanced order. Thus, the subject mice could distinguish stimulus differences between sessions and express preference over an empty bin.

Conclusion

Mouse models of ASD should be characterized according to behavioral deficits in social communicative behaviors between mice. Two inbred mouse strains of ASD models consistently demonstrate deficits in social preference in the social preference paradigm, in which mice can use only volatile social cues to assess the social sources. However, considering species-typical features of social communication, these two strains use different social strategies based on the availability of primary sensory modalities along with the 5-HT system underlying the mouse-specific social behaviors. The sensory communication of nocturnal mice heavily relies on the olfactory and tactile senses. BALB mice demonstrated strategic differences in sensorimotor communication, which rely on non-volatile olfactory investigation. The behavior of BTBR mice appears to be regulated by the 5-HT system. The present demonstration highlights the necessity for ASD translational studies to further investigate and focus on mechanistic properties underlying a deficiency in social communicative behaviors in mouse models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- BALB/cJ mice exhibited social contact but not approach to volatile cues
- BTBR mice failed to show social approach and contact
- BALB/cJ mice have tactile impairment linked to their social strategy
- BTBR mice release odor cues that induce avoidance in recipients
- 5HT agonist rescued social approach in BTBR but not in BALB/cJ mice

Figure 1.

Representative images of somatosensory barrel maps in barrel cortex of B6, BALB, and BTBR mice. Representative maps of CO-stained tangential sections through cortical layer IV prepared from B6 (A), BALB (B), and BTBR (C) mice (scale bar= 1000 μ m). The 20- μ m-thick belt encircling the hollow was defined as the barrel wall. The area between barrel walls was defined as the septum. CO-staining revealed whisker-related pattern in B6 and BALB mice, while the distinctive barrel walls were fuzzy in BTBR mice. VGluT2 (D, E, F) and NeuN (G, H, I) double labeling of tangential sections from the cortex of these three strains were applied to simultaneously visualize the distribution of thalamocortical axons clusters and cortical neurons (scale bar= 100 μ m). Unlike the discrete whisker-related VGIT2-positive patches in B6 mice, fuzzy VGluT2-staining patterns were found in BALB

and BTBR mice. NeuN staining revealed ring-like organizations of cortical neurons that form a border around the barrel clusters in B6 mice (**G**), while the ring-like organizations formed by cortical neurons cramming in the septum without border spaces between barrel clusters in BALB mice (**H**). No clear segregation of cortical neurons was observed in BTBR mice (**I**). VGluT2 staining of coronal sections prepared from these three mouse strains depicted enriched VGluT2-positive cells in cortical layer IV of B6 (**J**) and BTBR mice (**L**), while buzzy VGluT2-staining patterns were found in BALB mice (**K**). NeuN staining revealed discrete, sectioned structures encircled each hollow in B6 mice (**M**), while enriched neuronal wall (but relatively chunky inside the barrel patches) at the septum of BALB mice (**N**). These neural patterning structures shown by NeuN labeling were absent in BTBR mice (**O**).

Figure 2.

Somatosensory performances of female B6 (blue), BALB (red), and BTBR (yellow) mice (each N=12). **(A)** The logarithm thresholds for withdrawal responses to filament stimulations on the hindpaws and **(B)** the withdrawal responsivity to 1g filament stimulation on the hindpaws of B6, BALB, and BTBR mice in the Von Frey touch sense test. **(C)** Time latencies that the subject mice licked and removed a piece of adhesive tape attached on the hind paws. **(D)** The gap distance crossed in B6, BALB, and BTBR mice in the gap-crossing test. **(E)** Latencies of time that subject mice spent to cross a narrow bridge in the dark **(F)** Motor coordination and balance of these mice during each trial and the average time on the rotarod, manifested as the time that mice could remain on the accelerating rotating rod. All data are presented as means $\pm$ S.E.M.; * $p < 0.05$ compared to B6.

Figure 3.

Object and social recognition tests performed by female B6 (blue; N=14), BALB (red; N=12), and BTBR (yellow; N=12) mice using a habituation/dyshabituation paradigm throughout five trials. In the object recognition test (A), the subject mouse (B: B6, C: BALB, and D: BTBR) was confronted with an identical small object (Obj1) across the four trials (each 3 min) and then in the fifth trial, a novel object (novel) was placed. In the social recognition test (E), the subject mouse of either strain (F: B6, G: BALB, and H: BTBR) was confronted with a wire-mesh bin containing a stimulus mouse of either same strain with the subject (e.g., B6 subjects with B6 stimulus; circle) or different strain from the subject (e.g., B6 subjects with BALB stimulus; square). An identical stimulus mouse was exposed across the initial 4 trials and then in the fifth trial, a stimulus mouse was changed to a novel mouse (e.g., same: B6→B6, different: BALB→BTBR). Data are expressed as the mean investigation time $\pm$ S.E.M. Significant differences between trials compared with trial 1 (habituation), * $p < 0.05$, or trial 4 (dyshabituation), # $p < 0.05$, and between stimulus mouse (different vs. same), + $p < 0.05$.

Figure 4.

The percentages of social preference (time spent in social bin/total time spent in both empty and social bins; 50% = chance level) were calculated in B6 (**B**), BALB (**C**), or BTBR (**D**) mice that received a vehicle or buspirone injection (each N=12). Data are expressed as the mean percentages $\pm$ S.E.M. Significant differences between stimulus mouse (B6; blue, BALB; red, or BTBR; yellow) sessions compared to Empty session, $+p < 0.05$, and between vehicle (circle) vs. buspirone (square) injection, $\#p < 0.05$. n.s.; non-significant.

Figure 5.

Social interaction test performed by vehicle-injected subject mice of either strain, B6 (N=13), BALB (N=12), or BTBR (N=12) that were exposed to a stimulus mouse of either strain (B6; blue, BALB; red, or BTBR; yellow). Time spent sniff the stimulus mouse (**A**), time spent in physical contacts with each other (**B**), time spent sniff by the stimulus mouse (**C**), and the ratio that the subject mice exhibited evasion to the approaches of the stimulus mouse (**D**), in the social interaction test. Data are expressed as the mean values ± S.E.M. Significant differences between stimulus strains, * $p < 0.05$ and between subject strains, # $p < 0.05$. Significant main effects of stimulus (BALB) mice, + $p < 0.05$.

Figure 6.

Social interaction test performed by mouse pairs of either subject mouse, B6 (vehicle N=13 and buspirone N=12)(A, D, G, and J), BALB (each N=12) (B, E, H, and K), or BTBR (each N=12) (C, F, I, and L) that were injected with either vehicle (circle) or buspirone (square), and stimulus mouse (B6; blue, BALB; red, or BTBR; yellow). Time spent in sniff the stimulus mouse (A, B, and C), time spent in physical contacts each other (D, E, and F), time spent in sniff by the stimulus mouse (G, H, and I), and the ratio of evasion to the approaches of the stimulus mouse (J, K, and L). Data are expressed as the mean values ± S.E.M. Significant differences between stimulus mice compared to B6 stimulus mouse, # $p < 0.05$; between vehicle vs. buspirone * $p < 0.05$; and between stimulus mice, + $p < 0.05$. n.s.; non-significant.

Figure 7.

The percentages of odor preference (time spent in social/total time spent in both empty and social) in B6 subject mice (each N=12) were calculated by B6 (blue; **B**), BALB (red; **C**), adult BTBR (yellow; **D**), or juvenile BTBR (green; **E**) stimulus mice. Data are expressed as the mean percentages $\pm$ S.E.M. Significant differences between stimulus mice compared to empty (grey), # $p < 0.05$, and between stimulus mice (vehicle (circle) vs. buspirone (square)), + $p < 0.05$.