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Low Sociability is Associated with Reduced Size of the Corpus Callosum in the BALB/cJ Inbred Mouse Strain

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Abstract

The behavioral manifestations of autism, including reduced sociability (reduced tendency to seek social interaction), may be related to underdevelopment of the corpus callosum (CC). The BALB/cJ inbred mouse strain is a useful model system for testing the relationship between reduced sociability and CC underdevelopment. BALB/cJ mice show low levels of sociability, on average, but substantial intrastain variability in sociability, as well as striking variability in CC development. This study tested the hypothesis that sociability is positively correlated with CC size within the BALB/cJ inbred strain. 30-day-old BALB/cJ and C57BL/6J mice were tested for sociability towards gonadectomized A/J stimulus mice in a social choice task. The size of the corpus callosum was measured histologically at the mid-sagittal plane. BALB/cJ mice showed a significant positive correlation between the tendency to sniff the stimulus mouse and size of the CC relative to brain weight. C57BL/6J mice showed consistently high levels of sociability and normal corpus callosum development. These results suggest that abnormal white matter structure is associated with deficits in sociability in BALB/cJ mice. Additional studies are warranted to elucidate the relationship between brain connectivity and sociability in this model system.

Keywords

affiliative behavior; social approach; autism; brain; development; connectivity

1. INTRODUCTION

A core symptom domain of autism spectrum disorders is impairment in social behaviors, including reduced sociability (reduced tendency to seek social interactions). However, the

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biological basis of this reduced sociability is poorly understood (Hill and Frith 2003). A promising hypothesis is that long-range (e.g. interhemispheric) underconnectivity of the brain may underlie reduced sociability and other behavioral phenotypes of autism spectrum disorders (Belmonte et al., 2004; Geschwind and Levitt 2007). According to this hypothesis, underconnectivity may preferentially impair rapid processing of complex and novel stimuli that require coordination of lateralized brain functions, such as emotional and social behaviors (Paul et al., 2007). Reduced size of the corpus callosum (CC)—a type of structural brain underconnectivity—has been associated with impaired social (Alexander et al., 2007) and cognitive (Alexander et al., 2007; Just et al., 2007) functioning in humans. Moreover, reduced corpus callosum size, relative to total brain volume, is among the best-replicated structural brain phenotypes found in autism. (Alexander et al., 2007; Brambilla et al., 2003; Chung et al., 2004; Egaas et al., 1995; Hardan et al., 2000; Manes et al., 1999; Piven et al., 1997; Vidal et al., 2006; Waiter et al., 2005)

Given the experimental control they afford, mouse models are useful for testing hypotheses about the relationships between sociability and corpus callosum development. On average, BALB/cJ inbred mice display a low tendency to approach conspecifics for social interaction (i.e., low sociability) (Brodkin et al., 2004; Brodtkin 2007; Sankoorikal et al., 2006) and low levels of reward associated with social interaction (Panksepp and Lahvis 2007; Panksepp et al., 2007) when compared to other strains of mice, and especially compared to the highly sociable C57BL/6J strain. Moreover, the related BALB/cByJ inbred strain also shows comparatively low sociability (Moy et al., 2007).

Although BALB/cJ sociability is relatively low on average, sociability levels are highly variable within the strain, with particular BALB/cJ mice showing relatively high levels of sociability, and other BALB/cJ mice showing strikingly low levels (Brodtkin et al., 2004; Sankoorikal et al., 2006). Interestingly, BALB/cJ mice also show highly variable development of the corpus callosum, with some showing normal corpus callosum development, and others (~30-40% on average) showing either unusually small or no corpora callosa (Wahlsten 1974). The corpus callosum defect in a subset of BALB/cJ mice is due to a delay in formation of an interhemispheric bridge of tissue in the dorsal septal region during prenatal brain development (Wahlsten et al., 2006). This bridge is necessary for axons to cross the midline to form the corpus callosum.

In addition, other strains with corpus callosum (CC) defects have also shown relatively low sociability, in particular the BALB/cByJ, BTBR $T^+ tf/J$, and 129S1/SvImJ strains (Balogh et al., 1999; Moy et al., 2007; Wahlsten et al., 2003b). In contrast, the highly sociable C57BL/6J strain exhibits normally sized CC (Wahlsten et al., 2003b). The high variability of both CC size (Wahlsten 1974) and sociability (Brodtkin et al., 2004; Sankoorikal et al., 2006) in BALB/cJ mice make this inbred strain a useful model system in which to test hypotheses about the relationship between sociability and CC development in a uniform genetic background. We hypothesized that sociability, as measured in a social choice task (Sankoorikal et al., 2006), would be positively correlated with CC size within the BALB/cJ inbred strain. Based on our prior work, we also hypothesized that BALB/cJ mice, on average, would show lower levels of sociability than C57BL/6J mice (Brodtkin et al., 2004; Sankoorikal et al., 2006).

2. RESULTS

Consistent with previous reports (Brodtkin et al., 2004; Sankoorikal et al., 2006), BALB/cJ mice were less sociable than C57BL/6J mice. As reflected in the chamber preference change scores (behavioral measures defined below in “Experimental Procedures,” “Data Analysis” subsection), BALB/cJ mice showed a lower level of social approach behavior than C57BL/6J mice (Figure 1A), $t(22) = 3.27$, $p < 0.01$, one-tailed. BALB/cJ spent less time in direct olfactory

investigation of a stimulus mouse than C57BL/6J mice, as reflected in significantly lower cylinder sniffing preference change scores in BALB/cJ than in C57BL/6J mice (Figure 1B), $t(22) = 6.71$, $p < 0.000001$, one-tailed. The lower sociability of BALB/cJ mice was not attributable to any locomotor impairment in the BALB/cJ strain: the two strains did not show significant differences in the number of transitions in Phase 1A, Phase 1B, and Phase 2 (Figure 1C), $F(1,22) = 1.24$, $p = 0.27$ (Phase 1A, Phase 1B, and Phase 2 defined below in “Experimental Procedures,” “Behavioral Testing” subsection). Both strains showed a significant reduction in locomotor activity from the earlier to the later phases of the social choice test (significant effect of phase $F(1,22) = 198$, $p < 0.001$), which is consistent with our previous reports (Sankoorikal et al 2006). Additionally, the lower sociability of BALB/cJ mice can not be explained by a general avoidance of novelty, because there was no significant difference in the amount of time each strain spent sniffing the cylinder containing the novel object in Phase 2 (Figure 1D), $t(22) = 1.13$, $p = 0.14$, one-tailed.

During the free interaction period (Phase 3), none of the C57BL/6J or BALB/cJ mice attacked a stimulus mouse. One C57BL/6J female did display a tail rattle, an aggressive signal, toward a stimulus mouse, but no BALB/cJ mice did. Thus, with the exception of the single tail rattle, the test mice did not show aggressive behaviors toward the stimulus mice.

Of the 12 C57BL/6J mice whose social data are reported above, CC data was obtained on seven, due to technical difficulties on the other five. CC data was obtained on all 12 BALB/cJ mice. As shown in prior research (Wahlsten 1974; Wahlsten et al., 2003b), all C57BL/6J mice had normally sized CC, while some BALB/cJ mice had relatively small CC, as reflected by lower index of abnormality scores (Figure 2).

To determine whether lower levels of sociability are associated with smaller CC in BALB/cJ mice ($n = 12$), we assessed the correlation of both cylinder sniffing preference change scores and chamber preference change scores with the CC index of abnormality in BALB/cJ mice (not including C57BL/6J mice in the analysis). In BALB/cJ mice, the cylinder sniffing preference change score, the most specific measure of social investigation, correlated strongly with the CC index, Spearman correlation coefficient = 0.62, $p = 0.02$, one-tailed (data for BALB/cJ are gray circles in Figure 2A). Also in BALB/cJ mice, the chamber preference change score, a less specific measure of social investigation, was not significantly correlated with the CC index, Spearman correlation coefficient = 0.37, $p = 0.12$, one-tailed (data for BALB/cJ are gray circles in Figure 2B). The correlation between cylinder sniffing preference change score and CC index in BALB/cJ mice was not due to the effect of a particular litter. A comparison among the BALB/cJ litters by one-way ANOVA did not show any significant differences among the litters in cylinder sniffing preference change score, $F(2,9) = 0.09$, $p > 0.05$ or in the CC index of abnormality, $F(2,9) = 0.32$, $p > 0.05$. In fact, each of the three BALB/cJ mice with the lowest cylinder sniffing preference change scores and the lowest CC indices of abnormality came from three different litters. This is consistent with previous work that has found striking within-litter variability in CC development in the BALB/cJ strain, and that has found this variability to be due to stochastic events early in fetal development and intrinsic to the fetus (Bulman-Fleming and Wahlsten 1991).

3. DISCUSSION

This study provides evidence that an abnormally small CC is associated with reduced levels of social interaction in BALB/cJ mice. Consistent with our original hypothesis, in BALB/cJ mice we found a significant positive correlation between sociability, as measured by time spent in olfactory investigation of a stimulus mouse, and size of the corpus callosum. Previous reports indicate that time spent sniffing a stimulus mouse (e.g., cylinder sniffing preference score in this study) may be among the most direct and sensitive measures of sociability (Moy et al.,

2007). A significant correlation was not found between corpus callosum size and chamber preference change scores, a somewhat less sensitive and direct measure of sociability. This latter lack of significant correlation appeared to be attributable to a single male mouse with small corpus callosum that spent much time in the social chamber, but little time sniffing at the stimulus mouse during Phase 2.

The behavioral data in this study replicate our previous findings of reduced sociability in BALB/cJ mice relative to C57BL/6J mice (Sankoorikal et al., 2006). Two methodological differences from our previous studies are worth noting. First, this study used gonadectomized A/J mice as stimulus mice, as opposed to the prepubescent DBA/2J stimulus mice used in our previous studies (Brodkin et al., 2004; Sankoorikal et al., 2006). Also, test mice in this study were bred in our facility at University of Pennsylvania, whereas mice in previous studies were shipped from the vendor prior to behavioral testing. The fact that we found the same sociability difference between BALB/cJ and C57BL/6J using different types of stimulus mice, and using different locations of breeding, indicates that the strain difference in sociability is robust to changes in these experimental conditions.

Although other studies have found BALB/cJ mice to be generally neophobic (Tang et al., 2002) under the conditions of this study, BALB/cJ and C57BL/6J mice showed no significant difference in time spent sniffing a novel object (paperweight). Yet they did show a robust difference in time spent sniffing a stimulus mouse. This indicates that, under the conditions of this study, BALB/cJ mice seem to show a selective aversion toward other mice, rather than toward a novel object (paperweight). The very low lighting in our study may have reduced the generalized anxiety level of BALB/cJ mice, which may have helped to reduce any generalized neophobia, and helped to reveal a more selective aversion toward social stimuli.

Our and others' prior work, showing that BALB/c display low sociability (Brodkin et al., 2004; Moy et al., 2007; Sankoorikal et al., 2006), has been supported recently by research employing a different behavioral paradigm. These studies (Panksepp and Lahvis 2007; Panksepp et al., 2007), using a conditioned place preference paradigm, have found that BALB/cJ mice have reduced social reward relative to other strains, which is consistent with our results. Furthermore, the developmental pattern of results in the two paradigms is similar: the differences between BALB/cJ and C57BL/6J mice are greater at younger ages (approximately 4 weeks) than at older ages (6.5 or 9 weeks) (Panksepp et al., 2007; Sankoorikal et al., 2006). This similarity of results expands the experimental conditions in which the social impairment of BALB/cJ mice has been detected, and shows that this impairment is not attributable to the particular conditions of a single experimental paradigm.

Although we have now demonstrated low sociability of BALB/cJ mice toward both prepubescent DBA/2J mice (Brodkin et al 2004) and towards gonadectomized A/J mice (in the current report), one research group reported that BALB/cJ mice showed somewhat higher levels of sociability towards other BALB/cJ mice than towards mice from non-BALB/cJ strains (Logue et al., 2008). However, a different group has reported that BALB/cJ mice showed the same lack of social reward in response to other BALB/cJ mice as in response to mixed groups of BALB/cJ and C57BL/6J mice (Panksepp and Lahvis 2007; Panksepp et al., 2007), and other studies have found relatively low levels of affiliative social interaction among groups of BALB/cJ mice (Kalueff et al., 2006; Southwick and Clark, 1968). Thus, further research is warranted to determine whether BALB/cJ mice may be significantly more sociable with each other than with mice from other strains.

In addition to the evidence for underconnectivity of the autistic brain, the relationship between CC size and sociability may be evident in other disorders. Social abnormalities and a small CC size have been associated with each other in schizophrenia (Mitelman et al., 2007; Paul et al.,

2007), agenesis of the CC (Paul et al., 2007), fetal alcohol syndrome (Niccols 2007; Spadoni et al., 2007), and depression (Ballmaier et al., 2007).

Because only approximately 30–40% of BALB/c mice have been reported to show underdevelopment of the corpus callosum (Wahlsten 1974), future studies with larger sample sizes are needed to more fully characterize the effect of CC underdevelopment on social behaviors across various ages in this model system. The correlation reported here between sociability and CC size does not prove with certainty that the CC itself is the specific anatomical region most related to sociability. Reduced CC size in a subset of BALB/cJ mice is associated with other connectivity abnormalities that may be more proximally related to mediating reduced sociability in these BALB/c mice. In the subset of BALB/cJ mice with underdevelopment of the corpus callosum, those axons that fail to cross the midline re-enter the ipsilateral cerebral cortex (forming a histologically visible “Probst bundle”), and form aberrant synaptic connections (Lefkowitz et al., 1991; Wahlsten et al., 2006). Future studies are needed to identify the precise connectivity abnormalities that are relevant to sociability, and the mechanisms by which disrupted connectivity can influence sociability. These mechanistic studies may provide important information for future translational studies of neurodevelopmental disorders, such as autism.

4. EXPERIMENTAL PROCEDURES

Subjects

C57BL/6J ($n = 12$; 6 females, 6 males) and BALB/cJ ($n = 12$; 6 females, 6 males) mice were bred at the University of Pennsylvania. To ensure adequate nutrition for all pups and to ensure that litters were balanced in numbers of males and females, litters were culled to two males and two females at 2–4 days postnatally (P2–4). Following weaning on P25, litters were divided by gender, so that the mice were housed two male littermates or two female littermates per cage. “Stimulus mice” for the social choice test were adult (36–to–42-week-old) A/J mice that had been obtained from The Jackson Laboratory (Bar Harbor, ME). These mice had been gonadectomized prior to puberty to minimize the extent to which they would elicit sexual and aggressive motivations from the test mice. Stimulus mice were housed either five males or five females to a cage. Food and water were available *ad libitum*, and the housing room was maintained on a 12-h light-dark cycle (lights on at 7:00 a.m.). All mice were housed in cages with filter cages tops, and when cage tops needed to be opened, this was done under a hood, one cage at a time. Most mice were tested for sociability on P30 (range: P29–31), and all were perfused on P31. All animals were treated in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Behavioral Testing

C57BL/6J and BALB/cJ “test mice” were assessed for their tendency to approach a stimulus mouse using a slight modification of a social choice test that has been reported previously (Brodkin et al., 2004; Sankoorikal et al., 2006). The social choice test was carried out in a three-chambered apparatus with no top or bottom and that consisted of a center chamber and two end chambers, with dimensions that have been reported previously (Sankoorikal et al., 2006). Behavioral testing was videotaped with a Sony digital videocamera with NightShot (infrared) feature for recording in low light. To minimize the general stress level of the mice, the testing room was very dimly lit; the lighting within all chambers measured at 1 – 2 lux during testing. Prior to the start of the test, one end chamber was designated the “social chamber,” into which a stimulus mouse would be introduced, and the other end chamber was designated the “nonsocial chamber.” The end chamber designated as the social chamber was varied in a counterbalanced sequence among tests. Before each test, the apparatus was placed on a clean

mat and clean mouse bedding. Two identical clear, Plexiglas cylinders (each 7 cm in diameter, 12.2 cm tall) with removable black, Plexiglas lids were placed in the apparatus, one in each end chamber. The stimulus mouse could move around easily within the cylinder. The cylinders had multiple holes (1 cm in diameter) to allow for air exchange between the inside and outside of the cylinder. Auditory, visual, and olfactory investigation between a mouse inside and a mouse outside the cylinder was thus possible.

In Phase 1 of the test, the test mouse was placed in the center chamber and allowed to explore all three chambers for 10 minutes. During this acclimation period, three baseline measurements were collected: how much time the mouse spent in each of the three chambers (“chamber time”), how much time the mouse spent sniffing each of two Plexiglas cylinders placed in the two end chambers (“sniffing time”), and how many times the test mouse moved between chambers (“transitions,” a measure of locomotor activity).

Following Phase 1, the lids of the cylinders were simultaneously removed. A stimulus mouse was placed in the cylinder in the social chamber (“social cylinder”), while a “novel object” was simultaneously placed into the other cylinder (“nonsocial cylinder”) in the “nonsocial chamber.” The novel object was a 42.0 g black paperweight shaped as an upside-down, rounded cone (6.0 mm × 5.5 mm × 4.0 mm) that was heavy enough to prevent the mice from pushing it. During the 5-minute social choice period (Phase 2), chamber times, sniffing times, and numbers of transitions among chambers were again recorded. To determine whether the social approach and sniffing behaviors might be influenced by aggressive motivations, the cylinders then were removed simultaneously following Phase 2, and the test and stimulus mice were observed for 5 minutes for aggressive interactions, including tail rattles and attacks (i.e. bites and/or vigorous lunges).

After testing, the apparatus, cylinders, and paperweight were all washed with copious amounts of water and dried before testing the next mouse. All four mice from a single litter were tested on the same day between 12 noon and 5 p.m. in an order counterbalanced for gender. Stimulus mice were used on multiple testing days, but never more than once in a single day.

Tissue Processing

Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital. The mice were then transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (both solutions: room temperature, pH 7.4). Brains were post-fixed in 4% paraformaldehyde for 7 days. Brain weights and CC sizes were measured using procedures described previously (Wahlsten et al., 2003a). Brains were trimmed to a standard configuration by cutting off the olfactory bulbs anterior to the cortex, the paraflocculi of the cerebellum, and the spinal cord below the base of the medulla oblongata. Brains were then weighed, and bisected at the midsagittal line. The right hemispheres were stained with 0.2% gold chloride (Sigma-Aldrich, St. Louis, MO), then immersed in 2.5% sodium thiosulfate (Fisher Scientific) solution for 5 minutes. Following storage in 4% paraformaldehyde, the midsagittal plane of a brain was digitally imaged on a stereoscope. The image scale was calibrated to a micrometer prior to each session with the stereoscope. The area of the corpus callosum was then measured from the digital image with ImageJ software from NIH (<http://rsb.info.nih.gov/ij/index.html>). Measurement of CC size was made only at the midsagittal line (not at additional sections) because a large body of previous work has established that CC size at the midsagittal line is an excellent indicator of overall CC development in BALB/c strains (Livy et al., 1997; Wahlsten 1974; Wahlsten et al., 2003a; Wahlsten et al., 2003b; Wahlsten et al., 2006).

Data Analysis

Measures of sociability were analyzed as described previously (Sankoorikal et al., 2006). All times were measured in seconds. For each mouse, the time spent in the nonsocial chamber was subtracted from time spent in the social chamber, to yield a “chamber preference score.” Similarly, the time spent sniffing the nonsocial cylinder was subtracted from the time spent sniffing the social cylinder, to yield a “cylinder sniffing preference score.” These calculations were done separately for the first 5-minute period (Phase 1A) and for the second 5-minute period (Phase 1B) that made up the 10-minute Phase 1, as well as for the 5-minute period of Phase 2. For each mouse, the mean of the two chamber preference scores for Phase 1A and Phase 1B was calculated, as was the mean of the two cylinder sniffing preference scores for the Phase 1A and Phase 1B. Then, the mean chamber preference score for Phase 1 (mean of Phase 1A and Phase 1B scores) was subtracted from the chamber preference score for Phase 2, to yield a “chamber preference change score.” Similarly, the mean cylinder sniffing preference score for Phase 1 was subtracted from the cylinder sniffing preference score for Phase 2, to yield a “cylinder sniffing preference change score.” For each mouse, a positive “chamber preference change score” or a positive “cylinder sniffing preference change score” indicated a greater preference for the social chamber and social cylinder sniffing, respectively, during Phase 2 (presence of stimulus mouse) vs. Phase 1 (absence of stimulus mouse). As in our previous work, this “preference change” score was used for all subsequent analysis for the chamber time and sniffing time variables (Sankoorikal et al., 2006).

Between strain comparisons of chamber preference change scores, cylinder sniffing preference change scores, and time spent sniffing the paperweight were conducted using *t* tests. One-sided tests were used, because of our *a priori* hypotheses, based on previous work (Brodin et al., 2004; Sankoorikal et al., 2006; Tang et al., 2002), that BALB/cJ mice would show lower levels of sociability than C57BL/6J mice and lower levels of investigation of the novel object (paperweight). Between strain comparison of locomotor activity (numbers of transitions between chambers) in the consecutive phases of the social choice test (Phase 1A, Phase 1B, and Phase 2) was conducted using 2×3 analysis of variance (ANOVA) (strain of mouse $\times$ phase) with repeated measures on phase. Statistics were run on SPSS 15.0 for Windows, and alpha level was set at 0.05.

The “index of abnormality” of the corpus callosum size was calculated, as reported previously (Livy et al., 1997), as the ratio of the actual corpus callosum area to the expected area in mm^2 at the mid-sagittal section ($E(CC)$), given a particular brain weight in grams ($BrWt$). The expected area of the corpus callosum is calculated based on the equation $E(CC) = -0.42 + 2.7 (BrWt)$, which is based on data from juvenile mice less than 50 days old (Livy et al., 1997). Correlations between behavioral measures (chamber preference change score, cylinder sniffing preference change score) and the corpus callosum index of abnormality were calculated using Spearman rank correlation, because we did not assume a linear relationship between behavioral variables and corpus callosum size. For correlations, Bonferroni adjustment was made for two tests, and the threshold for significance was set at $p < 0.025$ ($0.05/2 = 0.025$).

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Fig. 1. BALB/cJ mice are less sociable than C57BL/6J mice

BALB/cJ ($n = 12$) and C57BL/6J ($n = 12$) test mice at ~30 days of age (range: 29 – 31 days of age) were tested for their tendency to approach and investigate a gonadectomized A/J stimulus mouse located in one chamber of a three-chambered apparatus. All results are displayed as $M \pm SEM$. A) BALB/cJ mice approach the stimulus mouse less than C57BL/6J mice do, as measured by the chamber preference change score ($t(22) = 3.27, p < 0.01$, one-tailed). B) BALB/cJ mice investigate the stimulus mouse less than C57BL/6J mice do, as measured by the cylinder sniffing preference change score ($t(22) = 6.71, p < 0.000001$, one-tailed). C) BALB/cJ and C57BL/6J mice show no significant differences in their locomotor activity, regardless of whether the stimulus mouse is absent (first (Phase 1A) and second (Phase 1B) 5-minute blocks of the 10-minute acclimation period) or present (social choice period (Phase 2)). D) BALB/cJ and C57BL/6J mice show no significant differences in time spent sniffing a novel object (paperweight) during Phase 2.

Fig. 2. Corpus callosum size correlates with sociability, as measured by the cylinder sniffing preference change score

The scatterplots display the relationships between measures of sociability and corpus callosum size for both BALB/cJ ($n = 12$) and C57BL/6J ($n = 7$) mice. Data for BALB/cJ mice are illustrated with gray circles, and data for C57BL/6J mice are illustrated with black triangles. A) The tendency of only BALB/cJ mice ($n = 12$) to sniff the social cylinder with the stimulus mouse inside (the cylinder sniffing preference change score) correlated with the size of their corpora collosa (corpus callosum index of abnormality), Spearman correlation coefficient 0.62, $p = 0.02$, one-tailed. B) The tendency of only BALB/cJ mice ($n = 12$) to spend time in the social chamber near the stimulus mouse (chamber preference change score) did not correlate with the size of their corpora collosa (corpus callosum index of abnormality).