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Sociability and brain development in BALB/cJ and C57BL/6J mice

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Abstract

Sociability—the tendency to seek social interaction–propels the development of social cognition and social skills, but is disrupted in autism spectrum disorders (ASD). BALB/cJ and C57BL/6J inbred mouse strains are useful models of low and high levels of juvenile sociability, respectively, but the neurobiological and developmental factors that account for the strains' contrasting sociability levels are largely unknown. We hypothesized that BALB/cJ mice would show increasing sociability with age but that C57BL/6J mice would show high sociability throughout development. We also hypothesized that littermates would resemble one another in sociability more than non-littermates. Finally, we hypothesized that low sociability would be associated with low corpus callosum size and increased brain size in BALB/cJ mice. Separate cohorts of C57BL/6J and BALB/cJ mice were tested for sociability at 19-, 23-, 31-, 42-, or 70-days-of-age, and brain weights and mid-sagittal corpus callosum area were measured. BALB/cJ sociability increased with age, and a strain by age interaction in sociability between 31 and 42 days of age suggested strong effects of puberty on sociability development. Sociability scores clustered according to litter membership in both strains, and perinatal litter size and sex ratio were identified as factors that contributed to this clustering in C57BL/6J, but not BALB/cJ, litters. There was no association between corpus callosum size and sociability, but smaller brains were associated with lower sociability in BALB/cJ mice. The associations reported here will provide directions for future mechanistic studies of sociability development.

Keywords

Autism; Mouse; Model; Juvenile; Social; Behavior

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1. Introduction

Developing children typically show a strong, preferential tendency to seek social interactions, starting in infancy [1,2]. Engaging in social interactions throughout childhood leads to development of expertise in social cognition and social skills that are vital for daily functioning [3,4]. However, most children with autism spectrum disorders (ASD) show a reduced tendency to seek social interactions, i.e., reduced sociability, starting in the first years of life [5,6]. Reduced sociability during this sensitive period for social behavior development contributes to impaired development of social reciprocity, social cognition, and social skills [3,4,7–11]. Because sociability is so important for propelling the many aspects of social behavior development, there is a strong need for improved understanding of the biological factors that influence sociability across development [12,13].

Mouse models are indispensable for studies of the fundamental neurobiology of sociability because of the experimental control and genetics resources that they afford [14]. The Social Approach Test (sometimes called the Social Choice Test) provides quantitative measurements of mouse sociability [15–18] and is perhaps the most well-established and widely used assay for assessing sociability in mouse models relevant to ASD [18–49]. In many studies, reduced sociability in the Social Approach Test has been associated with decreases in other measures of social interaction, including measures of direct, or free, social interaction [18,43], nest building [37], juvenile play [19,20,24], preference for social novelty[37,42,46,50–52], social transmission of food preference [19], and social behaviors in a visible burrow system [53]. Sociability measurements of mice in the Social Approach Test also tend to be highly replicable. Mice tested twice in the test exhibit broadly similar sociability in the repeated tests both on a group level [16,18] and on an individual level [54]. Moreover, relatively low levels of sociability in the Social Approach Test have been well replicated in certain inbred mouse strains, particularly the BTBR T^+ tf/J strain[19–21,24,53,55] and the BALB/cJ strain [15,18,25]. The low sociability of BALB/cJ mice in the Social Approach Test parallels their low levels of social behaviors during a free interaction [18] and their low levels of social reward [56]. The Social Approach Test is therefore an important and reliable assay for studying the development of sociability and is a useful indicator of social behavior impairments more generally, because reduced sociability in the test tends to be associated with reductions of various types of social behaviors in multiple contexts.

The neurobiological and developmental factors that account for mouse strain differences in sociability levels are largely unknown. As mentioned above, prepubescent BALB/cJ mice are less sociable than many other inbred strains, including the relatively sociable C57BL/6J strain [15,18,25] but a detailed study of sociability and brain development in BALB/cJ and C57BL/6J mice has not been reported previously. In this study, we sought to determine the effects of age, litter size and sex ratio, and brain and corpus callosum size on sociability in BALB/cJ and C57BL/6J mice. Motivations for sociability vary by age because affiliative motivations are more prominent in the juvenile period, whereas sexual and aggressive motivations become more prominent after the onset of puberty in humans [57,58] and mice [56]. Sociability during the juvenile period is particularly relevant to ASD, in which sociability tends to be markedly reduced starting in early childhood [3]. As they grow into later adolescence and adulthood, individuals with ASD, on average, tend to show somewhat increased interest in social interactions and modest declines in social impairment [59,60]. Prepubescent BALB/cJ mice at ~30 days of age are less sociable than C57BL/6J mice, as well as mice of several other inbred strains[15,18,25]. Based on the developmental pattern seen in ASD, as well as increases in sexual and aggressive motivations following puberty in mice, we hypothesized that the sociability of BALB/cJ mice would increase from prepubescence to adulthood.

A recent and rigorous study of a large sample of twins estimated that the genetic heritability of ASD (38%) was lower than previously thought, but still substantial, and that the contribution of the shared environment to ASD (58%) was much higher than previously thought [61]. Consistent with the influence of the common environment on ASD, we hypothesized that C57BL/6J and BALB/cJ littermates would be more alike in their degree of sociability than non-littermates of the respective strains. Numerous prenatal and perinatal factors have been identified as contributing risk factors for developing ASD [62,63] and include birth weight, multiple births, and higher levels of fetal testosterone [63–70]. We hypothesized that two perinatal factors in mice, litter size and litter sex ratio, would be associated with changes in sociability later in development. Moreover, the underconnectivity theory of ASD proposes that ASD deficits stem from long-range (e.g., interhemispheric) underconnectivity in the brain and short-range overconnectivity [71,72]. Accordingly, reduced corpus callosum (CC) size and enlarged brains are among the most replicated brain phenotypes in ASD [73,74] and may be related to reduced sociability. Adult BALB/cJ mice have large brains compared to other mouse strains [75,76] and some BALB/cJ mice have an unusually small or absent CC [77]. We previously reported a within-strain positive correlation between CC size and sociability in a small sample of 30-day-old BALB/cJ mice [25], which we hypothesized would be found in the much larger sample reported described here. Additionally, we hypothesized that larger brains would be associated with lower sociability.

2. Materials and methods

2.1. Animal husbandry

Progenitor C57BL/6J and BALB/cJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were mated at the University of Pennsylvania to produce C57BL/6J (n = 133) and BALB/cJ (n = 156) mice that were tested at various ages for sociability in the Social Approach Test, as described below. The first day following a litter's birth was considered postnatal day 1 (P1). Litters were culled to 4 pups usually on P2–P4 (and rarely on P5 or P6) to ensure sufficient nutrition for each pup and a more uniform postnatal social environment. The original, perinatal litter size and litter composition by sex were recorded at this time. Litters were culled as closely as possible to 2 females and 2 males. Mice were ear tagged on P14–P18 (and on P13, P20, and P24 for one litter each), and ear tagging always preceded testing for sociability by at least 2 days. Two cohorts of mice were tested for sociability at 19 or 23-days-of-age. The remaining cohorts of mice were weaned on P23–P26 (and on P27 for one litter) and, following weaning, same-sex littermates were housed together at 2 per cage.

Stimulus mice used in the Social Approach Test were adult, 22–54-week-old A/J mice obtained from The Jackson Laboratory. These mice had been gonadectomized before puberty to minimize aggressive and sexual behaviors directed toward them by the test mice in the Social Approach Test. The A/J mice were housed 4–5 same-sex mice per cage.

All mice had access to food and water ad libitum, and were maintained in a 12-h light–dark cycle (light began at 7:00 a.m.). 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.

2.2. Social Approach Test

Separate cohorts of mice underwent the Social Approach Test at ages of 19, 23, 31, 42, or 70 days. These ages were selected as potentially interesting times for the development of sociability. Mice at 19 days of age are about the youngest mice for which valid data in the

Social Approach Test can be obtained, because mice display their first active social interactions around 17 days of age [78,79]. Mice in our colony are weaned near 23 days of age. Mice at 31 days of age are about the oldest mice that can be safely assumed to be prepubescent, as early signs of pubescence in inbred strains may occur shortly thereafter [80]. Additionally, we have conducted prior studies of sociability near this age [15,18,25,81]. Mice at 42 days are pubescent, and 70-day-old mice are adults [80]. Separate cohorts of mice were used for Social Approach Testing at the 5 different ages in order to maximize statistical power and in order to avoid the potential confounding effects of repeated Social Approach Testing on later tests.

A minority of mice were tested on the day before or after the planned day for logistical reasons. The evening prior to testing, mice were temporarily moved to a dark testing room so that their social behaviors could be video-recorded in their home cages. This procedure was designed to be minimally disruptive: mice were never removed from their home cages and were in the testing room for less than 2 h. The results of these home cage social behavior observations will be reported elsewhere.

The Social Approach Test procedure matched that reported previously[15,18,25]. Briefly, each mouse was initially allowed to explore and habituate to a 3-chambered Plexiglas box under dim lighting (<2 lx) for 10 min (Phase 1). The two end chambers, which were separated by a middle chamber, each contained an empty transparent Plexiglas cylinder in its center. Following Phase 1, an experimenter (A.F.) placed a gonadectomized A/J stimulus mouse into one cylinder (the “social cylinder”) while simultaneously placing a novel object (a paper weight) into the other, identical cylinder (the “nonsocial cylinder”). For the next 5 min (Phase 2), the test mouse could continue to move freely throughout the box and investigate the cylinders. Both cylinders had many holes (1 cm in diameter) in their walls so that the test mouse could sniff the stimulus mouse or novel object inside. For both Phases 1 and 2, the experimenter(s) recorded (either live or later from video) the amount of time the test mouse spent investigating (including sniffing) each cylinder, the amount of time the test mouse spent in each chamber, and the number of times the mouse moved from one chamber to another (i.e., “transitions”). The amount of time that the test mouse sniffed, reared against, and climbed on the social cylinder during Phase 2 was considered an index of sociability, and the sum of these behaviors is referred to below as “social cylinder investigation.” Sniffing of the social cylinder was the overwhelmingly predominant behavior included in social cylinder investigation. Climbing on the walls of the cylinder occurred very rarely. We used social cylinder investigation as the index of sociability because we established previously that sociability scores based on cylinder investigation are more reliable and ecologically valid than those based on how much time the test mouse spends in each chamber of the box [54]. The number of transitions was considered an index of locomotor activity.

Following Phase 2, the cylinders were removed so that the two mice could interact freely for 5 min and be observed for aggressive interactions (Phase 3). Of the 289 mice tested for sociability, 13 appeared to attack the stimulus mouse during Phase 3. In many cases, the observed incident was not definitively established as an attack, but a liberal criterion of “attack” was employed to minimize the effects of aggressive motivations for social approach and investigation in the data. These 13 mice were eliminated from any further analysis. The numbers of these mice eliminated from further analysis and the remaining 276 mice are reported in Table 1.

2.3. Tissue processing

On the day following the Social Approach Test, mice were weighed and anesthetized with an intraperitoneal injection of ketamine–xylazine solution (ketamine, 100 mg/kg; xylazine,

20 mg/kg). They were then transcardially perfused with icecold 0.9% saline (pH 7.4), then with ice-cold 4% paraformaldehyde (PFA, pH 7.4). Whole brains were dissected out and placed in 4% PFA for 4 days until weighing. Brain weights and CC size were measured as described previously [25,82]. Brains were briefly blotted on a paper towel to remove excess PFA and then 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 weighed and next bisected at the midsagittal line with the use of a sagittal cutting block. The right hemispheres were stained in 0.2% gold chloride solution (Sigma–Aldrich, St. Louis, MO), then immersed in 2.5% sodium thiosulfate (Fisher Scientific) solution for 5 min. Following storage in 4% PFA, 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 each corpus callosum was outlined 3 times from the digital image with ImageJ software from NIH (http://rsb.info.nih.gov/ij/index.html). The average of these 3 outlines was used as the final CC measurement. Measurement of CC size was made only at the midsagittal line because this is a reliable indicator of overall CC development in BALB/c strains, as established in a large body of previous work [77,82–85].

2.4. Data analysis

The data for social cylinder investigation (the index of sociability) violated several assumptions of the conventional analysis of variance (ANOVA) and analysis of covariance (ANCOVA). The data showed heterogeneity of variances among the experimental groups and some groups showed non-normal distributions. To prevent Type I error rates from exceeding the nominal α level, we implemented robust statistical procedures alongside conventional analyses. These robust procedures are described in Wilcox [86], which also describes functions written for the statistical software R [87] that implement these procedures. The 20% trimmed means ANOVAs were implemented by the R functions ‘t2way’ and ‘t3way’; an approach to Theil-Sen regression analysis (called “OP regression”), by ‘opreg’; hypothesis tests of the regression coefficients, by ‘regtest’; a least median squares (LMS) method to detect regression outliers, by ‘reglev’; and OP correlation, by ‘mscor’. Some of these robust functions use bootstrapping techniques and were run at 1000 iterations, so that the lowest possible p value was 0.001. Conventional ANOVA, separate-slopes ANCOVA, and ordinary least squares (OLS) regression were implemented as cases of the general linear model by the R functions ‘lm’ and ‘aov’.

For an effect to achieve statistical significance, both the conventional and the robust tests had to produce a p value below the α level. If either test yielded a p value between α and 2α, the effect is reported as a “trend.” In some cases (i.e., factorial designs with more than 3 independent variables and designs that mixed categorical and continuous variables), R functions for robust procedures were not available. In these cases, conventional analyses were relied upon exclusively, but when any significant effects were followed by simpler post-hoc tests, robust statistics were again employed wherever possible to verify the effects. The α level was set at 0.05. As omnibus effects were followed by post-hoc tests of experimental subgroups, the Bonferroni correction for multiple testing was employed across all subgroups at each stage.

Conventional and linear mixed effects models were compared using Akaike’s Information Criterion (AIC), which provides an estimate of the distance (or the information lost) between the model and the unknown process (i.e., reality) that produced the observed data [88]. The model with the lower AIC is preferred. The AIC was used as an alternative to hypothesis testing because of the difficulties in calculating valid p values for testing whether the effect of a random effects variable is significant [89]. Intracluster (or intraclass) correlation coefficients were calculated as the between-litter variance as a proportion of the

sum of the between and within-litter variances $\left(\sigma_b^2/\left(\sigma_b^2+\sigma_w^2\right)\right)$ and could range from 0 to 1 [89]. All analyses were run on the statistical software R [87] with functions described in Wilcox [86] and the software package lmer [90].

CC size was classified as normal or abnormally small based on an "index of abnormality" [83]. The CC index was calculated as the ratio of the actual corpus callosum area to the expected area (E(CC)) in mm^2 at the midsagittal section, given a particular brain weight in grams (BrWt). For mice tested at 20-, 24-, 32-, and 43- days-of-age, the expected area of the corpus callosum was calculated based on the equation E(CC) = −0.42 + 2.7(BrWt), which was based on data from juvenile mice less than 50-days-old [83]. For mice tested at 71 days, the expected area of the corpus callosum was calculated based on the equation E(CC) = −0.1 + 2.2(BrWt), which was based on data from mice older than 50 days [83]. A CC with an index less than 0.65 was considered abnormally small.

3. Results

3.1. Development of sociability

To examine sociability across postnatal development, we tested different groups of C57BL/6J and BALB/cJ mice in the Social Approach Test at various ages (Fig. 1). An ANCOVA modeled mouse strain (C57BL/6J vs. BALB/cJ) and sex (female vs. male) as factors of social cylinder investigation (which consisted predominantly of sniffing of the social cylinder) during Phase 2 of the Social Approach Test and age (at 19, 23, 31, 42, and 70 days) as a continuous covariate of social cylinder investigation during Phase 2. The analysis showed a main effect of strain, $F(1, 268) = 8.35$, $p = 0.004$, and a strain by age interaction, $F(1, 268) = 13.67$, $p = 0.0003$. A 20% trimmed means ANOVA, which modeled age as a factor, verified these effects: strain, $Q(1, 256) = 5.99$, $p = 0.018$; strain by age, $Q(4, 256) = 31.08$, $p = 0.001$. Additionally, the analyses identified a trend for a main effect of sex, $F(1, 268) = 3.84$, $p = 0.051$ ($Q(1, 256) = 5.70$, $p = 0.02$), and for a sex by age interaction, $F(1, 268) = 3.30$, $p = 0.071$ ($Q(4, 256) = 8.47$, $p = 0.105$). All α levels were 0.05. In the following paragraphs, the statistically significant effects (the main effect of strain and the strain-by-age interaction) are investigated in more detail, while the trends toward effects (the main effect of sex and the sex-by-age interaction) are not (Fig. 1a and b).

To further investigate how the strains differed in their sociability across development, we followed with analyses at each age group. Alpha levels were corrected to 0.01 (0.05/5 age groups). At 19 days of age, 2 × 2 (strain by sex) ANOVAs (conventional and 20% trimmed means) showed that BALB/cJ mice were less sociable than C57BL/6J mice, $F(1, 41) = 21.06$, $p = 0.00004$ ($Q(1, 41) = 28.62$, $p = 0.001$). No differences between the strains were found at 23 days of age, $F(1, 43) = 2.54$, $p = 0.12$ ($Q(1, 43) = 2.42$, $p = 0.14$). At 31 days of age, BALB/cJ mice were less sociable than C57BL/6J mice, $F(1, 99) = 8.31$, $p = 0.0048$ ($Q(1, 99) = 9.57$, $p = 0.004$), which was consistent with our prior experiments at this age [15,18,25]. No strain differences were found at 42 days, $F(1, 32) = 4.20$, $p = 0.049$ ($Q(1, 32) = 3.82$, $p = 0.066$), nor at 70 days, $F(1, 41) = 1.44$, $p = 0.24$ ($Q(1, 41) = 0.32$, $p = 0.58$).

To determine when during development strain and age interacted, we analyzed each of the four intervals between age groups. Alpha levels were corrected to 0.0125 (0.05/4 intervals). From 19 to 23 days, 2 × 2 × 2 (strain by sex by age) ANOVAs indicated a trend for a strain by age interaction, $F(1, 84) = 5.24$, $p = 0.0245$ ($Q(1, 84) = 8.25$, $p = 0.008$). From 23 to 31 days, no interaction was found, $F(1, 142) = 0.32$, $p = 0.57$ ($Q(1, 142) = 0.90$, $p = 0.35$). From 31 to 42 days, strain and age did interact, $F(1,131) = 11.16$, $p = 0.001$ ($Q(1, 131) = 10.1$, $p = 0.004$). No interaction was found from 42 to 70 days, $F(1,73) = 2.24$, $p = 0.14$ ($Q(1, 73) = 2.27$, $p = 0.14$).

To examine the overall developmental trajectory of sociability in each strain, regression analyses modeled the social cylinder investigation during Phase 2 as $y = \beta_0 + \beta_1$ (age) + $\beta 2$ (sex) where β_0, β_1, and β_2 are the regression coefficients. All α levels were 0.025 (0.05/2 mouse strains). A conventional ordinary least squares (OLS) regression of the BALB/cJ data yielded $\beta 0 = 38.51$, $\beta 1 = 0.50$, and $\beta 2 = 2.96$. The slope of the regression line, 0.50, was significantly different from zero, $t(150) = 2.3$, $p = 0.023$, and positive, showing that the BALB/cJ mice increased in sociability from 19 to 70 days of age. This result was verified by the robust OP regression, which yielded $\beta 0 = 9.23$, $\beta 1 = 1.45$, and $\beta 2 = -0.27$ and $\beta 1$ was greater than zero, D = 0.014, $p = 0.010$. An OLS regression of the C57BL/6J data yielded $\beta 0 = 83.40$, $\beta 1 = -0.55$, and $\beta 2 = 17.56$ (OP: $\beta 0 = 82.98$, $\beta 1 = -0.85$, and $\beta 2 = 16.66$) and showed a decrease in sociability across development, $t(120) = -2.95$, $p = 0.004$, but OP regression did not confirm this result, D = 0.00004, $p = 0.109$.

The strain, sex, and age effects on sociability reported above were not substantially altered by the inclusion of litter effects, as described below. The amounts of time that the mice spent in the social, center, and nonsocial chambers are reported in Fig. 1c and d. Locomotor activity and investigation of the nonsocial cylinders in Phases 1 and 2 of the Social Approach Test are reported in Fig. 2, as is social investigation by the test mouse toward the stimulus mouse in Phase 3.

3.2. Litter effects

We investigated whether characteristics of an entire litter affected sociability of mice from that litter. We first investigated litter membership by asking whether littermates were more alike in their degree of sociability than non-littermates, after controlling for strain, sex, and age. In other words, does the sociability of mice cluster according to litter membership? Two 43-day-old C57BL/6J males were excluded from analyses of litter membership because each was the sole representative of its litter. Thirty-four C57BL/6J and 46 BALB/cJ litters remained and ranged from 2 to 4 pups. We analyzed social cylinder investigation (which consisted predominantly of sniffing of the social cylinder) in a linear mixed effects model for each strain as $y = \beta_0 + \beta_1$ (sex) + β_2 (age) + $\beta_{1,2}$ (sex, age) + b_1 (litter) where sex, age, and their interaction were modeled as fixed effects and litter was modeled as a random effect. This ‘litter’ model was compared to a ‘null’ model, which differed from the ‘litter’ model only by fictionally assigning all mice to the same litter.

For C57BL/6J mice, the large difference (1210–1161 = 49) between the null model AIC (1210) and the litter model AIC (1161) indicated that the null model received little support compared to the litter model. This difference translated into a >99.99% (i.e., $1–(2 \times 10^{-11})$) probability that the litter model was preferred to the null model, or that the litter model was 6×10^{10} times more likely to be correct than the null model [91]. BALB/cJ mice also showed a large difference (1583–1569 = 14) between null (1583) and litter (1569) model AIC, so that the litter model was 99.88% likely – or 802 times more likely – to be correct, compared to the null model (0.12%) (Fig. 3a). The intracluster correlation coefficients for C57BL/6J and BALB/cJ mice were 0.60 and 0.30, respectively. Thus for both strains, some factors that affect the litter as a whole cause the littermates to resemble each other in sociability more closely than do non-littermates.

We investigated whether the factors by which litter membership affects sociability included perinatal litter size and sex ratio. These two characteristics pertained to the litter prior to culling the litter to 4 pups on postnatal day 2–4. These data were missing for one 31-day-old BALB/cJ litter (2 females, 2 males), which was omitted from these analyses, but the two mice excluded from the litter membership analyses were included.

Forty-five BALB/cJ litters produced a median of 7 pups (interquartile range: 5–9) and the 36 C57BL/6J litters produced a median of 7 pups (interquartile range: 5.75–8). An ANCOVA (litter size as covariate; strain, sex, age (2 × 2 × 5) as factors) of social cylinder investigation revealed a main effect of litter size, $F(1, 232) = 12.6$, $p = 0.0005$, and a strain by litter size interaction, $F(1, 232) = 4.6$, $p = 0.032$. The α level was 0.05. To further investigate the interaction, we executed an ANCOVA (litter size as covariate; sex and age as factors) on each strain with $\alpha = 0.025$ (0.05/2 mouse strains). For BALB/cJ mice, there was a trend toward an age by sex by litter size interaction, $F(1, 129) = 2.5$, $p = 0.045$, but no other effects (all other ps > 0.17) (Fig. 3b). For C57BL/6J mice, there was a main effect of litter size such that larger perinatal litter sizes were associated with lower sociability, $F(1, 103) = 18.6$, $p = 0.00004$. This result was confirmed by a simple OP regression of litter size and the residuals of a 2 × 5 (sex by age) trimmed means ANOVA of social cylinder investigation, which yielded a slope significantly less than zero, D = 0.004, p < 0.001 (Fig. 3c).

We examined whether the perinatal sex ratio of the litter influenced sociability. The median ratio of the number of females in a litter to the entire litter size was 0.50 for both BALB/cJ (interquartile range: 0.33–0.63) and C57BL/6J (interquartile range: 0.40–0.57) litters. An ANCOVA (litter sex ratio as covariate; strain, sex, age (2 × 2 × 5) as factors) of social cylinder investigation revealed a main effect of litter sex ratio, $F(1, 232) = 9.5$, $p = 0.002$, and a trend toward a strain by litter sex ratio interaction, $F(1, 232) = 3.7$, $p = 0.056$. The α level was 0.05. Because the interaction was so close to significance, we implemented an ANCOVA (litter sex ratio as covariate; sex and age as factors) on each strain with $\alpha = 0.025$ (0.05/2 mouse strains). Litter sex ratio did not affect the sociability of BALB/cJ mice (all ps > 0.37) (Fig. 3d). A higher ratio of females in a C57BL/6J litter was associated with higher social cylinder investigation, $F(1, 103) = 7.2$, $p = 0.009$. This result was verified by a simple OP regression of litter sex ratio and the residuals of a 2 × 5 (sex by age) trimmed means ANOVA of social cylinder investigation, which yielded a slope significantly greater than zero, D = 0.097, $p = 0.011$ (Fig. 3e).

The effects of litter size and litter sex ratio on social cylinder investigation during Phase 2 appeared to be independent. OP correlation – which provides a robust estimate of Pearson's population correlation coefficient ρ – of litter size and sex ratio in C57BL/6J mice (counting each litter as a single n) showed that $r_p = -0.13$, which was not statistically significant (T_p (test statistic) = 1.14 < c (critical value) = 2.44) (Fig. 3f). This was consistent with prior studies that did not find a relationship between litter size and sex ratio[92,93], including one on C57BL/6J mice [94].

Litter size and litter sex ratio were two factors by which litter membership affected sociability in C57BL/6J mice. We asked whether these two factors entirely accounted for the clustering of C57BL/6J sociability by litter membership. We incorporated these factors into the model $y = \beta_0 + \beta_1$ (sex) + β_2 (age) + β_3 (litter size) + β_4 (litter sex ratio) + $\beta_{1,2}$ (sex,age) + b_1 (litter) where sex, age, litter size, litter sex ratio, and the sex by age interaction were modeled as fixed effects and litter was modeled as a random effect (all 6 mice excluded from prior litter-related analyses were excluded here). The comparison of this 'litter' model (AIC = 1146) to a 'null' model (AIC = 1188) that fictionally assigned all mice to the same litter, as described previously, yielded a large AIC difference (1188–1146 = 42). This result favored the litter model as >99.99% (i.e., $1–(9 \times 10^{-10})$) probable – or 1×10^9 times more likely – to be preferred to the null model. Hence, litter size and sex ratio could not entirely account for the clustering of C57BL/6J sociability by litter membership; some clustering remained unexplained even after accounting for litter size and sex ratio. Thus, there are very likely to be factors in addition to litter size and sex ratio that affect C57BL/6J sociability at the litter level.

3.3. Corpus callosum

Out of the 140 BALB/cJ mice bred and used for analysis, only 16 (11.4%) were identified as having an abnormally small CC size, which was defined as a CC index of abnormality less than 0.65 (i.e., less than 65% of the expected CC size, based on the brain weight, as described in Section 2.4). Because the regression equations used to calculate the CC index were based on previously reported F_1 and F_2 hybrid crosses of the C57BL/6J and DBA/2J strains [83], we were concerned that the equations might not apply well to the BALB/cJ strain. We therefore used OP regression to model CC area as a function of brain weight, $y = \beta_0 + \beta_1$ (brain weight), which yielded $\beta_0 = -0.82$ and $\beta_1 = 3.64$. Application of the same 0.65 criterion identified the same 16 BALB/cJ mice (Fig. 4a). Finally, a LMS method of detecting outliers also identified the same 16 mice as having an abnormally small CC area. All three methods therefore agreed and showed that our identification of mice with an abnormally small CC was robust.

The 2 (CC group: normal size vs. abnormally small) × 5 (age) ANOVAs showed no differences in social cylinder investigation (which consisted predominantly of sniffing of the social cylinder) between the normal-sized and abnormally small CC groups, $F(1, 136) = 0.19$, $p = 0.66$ ($Q(1, 136) = 0.57$, $p = 0.46$), nor did they show an interaction between CC size and age, $F(1, 136) = 2.60$, $p = 0.11$ ($Q(1, 136) = 5.77$, $p = 0.38$) (Fig. 4b). Including sex as a factor in the analyses made no substantial difference in the results. The α level was 0.05.

3.4. Brain weight

Brain weight was analyzed as a proportion of body weight (called “relative brain weight”) (Fig. 5a). To examine strain differences in relative brain weight at each age, 2 × 2 (strain by sex) ANOVAs were executed with α levels set to 0.01 (0.05/5 age groups). C57BL/6J mice had larger relative brain weight than BALB/cJ mice at 20 days, $F(1, 41) = 51.5$, $p = 9.3 \times 10^{-9}$ ($Q(1, 41) = 35.7$, $p = 0.001$), and at 24 days, $F(1, 43) = 26.7$, $p = 5.9 \times 10^{-6}$ ($Q(1, 43) = 22.8$, $p = 0.001$). At 32 days, there was a trend toward larger relative brain weights for C57BL/6J vs. BALB/cJ mice, $F(1, 80) = 24.3$, $p = 4.4 \times 10^{-6}$ ($Q(1,80) = 5.3$, $p = 0.04$), but this trend was exaggerated by 6 outlying values (3 C57BL/6J females, 3 C57BL/6J males) that made the noneffects of the trimmed means analysis more trustworthy. C57BL/6J and BALB/cJ relative brain weights did not differ at 43 days, $F(1, 31) = 0.9$, $p = 0.36$ ($Q(1, 31) = 3.2$, $p = 0.098$). C57BL/6J mice at 71 days trended toward having larger relative brain weights than 71-day- old BALB/cJ mice, $F(1, 41) = 13.0$, $p = 0.0008$ ($Q(1, 41) = 7.3$, $p = 0.012$).

An ANCOVA of social cylinder investigation (which consisted predominantly of sniffing of the social cylinder) modeled relative brain weight as a covariate and strain, sex, and age (2 × 2 × 5) as factors, and revealed a strain by age by brain weight interaction, $F(4, 216) = 3.1$, $p = 0.017$, $\alpha = 0.05$. Subsequent ANCOVAs (relative brain weight as covariate; strain and sex as factors) at each age showed a strain by relative brain weight interaction at 31 days, $F(1, 76) = 7.3$, $p = 0.0086$, $\alpha = 0.01$ (0.05/5 age groups). No other effects of relative brain weight were found at any other ages (all ps > 0.04, $\alpha = 0.01$).

At 31 days of age, an ANCOVA (relative brain weight as covariate; sex as factor) for each strain showed that BALB/cJ mice with larger brains were more sociable than mice with smaller brains, $F(1, 50) = 6.2$, $p = 0.016$, $\alpha = 0.025$ (0.05/2 mouse strains) (Fig. 5b), but showed no effect in the C57BL/6J mice (both ps > 0.08, $\alpha = 0.025$) (Fig. 5c). An OP regression modeled social cylinder investigation of the 31-day-old BALB/cJ mice as $y = \beta_0 + \beta_1$ (relative brain weight) + β_2 (sex), which showed that β_1 trended toward being greater

than zero, D = 0.058, $p = 0.039$, $\alpha = 0.025$. An analogous OP regression for the 31-day-old C57BL/6J mice showed no effect, D = 0.008, $p = 0.52$, $\alpha = 0.025$.

4. Discussion

Our results show not only that C57BL/6J and BALB/cJ mice differ in their development of sociability, but also that the strains differ in the factors that influence intra-strain variability of sociability. BALB/cJ mice were generally less sociable than C57BL/6J mice at prepubescent ages and increased in sociability as they aged, whereas the sociability of C57BL/6J mice did not change significantly across development. Litter size and sex ratio were associated with alterations in sociability of C57BL/6J mice, but did not clearly show effects in BALB/cJ mice. Conversely, brain weight correlated with BALB/cJ sociability, but did not affect C57BL/6J sociability.

The development of C57BL/6J and BALB/cJ sociability shows that strain differences are generally more prominent at younger ages whereas sex differences appear to be more prominent at older ages. BALB/cJ mice were less sociable than C57BL/6J mice before pubescence (except at 23 days of age), but this strain difference was not evident at older ages. By adulthood, the sociability differences between the strains were small compared to the sociability differences between the sexes. This pattern agrees with Panksepp et al. [95], who showed that social behaviors of C57BL/6J and BALB/cJ mice were increasingly sex-sensitive from 25 to 45 days of age, likely due to the effects of puberty on behavior.

The importance of early adolescence to the development of social behaviors, which was emphasized by Panksepp and Lahvis [56], was highlighted by the strain by age interaction in our data. From 31- to 42-days-of-age, the sociability of C57BL/6J mice appeared to decrease whereas the sociability of BALB/cJ increased,more so in male than in female mice. Panksepp et al. [95] did not identify a similar pattern in same-sex social behaviors, which may be attributable to differing social test situations. Our Social Approach Test in littermate-housed mice occurred during the light cycle in a neutral arena with an adult gonadectomized A/J stimulus mouse whose movement was restricted. By contrast, Panksepp et al. [95] tested two mice of the same strain and age in the home cage of one of those mice following 24 h of social isolation. Both mice could move freely and testing was during the dark cycle.

The importance of puberty to sociability suggests that the biological mechanisms for social approach and investigation are quite different before vs. after the onset of puberty. Adolescence marks the start of more pronounced sexual and aggressive motivations, indicating that earlier ages are of particular interest for the study of social affiliation. Social affiliation at prepubescent ages in mice may be particularly relevant to neurodevelopmental disorders, such as idiopathic and syndromic forms of ASD, which are characterized by childhood-onset impairments in social affiliative behaviors. We have repeatedly shown that BALB/cJ mice are less sociable than C57BL/6J mice at about 30 days of age [15,18,25]. Interestingly, this strain difference appeared to be larger at 19 days. Panksepp et al. [95] also found a larger strain difference at younger ages between same-sex mice, but their difference was at 25–26 days, near the 23-day age when we found no significant difference.

In addition to sexual and aggressive motivations, anxiety may also affect the tendency to seek social interaction, or sociability. Heightened anxiety has been thought to reduce social interaction in certain contexts. In the Social Interaction Test, two rodents are allowed to move freely about an arena and the duration of their active social investigation is recorded. Factors that increase anxiety – such as anxiogenic drugs, bright lighting, and unfamiliarity with the arena – tend to decrease social interaction, whereas anxiolytics, dim lighting, and

familiarity with the arena tend to increase social interaction [96–98]. In nonsocial contexts, adult BALB/cJ mice usually exhibit high levels of anxiety-related behaviors compared to other mouse strains, including C57BL/6J mice[99–104]. Thus, one might have predicted that adult BALB/cJ mice would show lower sociability than adult C57BL/6J mice. Instead, the sociability of 70-day-old adult BALB/cJ mice was about equal to or even slightly higher than that of adult C57BL/6J mice. This result suggests that anxiety was not the predominant factor affecting adult sociability. Alternatively, the traditional interpretation of the anxiety–sociability relationship may be incomplete, perhaps because anxiety in nonsocial contexts does not correlate well with anxiety measured in the Social Interaction Test [105,106] and may not correlate well with behavior in the Social Approach Test. In any case, little is known about anxiety and how it relates to sociability in prepubescent mice. This will be an important area for future research because autistic children exhibit elevated levels of anxiety, and the relationship between anxiety and social deficits in ASD is poorly understood [107,108].

Sociability clustered according to litter membership, so that there was less difference in the sociability among littermates, compared to the larger differences in sociability among non-littermates. Future research on mouse sociability should account for this clustering effect, and Galbraith et al. [89] compare methods for doing so. The strength of clustering appeared to be stronger in C57BL/6J mice than in BALB/cJ mice. The clustering may be attributable to pre- or postnatal maternal factors, epigenetics, littermate social interactions [109], and/or other aspects of the litter environment. We identified two factors, litter size and sex ratio, that accounted for some of this clustering in C57BL/6J mice.

Litter size can affect the subsequent behaviors of pups in the open field and in tests of aggression [93,110,111], but no effects have been previously identified on social affiliative behaviors to our knowledge. Panksepp et al. [95] did not find any such effects, but this discrepancy may be due to different social test conditions between their study and ours, as noted previously. C57BL/6J mice from larger litters may be less sociable due to prenatal effects. It is not clear whether pups in large litters compete for nutritional resources in utero [112,113], but they do have relatively low birth weights [114,115] and they behaviorally and physiologically resemble undernourished mice in some ways [116]. In humans, both multiple concurrent births [64,68] and low birth weight [65,67,117,118] have been identified as risk factors for ASD. These findings are not entirely consistent [64–68], but were validated in an extensive meta-analysis of perinatal factors that predict ASD onset [63].

Because we culled our litters within four days of birth, postnatal effects of litter size on C57BL/6J sociability were probably minimal, but such effects cannot be excluded from consideration. Maternal milk production and quality might not be a limiting factor to pups shortly after birth [119], but maternal behaviors could play a role. Larger litter sizes in rats have been associated with less maternal licking and grooming toward each pup [120], and these maternal behaviors are associated with differences in pups' subsequent behaviors in rats [121,122] and mice [123,124]. Additionally, litter size affects how pups interact in the nest [125], which may influence subsequent development. The potential influence of these postnatal factors also raises the possibility that our results might not apply to litters in other studies that are not culled shortly after birth. However, the culling in the current study ensured that pups received adequate nutrition after the first postnatal week [119] and were reared in a more uniform social environment, which likely reduced the variability in our measurements of sociability.

C57BL/6J pups born in litters with a higher ratio of females were more sociable than mice from male-predominant litters. Notably, litters cross-fostered to be predominantly female showed higher levels of social play [126], but these postnatally manipulated litter ratios

overlapped our pre-culling period by only 1–3 days. More relevantly, a gestating pup is exposed to more estradiol and less testosterone when the adjacent pup(s) is female, and such pups display more “female-typical” physiology and behaviors [127]. Thus, hormones in utero may influence C57BL/6J sociability.

This finding in C57BL/6J mice may be consistent with human studies suggesting that higher levels of fetal testosterone are associated with reduced sociability and increased risk of ASD [69,70]. Interestingly, our data suggest that the effect of litter sex ratio (and perhaps prenatal testosterone exposure) vary by strain, implicating possible gene by hormone–exposure interactions in their effects on sociability. Interactions between gene variants and prenatal testosterone exposure in their effects on sociability may be worthy of future study in humans and animal models.

Our results did not confirm our hypothesis that abnormally small CC would be associated with low sociability in BALB/cJ mice. The sociability–CC result was inconsistent with a prior experiment in 30-day-old mice that used the same methods, but a smaller sample size, and found a positive correlation between CC size and sociability [25]. Examination of the data (Fig. 3b) suggested that any undetected effects of CC size on sociability may have varied with development. The sociability means of small-CC mice were consistently higher than those of normal-CC mice at younger ages, but lower at older ages. Unfortunately, the low numbers of small-CC mice at each age limited our statistical power to detect any potential CC-size-by-age interaction. Notably, other studies [20,81] have not detected an association between the corpus callosum and social behaviors in mice.

We originally predicted that BALB/cJ mice with larger brains would be less sociable than smaller-brained mice, in parallel with humans with ASD who have larger brains on average. Instead, 31day-old BALB/cJ mice with larger relative brain weights trended toward being more sociable. Smaller brain size correlated with lower sociability not only within the BALB/cJ strain, but also between strains. BALB/cJ mice generally had smaller relative brain weights than C57BL/6J mice at younger ages, when BALB/cJ mice were generally less sociable than C57BL/6J mice, and the largest difference in brain size – at 20 days of age – corresponded to the largest difference in sociability.

Although, on average, children with ASD have larger brains than normal children, ASD is highly heterogeneous, and a subset of individuals with ASD-like social behavior phenotypes have smaller than average brains. As examples, children with certain syndromic forms of ASD (e.g., Rett syndrome and Angelman syndrome) have sociability disruptions and relatively small brains[128]. Thus, although the association of smaller brain size with lower sociability in BALB/cJ mice may not be relevant to the most common, idiopathic forms of ASD, it may be relevant to smaller subsets of individuals with ASD or with ASD-like syndromes that are associated with smaller brain size.

A complex and diverse set of gene-environment interactions influence both the BALB/cJ-C57BL/6J strain difference and intrastrain variability in sociability development. Additional study of these and other mouse models will be crucial to discovering the many interwoven factors and molecular mechanisms that affect the developmental neurobiology of sociability. This research is likely to reveal important insights into the neural mechanisms of typical and atypical social behavior development.

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Abbreviation

CC corpus callosum

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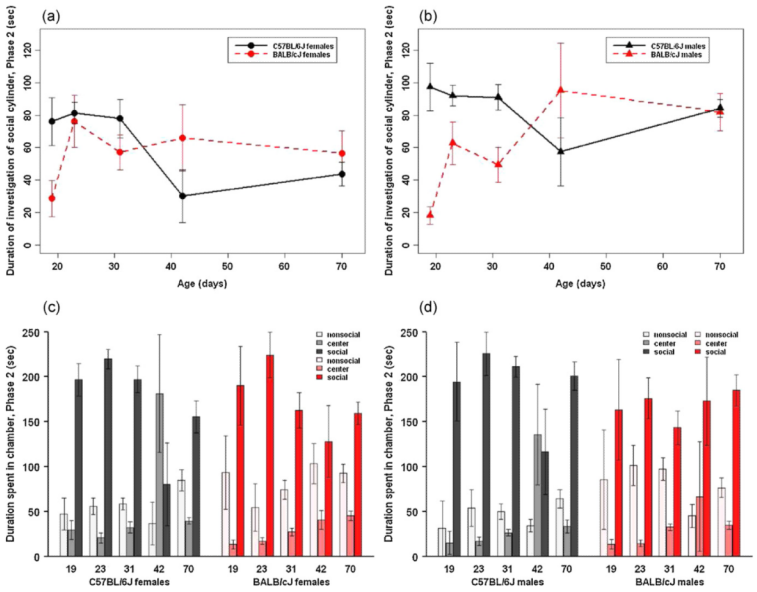

Fig. 1.

Development of social behaviors in the Social Approach Test. Values are 20% trimmed means ± SE of the trimmed means. (a and b) Investigation of (including, primarily, sniffing of) the social cylinder during Phase 2. (a) Females. (b) Males. Analyses of social cylinder investigation (a and b) showed that BALB/cJ mice were less sociable than C57BL/6J mice at 19 and 31 days of age, strain and age interacted between 31 and 42 days of age, and the sociability of BALB/cJ mice increased from 19 to 70 days of age. (c and d) Amount of time spent in each chamber during Phase 2. (c) Females. (d) Males.

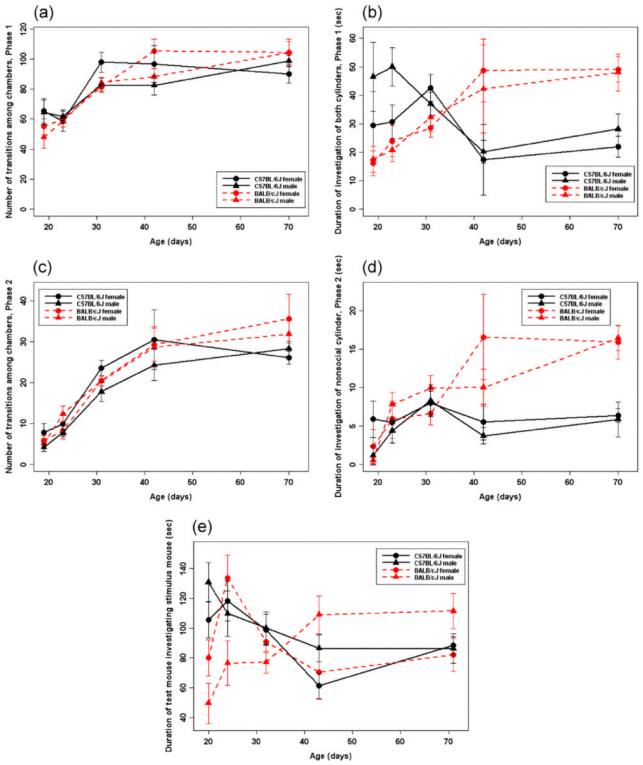

Fig. 2.

Development of locomotor activity, nonsocial cylinder investigation, and free social interaction in the Social Approach Test. Values are 20% trimmed means ± SE of the trimmed means. (a) Number of times test mouse moved among chambers (i.e., number of transitions) during Phase 1. (b) Amount of time test mouse investigated (primarily sniffed) both empty cylinders during Phase 1. (c) Number of times test mouse moved among chambers (i.e., number of transitions) during Phase 2. (d) Amount of time test mouse investigated (primarily sniffed) nonsocial cylinder containing a novel object during Phase 2. (e) Amount of time test mouse investigated (primarily sniffed) the stimulus mouse during Phase 3.

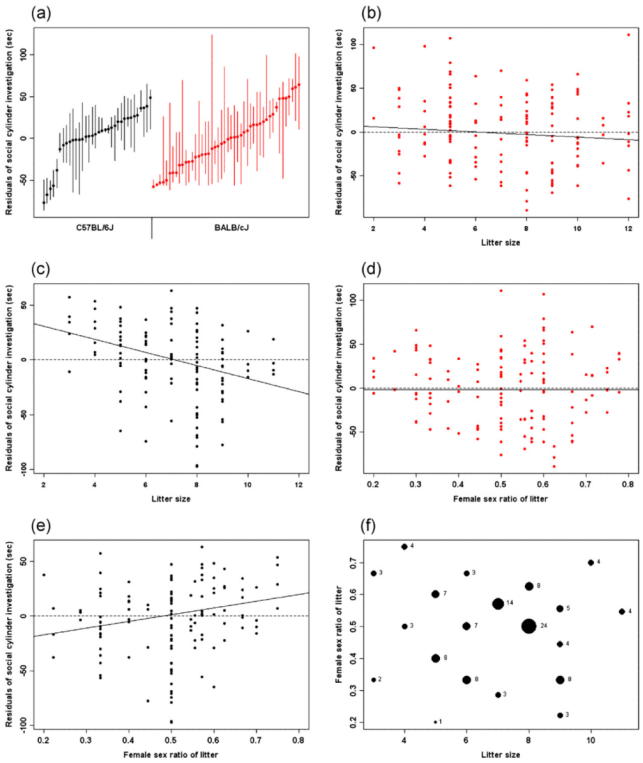

Fig. 3.

Litter effects in C57BL/6J and BALB/cJ mice. (a) Clustering of residuals of social cylinder investigation (includes, predominantly, sniffing) by litter membership. Each point denotes the median for all mice in a litter. Each vertical line denotes the range for all mice in a litter. Analyses showed that social cylinder investigation did cluster by litter membership in C57BL/6J and BALB/cJ mice (i.e., the differences between the litters were large relative to the within-litter variability). (b) Residuals of social cylinder investigation as a function of BALB/cJ litter size. (c) Residuals of social cylinder investigation as a function of C57BL/6J litter size. (d) Residuals of social cylinder investigation as a function of female sex ratio of BALB/cJ litters. (e) Residuals of social cylinder investigation as a function of female sex ratio of C57BL/6J litters. For (b–e), y axes plot residuals of social cylinder investigation following 20% trimmed means ANOVAs that took sex and age as factors. Solid lines denote OP regression lines. For (b and d) BALB/cJ mice, the slope of the OP regression line was not significantly different from zero (dotted horizontal line). For (c and e) C57BL/6J mice, the slope of the OP regression line was significantly different from zero (dotted horizontal line): higher sociability correlated with (c) smaller litter size and (e) higher female sex ratio. (f) Female sex ratio of litter vs. litter size for C57BL/6J litters. Area of points is proportional to number of litters, which is printed next to corresponding point. C57BL/6J female sex ratio and litter size did not correlate.

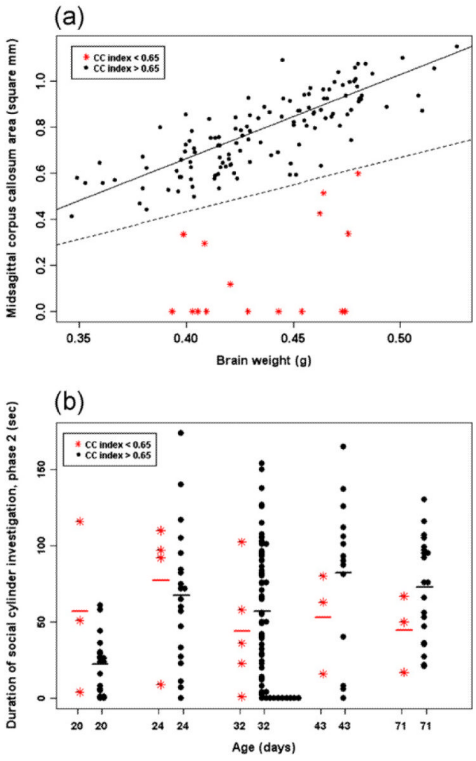

Fig. 4.

Normal vs. abnormally small corpus callosum (CC) size in BALB/cJ mice. A CC index <0.65 is considered abnormally small. (a) Area of the CC at the midsagittal plane as a function of brain weight. Solid line denotes the OP regression line. Dotted line denotes CC index = 0.65, based on the 0.65 criterion of the OP regression line. Red stars indicate data of mice with CC index <0.65. (b) Development of social cylinder investigation (includes, predominantly, sniffing) of mice with normal and abnormally small CC. Horizontal bars denote means of each group. Red stars indicate data of mice with CC index <0.65. The sociability of mice with abnormally small CC did not differ significantly from that of mice with normal-sized CC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

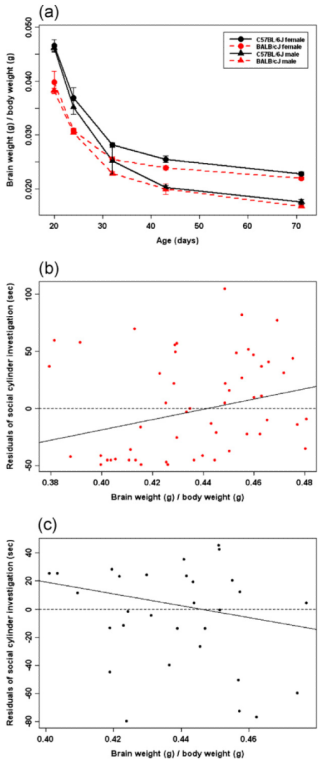

Fig. 5.

Development of relative brain weight and sociability in C57BL/6J and BALB/cJ mice. (a) Perfused brain weight as a proportion of body weight (relative brain weight). Values are 20% trimmed means ± SE of the trimmed means. C57BL/6J mice had larger relative brain weights than BALB/cJ mice at 20 and 24 days. (b) Residuals of social cylinder investigation (includes, predominantly, sniffing) as a function of relative brain weight in 31-day-old BALB/cJ mice. (c) Residuals of social cylinder investigation (includes sniffing) as a function of relative brain weight in 31-day-old C57BL/6J mice. For (b and c), y axes plot residuals of social cylinder investigation (includes sniffing) following 20% trimmed means ANOVAs that took sex as a factor. Solid lines denote OP regression lines. For (b) BALB/cJ mice, the slope of the OP regression line trended toward a significant difference from zero (dotted horizontal line): higher sociability trended toward a correlation with larger relative brain weight. No correlations were detected for (c) C57BL/6J mice.

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Table 1

Number of mice in each experimental group. Numbers in parentheses denote additional mice that were excluded from further analyses due to aggression displayed during Phase 3 of the Social Approach Test.

	Age				
	19 days	**23 days**	**31 days**	**42 days**	**70 days**
C57BL/6J females	13	11	17 (1)	8 (1)	13
C57BL/6J males	10	12	19	11 (3)	9 (5)
BALB/cJ females	10	10	35	9	12
BALB/cJ males	12	14	32	8 (2)	11 (1)