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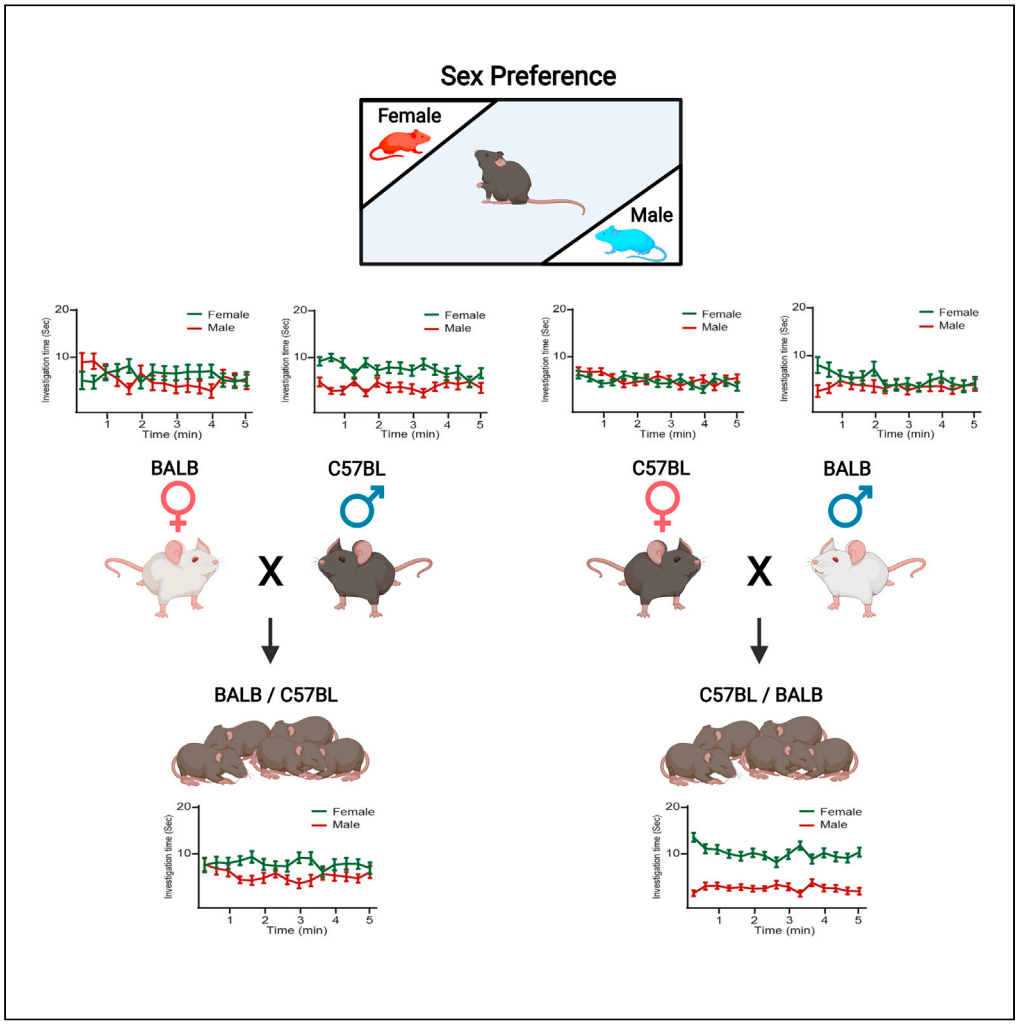

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Highlights

Social investigation behavior of laboratory mice is highly strain- and sex-specific

Some behavioral aspects are either strain- or sex-specific, but not both

Mixed offspring of distinct strains behave differently from both parental strains

The behavior of mixed offspring depends on the specific combination of parents

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Sex-dependent features of social behavior differ between distinct laboratory mouse strains and their mixed offspring

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SUMMARY

The survival of individuals of gregarious species depends on their social interactions. In humans, atypical social behavior is a hallmark of several psychopathological conditions, many of which have sex-specific manifestations. Various laboratory mouse strains are used to reveal the mechanisms mediating typical and atypical social behavior in mammals. Here, we used three social discrimination tests to characterize social behavior in males and females of three widely used laboratory mouse strains (C57BL/6J, BALB/c, and ICR). We found marked sex- and strain-specific differences in the behavior exhibited by subjects, in a test-dependent manner. Interestingly, some characteristics were strain-dependent, while others were sex-dependent. We then crossbred C57BL/6J and BALB/c mice and found that offspring of such crossbreeding exhibit social behavior which differs from both parental strains and depends on the specific combination of parental strains. Thus, social behavior of laboratory mice is sex- and strain-specific and depends on both genetic and environmental factors.

INTRODUCTION

The survival and success of individuals of gregarious mammalian species depend on their ability to form social interactions properly (Robinson et al., 2008; Stanley and Adolphs, 2013). In humans, atypical social behavior is a hallmark of several psychopathological conditions and neurodevelopmental diseases (NDDs) (Porcelli et al., 2019), such as social anxiety disorder (Leichsenring and Leweke, 2017), autism spectrum disorder (de la Torre-Ubieta et al., 2016), and schizophrenia (Mier and Kirsch, 2017). Notably, many of these conditions have sex-specific manifestations and exhibit a robust diagnostic sex-bias (Gobinath et al., 2017; Palanza and Parmigiani, 2017). However, unraveling the biological basis of sex-specific manifestations in human NDDs is extremely difficult due to the strong influence of culture, education, and living style, all of which are heavily sex-biased (Grabowska, 2017). One way to overcome this difficulty is by using animal models, which are not affected by cultural factors (Bredewold and Veenema, 2018; Palanza and Parmigiani, 2017). Animal models are an important tool for exploring the biological basis of social behavior in general and particularly are used to unravel impaired mechanisms which underlie atypical social behavior in NDDs (Crawley, 2012; Fernando and Robbins, 2011; Kaiser et al., 2017; Nestler and Hyman, 2010). Specifically, genetically modified mouse models carrying mutations in NDD-associated genes are widely used for such research (McGraw et al., 2017). Notably, different laboratories use distinct laboratory mouse strains, some of which are inbred strains (Casellas, 2011), for their research, and these strains also serve as a genetic background for the various mouse models of NDDs (Tam and Cheung, 2020; Wade and Daly, 2005). While multiple previous studies have explored inter-strain differences in murine social behavior (Moy et al., 2007, 2008; Netser et al., 2020), the effect of sex on aspects of social behaviors which are not associated with aggression, parenting, or sexual behavior has been poorly studied. Moreover, nothing is known about the consequences of mixing distinct strains by crossbreeding, regarding the social behavior of the offspring.

Several behavioral paradigms have been developed for assessing social behavior in mouse models. Of these, the most famous is the three-chamber test (Moy et al., 2004), which employs two types of social discrimination tasks: social preference (SP) and social novelty preference (SNP). We have previously presented a computational experimental system for automatic assessment of murine social discrimination behavior (Netser et al., 2019) and used it to analyze the behavior of C57BL/6J mice in the SP and SNP paradigms (Netser et al., 2017, 2020). Here, we added

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a third paradigm of social discrimination, the sex-preference (SxP) paradigm (Jabarin et al., 2021), in order to systematically examine sex- and strain-dependent behavioral parameters in the three most commonly used (estimated by publications, see https://www.labome.com/method/Laboratory-Mice-and-Rats.html) laboratory mouse strains (C57BL/6J, BALB/c, and ICR). Moreover, we characterized the behavior of offspring generated by crossbreeding of C57BL/6J and BALB/c mice.

RESULTS

Sex- and strain-dependent differences in social discrimination behavior

To assess sex- and strain-specific differences in social behavior, we employed our computerized behavioral system (Netser et al., 2017, 2019) to analyze the level and dynamics of investigation behavior exhibited by mice in three distinct social discrimination tests: SP, SNP, and SxP (see Figure S1A for the timeline). We used all three tests to systematically characterize the behavior of male and female subjects of three distinct laboratory strains: C57BL/6J, BALB/c, and ICR (CD-1). Of these strains, the former two are inbred while the latter is an outbred strain. In all tests, we automatically measured the time dedicated by the subject to investigating each of two stimuli, simultaneously presented in distinct chambers, which are located at the opposite corners of the experimental arena. A statistically significant difference in investigation time between the two stimuli was considered to reflect a preference for the stimulus investigated by the subject for a longer duration. Importantly, all experiments were repeated using at least two distinct cohorts of animals (see Table S1), and the minimum number of mice subjected to each test was nineteen. It should be noted, however, that not all mice went through all three tests.

As apparent in Figure 1, we found sex- and strain-specific differences in the performance of the subjects in a test-dependent manner. While a preference for the social stimulus compared to the object stimulus was observed for all strains and both sexes (Figure 1A. *C57: Males:* $t_{57} = 7.754$, $p < 0.0001$; *Females:* $t_{26} = 4.044$, $p < 0.0001$. *BALB/c: Males:* $t_{20} = 10.210$, $p < 0.0001$; *Females:* $t_{18} = 3.080$, $p = 0.006$. *ICR: Males:* $t_{22} = 9.496$, $p < 0.0001$; *Females:* $t_{34} = 6.110$, $p < 0.0001$, *paired t test*), the results of the other two tests were different. A preference for the novel social stimulus over the familiar one in the SNP test was observed for females of all strains. However, when examining males, only C57BL/6J mice showed such a preference (Figure 1B. *C57: Males:* $t_{57} = 5.780$, $p < 0.0001$; *Females:* $t_{24} = 3.088$, $p < 0.005$. *BALB/c: Males:* $t_{20} = 1.423$, *n.s.; Females:* $t_{19} = 4.287$, $p < 0.0001$. *ICR: Males:* $t_{23} = 1.199$, *n.s.; Females:* $t_{34} = 3.774$, $p < 0.001$, *paired t test*). The most variable pattern was found in the SxP test (Figure 1C), where C57BL/6J and ICR males showed a clear preference for the opposite-sex stimulus, while BALB/c males did not show any preference between the stimuli (*C57:* $t_{44} = 4.281$, $p < 0.0001$; *BALB/c:* $t_{21} = 1.812$, *n.s.; ICR:* $t_{37} = 9.508$, $p < 0.0001$, *paired t test*). In contrast, in none of the strains did females show a preference for the opposite-sex stimulus. However, ICR females showed a clear preference for the same-sex stimulus (*C57:* $t_{49} = -0.982$, *n.s.; BALB/c:* $t_{19} = 0.739$, *n.s.; ICR:* $t_{34} = 5.833$, $p < 0.0001$, *paired t test*).

For direct statistical comparison between animal groups, we calculated the difference in investigation time between the two stimuli (henceforth termed ΔIT) and compared it across sexes and strains separately for each test. For the SP test (Figure 2A), we found a significant main effect of sex only ($F_{1,177} = 19.241$, $p < 0.0001$, *two-way ANOVA*). *Post hoc* analysis revealed a significantly higher ΔIT for males, as compared to females, for all three strains (*C57:* $t_{83} = -2.085$, $p = 0.040$; *BALB/c:* $t_{38} = -2.113$, $p = 0.041$; *ICR:* $t_{56} = -3.787$, $p < 0.0001$, *independent t test*). For the SNP test (Figure 2B), there was a significant interaction between sex and strain ($F_{2,177} = 4.864$, $p < 0.009$, *two-way ANOVA*). *Post hoc* analysis revealed a borderline significant differences between female and male ICR mice ($t_{177} = 2.009$, $p < 0.049$, *Holm-Sidak test*), while no significant difference was observed between male and female C57BL/6J and BALB/c mice. Between strains, C57BL/6J males showed significantly higher ΔIT than ICR males ($t_{177} = 2.444$, $p = 0.046$, *Holm-Sidak test*), and borderline significantly higher ΔIT than BALB/c males ($t_{177} = 2.102$, $p = 0.073$, *Holm-Sidak test*). As for the SxP test (Figure 2C), we observed again a significant interaction between sex and strain ($F_{2,204} = 12.705$, $p < 0.0001$, *two-way ANOVA*). *Post hoc* analysis revealed a significantly or borderline significantly higher ΔIT for males, as compared to females, for all three strains (*C57:* $t_{204} = 3.687$, $p = 0.0006$; *BALB/c:* $t_{204} = 1.777$, $p = 0.077$; *ICR:* $t_{204} = 9.164$, $p < 0.0001$, *Holm-Sidak test*). Across strains, significantly lower ΔIT values were found for ICR females compared to both C57BL/6J and BALB/c females (C57: $t_{204} = 4.489$, $p < 0.0001$; BALB/c: $t_{204} = 2.460$, $p = 0.029$, *Holm-Sidak test*), while ICR male mice showed significantly higher ΔIT than BALB/c male mice ($t_{204} = 3.391$, $p = 0.003$, *Holm-Sidak test*). Overall, these data suggest sex- and strain-specific differences in the preference exhibited by subjects during the SP, SNP, and SxP tests, in a test-dependent manner.

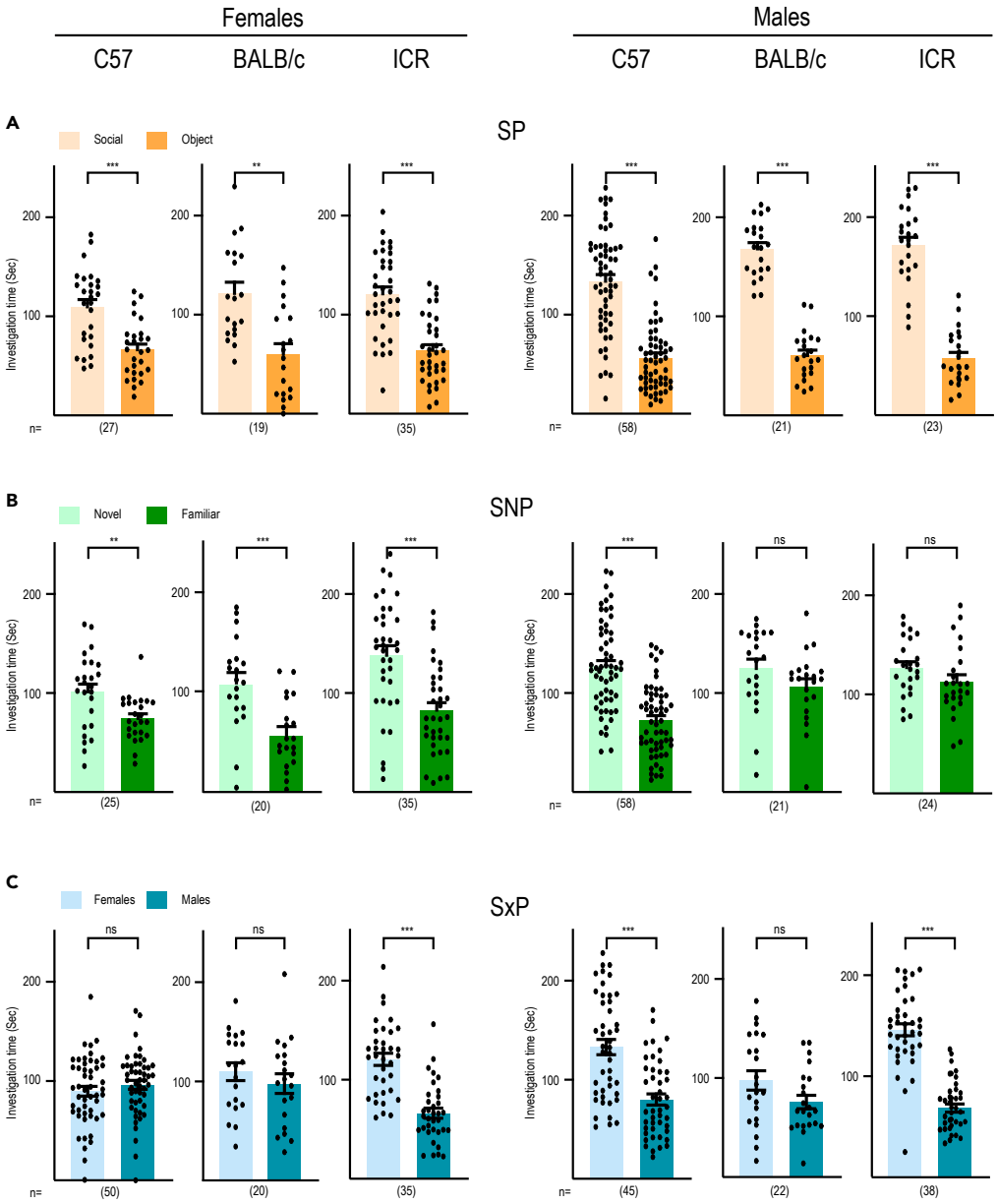

Figure 1. Sex- and strain-specific differences in social behavior during three distinct social discrimination tests: SP, SNP, and SxP

Mean investigation time (±SEM) measured separately for each stimulus across the SP (A), SNP (B), and SxP (C) tests, performed by females (left) and males (right) of all three mouse strains (denoted above). Sample size is denoted below the bars (#p< 0.1, **p< 0.01, ***p< 0.001, ns – not significant, paired t test).

We also examined if there are correlations between ΔIT in the various tests, separately for each group (Figure S2). In most cases, we found no significant correlation and in the few cases where a significant correlation was found (SP vs. SxP for ICR males, SNP vs. SxP for C57BL/6J females, and SNP vs. SxP for BALB/c males, $p < 0.05$, *Pearson's correlation*), this correlation was weak ($R < 0.5$).

Sex- and strain-dependent differences in the distance traveled during social interactions

We next compared the distance traveled by the subjects during each test between the various animal groups (Figure S3). For the SP test, we found a significant interaction between sex and strain ($F_{2,177} = 3.932$, $p = 0.0213$, *two-way ANOVA*, Figure S3A). *Post hoc* analysis revealed a significantly longer distance

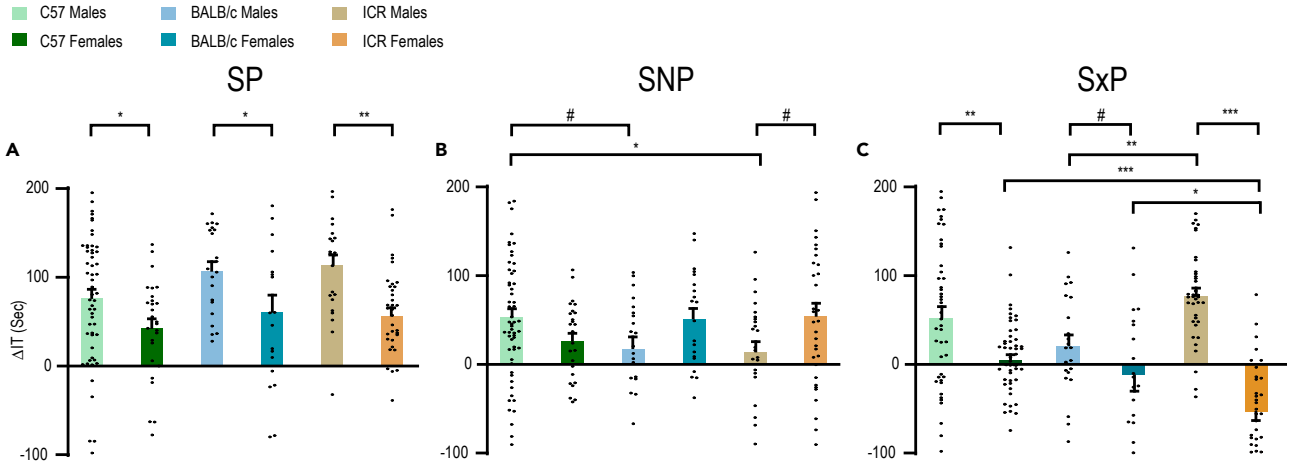

Figure 2. Sex- and strain-dependent difference in investigation time between the two stimuli in the SP, SNP, and SxP tests

Mean (±SEM) difference in investigation time (ΔIT) between the two stimuli, measured separately for each animal group during the SP (A), SNP (B), and SxP (C) tests. *p< 0.05, **p< 0.01, ***p< 0.001, ns – not significant, *post hoc* t test in cases of no multiple comparisons (A) and *post hoc* Holm-Sidak test, following main effect in two-way ANOVA test.

traveled by ICR females as compared to males (t_{177} = 3.426, p = 0.002, *Holm-Sidak test*). Between strains, we found a significantly shorter distance traveled by C57BL/6J females, as compared to ICR and BALB/c females (ICR: t_{177} = 6.209, p <0.0001; BALB/c: t_{177} = 3.575, p = 0.0009, *Holm-Sidak test*). For the SNP test, we found main effects of both sex ($F_{1,177}$ = 4.694, p = 0.0316) and strain ($F_{2,177}$ = 22.13, p <0.0001, *two-way ANOVA*, Figure S3B), but no interaction between them. *Post hoc* analysis revealed a borderline significantly lower distance traveled by males than by females for both C57BL/6j and ICR mice (C57BL/6J: t_{177} = 2.223, p <0.0542, ICR: t_{177} = 2.409, p <0.0502, *Holm-Sidak test*). Across trains, we revealed that ICR females traveled significantly longer distances from their counterparts of both other strains (C57BL/6J: t_{177} = 4.666, p <0.0001; BALB/c: t_{177} = 2.630, p = 0.008, *Holm-Sidak test*). In contrast, C57BL/6J males traveled significantly shorter distances than males of both other sexes (BALB/c: t_{177} = 4.221, p <0.0001; ICR: t_{177} = 4.595, p <0.0001, *Holm-Sidak test*). Finally, for the SxP test, we found a main effect only for the strain ($F_{2,204}$ = 85.61, p <0.0001, *two-way ANOVA*, Figure S3C), with C57BL/6J females traveling shorter distance than their counterparts of both other strains (BALB/c: t_{204} = 2.476, p = 0.014; ICR: t_{204} = 10.430, p <0.0001, *Holm-Sidak test*) and BALB/c females traveling less than ICR females (t_{204} = 5.861, p <0.0001, *Holm-Sidak test*). Similarly, C57BL/6J males traveled shorter distance than their counterparts of both other strains (BALB/c: t_{204} = 3.975, p = 0.0002; ICR: t_{204} = 8.075, p <0.0001, *Holm-Sidak test*) and BALB/c males traveling less than ICR males (t_{204} = 2.781, p = 0.0059, *Holm-Sidak test*). Notably, the differences between the groups in the distance traveled by the subjects were very similar between the various tests, suggesting that this parameter is test-independent. These results point to a general tendency of C57Bl/6J mice (both sexes) to travel less than BALB/c mice and a tendency of BALB/c mice to travel less than ICR mice, with almost no sex-dependent differences.

Strain-, but not sex-dependent differences in the dynamics of transitions between stimuli

In a previous study, we found that C57BL/6J mice and Sprague-Dawley (SD) rats exhibit distinct dynamics of transitions between the two stimuli during the SP test and linked these differences to their distinct dynamics of social motivation (Netser et al., 2020). Therefore, in the current study, we investigated the dynamics of transitions during all three tests for all animal groups. Interestingly, unlike the differences in investigation time (Figure 1), we found no difference between males and females of any of the strains in the dynamics of transitions between stimuli (Figure 3). We observed, however, marked differences among the various strains in a test-dependent manner. This difference was most notable between C57BL/6J mice and BALB/c mice. In accordance with our previous report, both male and female C57BL/6J mice exhibited high levels of transitions at the beginning of the test, which gradually declined during later stages, in all tests. In contrast, BALB/c mice exhibited a rather constant pattern of transitions, except for the SxP test, where a lower level at the beginning of the test was observed. For ICR mice, the pattern of transitions was rather constant across the various tests with one exception: in the SxP test, both male and female

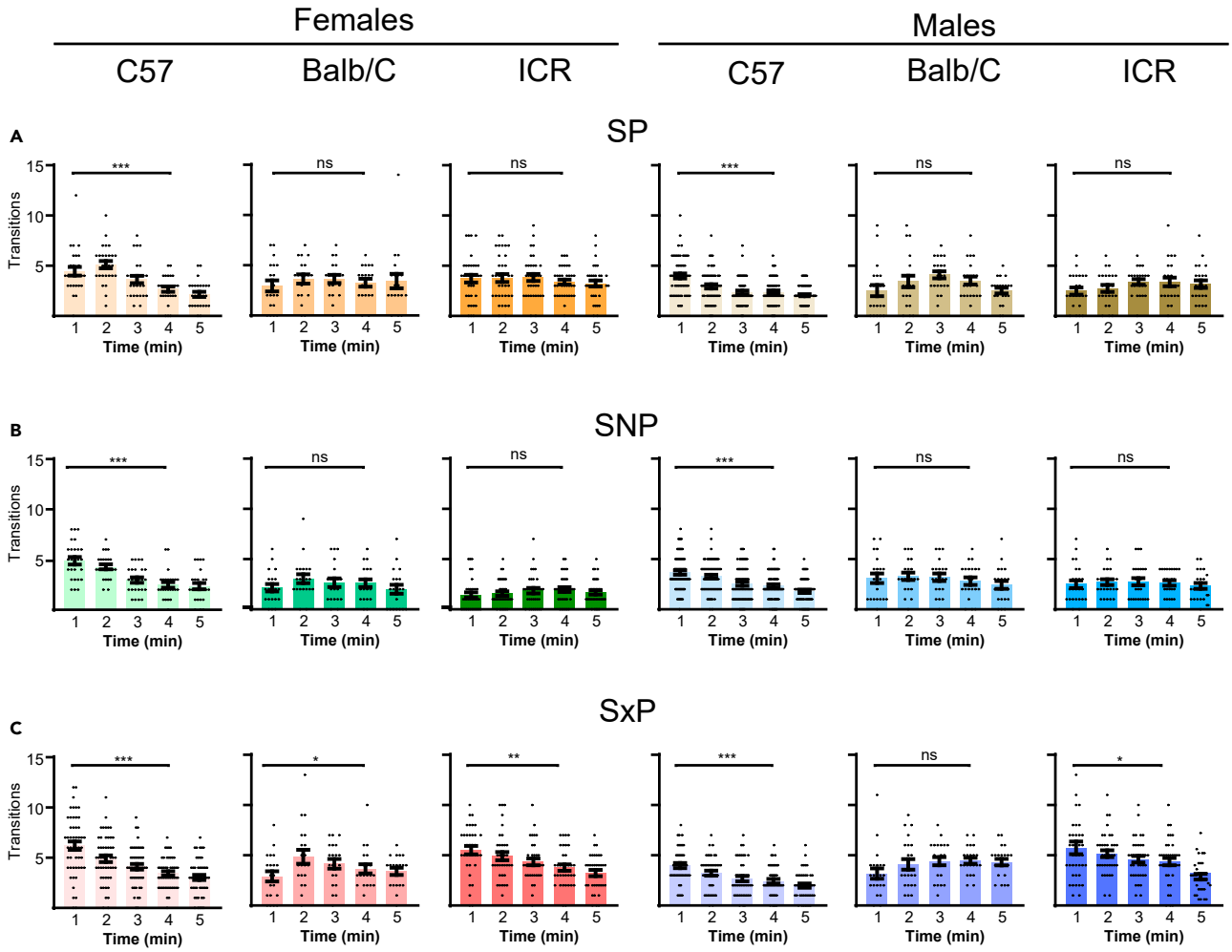

Figure 3. The dynamics of transitions between stimuli, across the three behavioral tests

Mean number of transitions (±SEM), 1-min bins) made by female (left) and male (right) subjects of the distinct mouse strains (denoted above), during the SP (A), SNP (B), and SxP (C) tests. *p < 0.05, **p < 0.1, ***p < 0.01, one-way repeated ANOVA.

ICR mice exhibited a pattern of transitions which was similar to the one displayed by C57BL/6J mice (Figure 3).

For statistical comparison between the groups, we calculated the difference in transition number between the first and fourth minute of the test (henceforth termed ΔTransition), for each animal group. These time bins were selected since they displayed the greatest difference in transition number in most cases. For the SP test (Figure 4A), we found a significant main effect of the strain ($F_{2,177}$ = 14.330, p <0.0001, *two-way ANOVA*), but not sex. *Post hoc* analysis revealed a significant difference between C57BL/6J males and males of both other strains (*BALB/c:* t_{177} = 3.955, p = 0.0003; *ICR:* t_{177} = 3.958, p = 0.0003, *Holm-Sidak test*), while C57BL/6J female mice showed a significant or borderline significant difference from females of the other strains (*BALB/c:* t_{177} = 2.689, p = 0.0233; *ICR:* t_{177} = 2.155, p = 0.0640, *Holm-Sidak test*). A very similar pattern was found for the SNP test (Figure 4B), with *post hoc* analysis following main effect of strain ($F_{2,177}$ = 19.962, p <0.0001, *two-way ANOVA*), revealing significant differences between C57BL/6J male mice and males of both other strains (*BALB/c:* t_{177} = 2.329, p = 0.0415; *ICR:* t_{177} = 3.186, p = 0.0051, *Holm-Sidak test*), and female C5BL/6J mice also showing significant difference from females of both other strains (*BALB/c:* t_{177} = 4.167, p <0.0001; *ICR:* t_{177} = 4.930, p <0.0001, *Holm-Sidak test*). For the SxP test (Figure 4C), *post hoc* analysis following main effect of strain ($F_{2,204}$ = 11.639, p <0.0001, *two-way ANOVA*) revealed that BALB/c males differed from males of both other strains (*C57:* t_{204} = 3.696, p = 0.0008; *ICR:* t_{177} = 3.351, p = 0.0019, *Holm-Sidak test*), and BALB/c females differed females of both

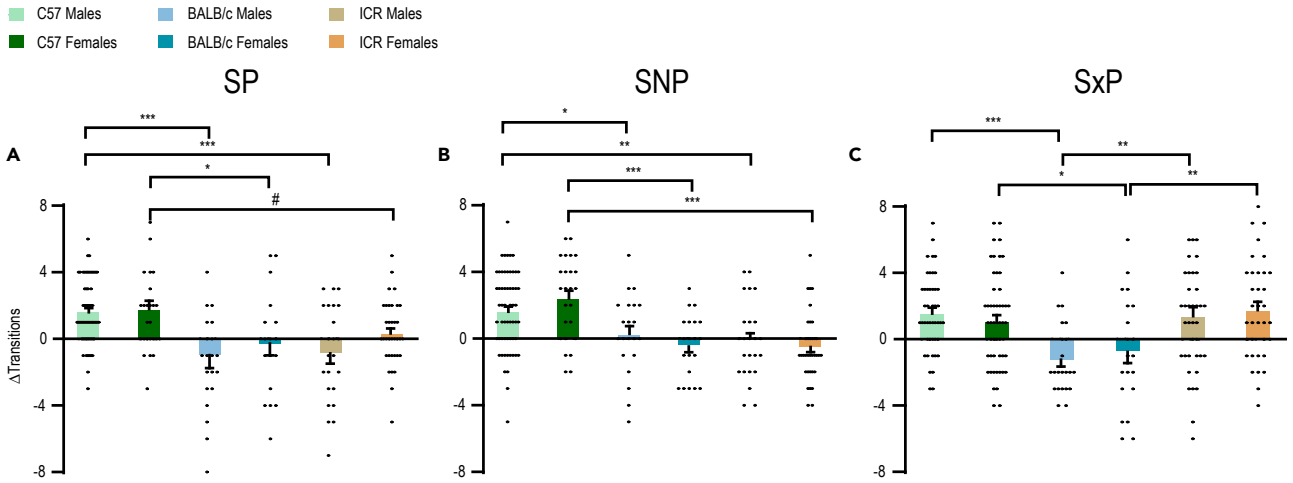

Figure 4. Strain-, but not Sex-dependent difference in the dynamics of transitions between the two stimuli

Mean (±SEM) difference in the number of transitions from one stimulus to the other between the first and fourth minute of each test (ΔTransition), measured separately for each animal group during the SP (A), SNP (B), and SxP (C) tests. *p< 0.05, **p< 0.01, ***p< 0.001, *post hoc* Holm-Sidak test, following main effect in two-way ANOVA test.

other strains (*C57:* $t_{204} = 2.377$, p = *0.0364; ICR:* $t_{177} = 3.023$, p = *0.0084, Holm-Sidak test*). Overall, these results suggest that the transition dynamics are a strain-, but not sex-specific feature, which is kept rather unchanged across the various tests, at least for the inbred C57BL/6J and BALB/c mice.

Sex-dependent differences in the dynamics of social preference behavior

After establishing the differences in the dynamics of transitions between the various animal groups, we examined whether the dynamics of the investigation behavior itself may differ between the various strains and sexes. While we have analyzed the dynamics of the SNP and SxP tests as well (Figure S4), we focused on the SP test for two reasons. First, since all groups showed a significant preference of the social stimulus over the object, we reasoned that differences in behavioral dynamics during this test could not be attributed to variations in preference between the groups. Second, as this test was always the first in the test series (Figure S1A), its results could not be affected by the other tests.

We observed an apparent difference between males and females in the behavioral dynamics of the SP test (Figure 5A). While males of all strains showed social preference that was very strong at the beginning of the test and declined over time, females showed a relatively stable preference throughout the test. To statistically analyze these apparent differences, we compared ΔIT between the first and last two minutes of the test, across sex and strains (Figure 5B). We found an interaction between time and sex ($F_{1,177} = 16.468$, p = *0.0001, mixed-model ANOVA*). *Post hoc* analysis revealed a significant difference between the first and last two minutes only for males, but not for females of all strains (*C57:* $t_{83} = 6.419$, p <*0.0001; BALB/c:* $t_{38} = 2.566$, p = *0.029; ICR:* $t_{56} = 2.691$, p = *0.019, Holm-Sidak test*). Thus, the investigation behavior during the SP test was changing in time in a sex-dependent manner, which further emphasizes the importance of behavioral dynamics in murine social behavior.

Sex- but not strain-dependent differences in bout duration during the SP test

We have previously shown that in C57BL/6J mice the difference in investigation time between the social stimulus and the object during the SP test is reflected only in long (>6 s) investigation bouts, while shorter bouts (≤6 s) do not differ between the two stimuli (Netser et al., 2017). To examine this point across sexes and strains, we plotted the fraction of ΔIT as a function of investigation bout duration for all strains and compared it between males and females. In order to avoid bias due to the fact that long bouts starting shortly before the end of the experiment are truncated, we calculated only the bouts that took place during the first four minutes of each trial. As apparent in Figure 6A, in all strains there seems to be a clear difference between males and females. Males of all strains showed longer bouts than females. In addition, BALB/c and ICR females showed more short bouts than males. We then statistically compared ΔIT separately for short and long bouts across sexes and strains. For short bouts (Figure 6B), we found a significant interaction

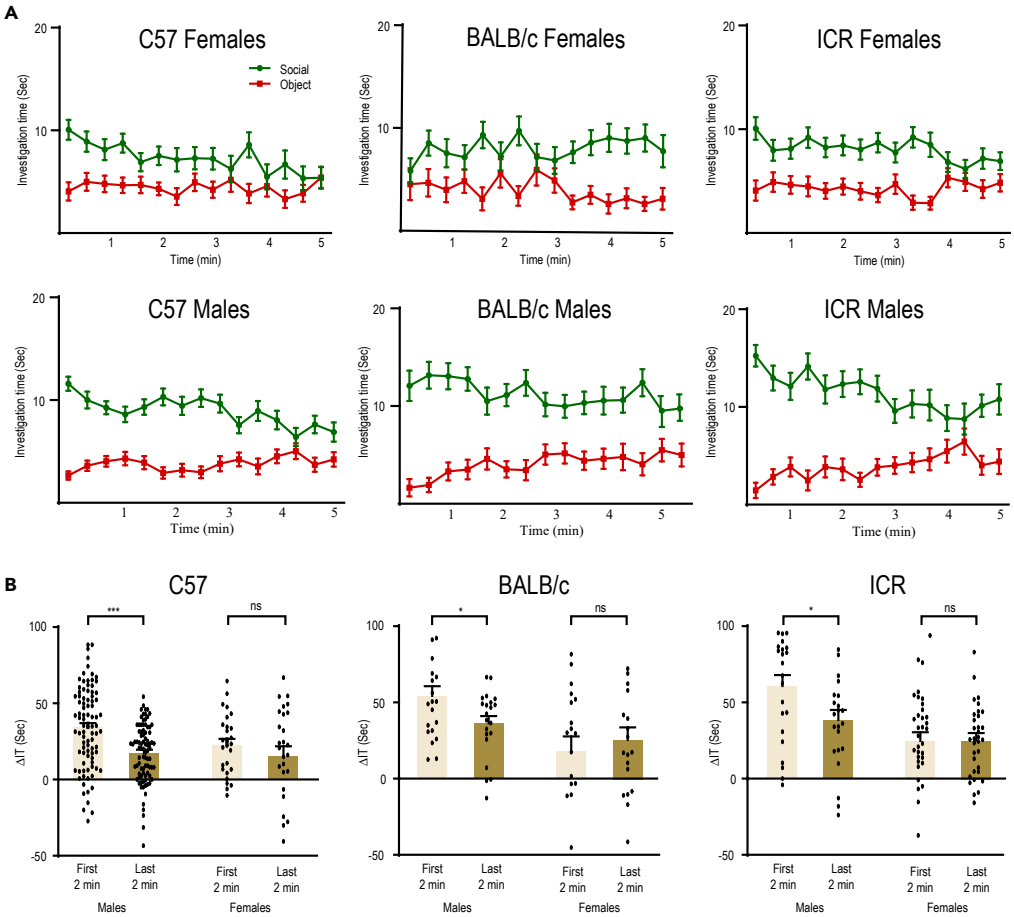

Figure 5. Sex-dependent dynamics of investigation behavior during the SP test

(A) Mean investigation time (±SEM), measured separately for each stimulus along the time course (20-s bins) of the SP test session (see Figure S4 for SNP and SxP) for females (upper panels) and males (lower panels) of the three strains. Note the transiently strong preference of males at the beginning of the test, as compared to the stable but weaker preference of females.

(B) Mean difference in investigation time (±SEM), between the stimuli (ΔIT), measured separately for the first and last two minutes of SP test. *p<0.05, ***p<0.001, ns – not significant, *post hoc* paired t test following the main effect in mixed-model ANOVA test.

between sex and strain ($F_{2,177}$ = *6.834*, p *<0.001, two-way ANOVA*), with a *post hoc* analysis showing a higher ΔIT for female BALB/c and ICR, but not C57BL/6J mice, as compared to males (*BALB/c:* t_{177} = *4.715*, p *<0.0001; ICR:* t_{177} = *2.311*, p = *0.0435, Holm-Sidak test*). In addition, C57BL/6J females showed a significantly lower ΔIT as compared to both BALB/c and ICR females (*BALB/c:* t_{177} = *3749γ*, p = *0.0007; ICR:* t_{177} = *2.610*, p = *0.0196, Holm-Sidak test*), while no difference was found between the males. In contrast, long bouts (Figure 6C) showed the main effect in ΔIT only for the sex ($F_{1,177}$ = *36.022*, p *<0.0001*), with a *post hoc* analysis revealing a significantly higher ΔIT for males as compared to females in all strains (*C57:* t_{177}= *−2.293*, p = *0.023; BALB/c:* t_{177}= *−3.897*, p = *0.0003; ICR:* t_{177}= *−4.056*, p = *0.0002, Holm-Sidak test*). Thus, social preference of males is generally expressed by longer bouts, while females express their social preference using shorter investigation bouts.

Offspring crossbreeding between C57BL/6J and BALB/c mice exhibit distinct social behavioral characteristics which depend on their parental combination

Finally, as we found significant differences in social behavior between the distinct strains, we sought to explore the effect of crossbreeding two distinct strains. For this purpose, we focused on male offspring generated by crossing C57BL/6J and BALB/c mice. These two strains were chosen because both are inbred

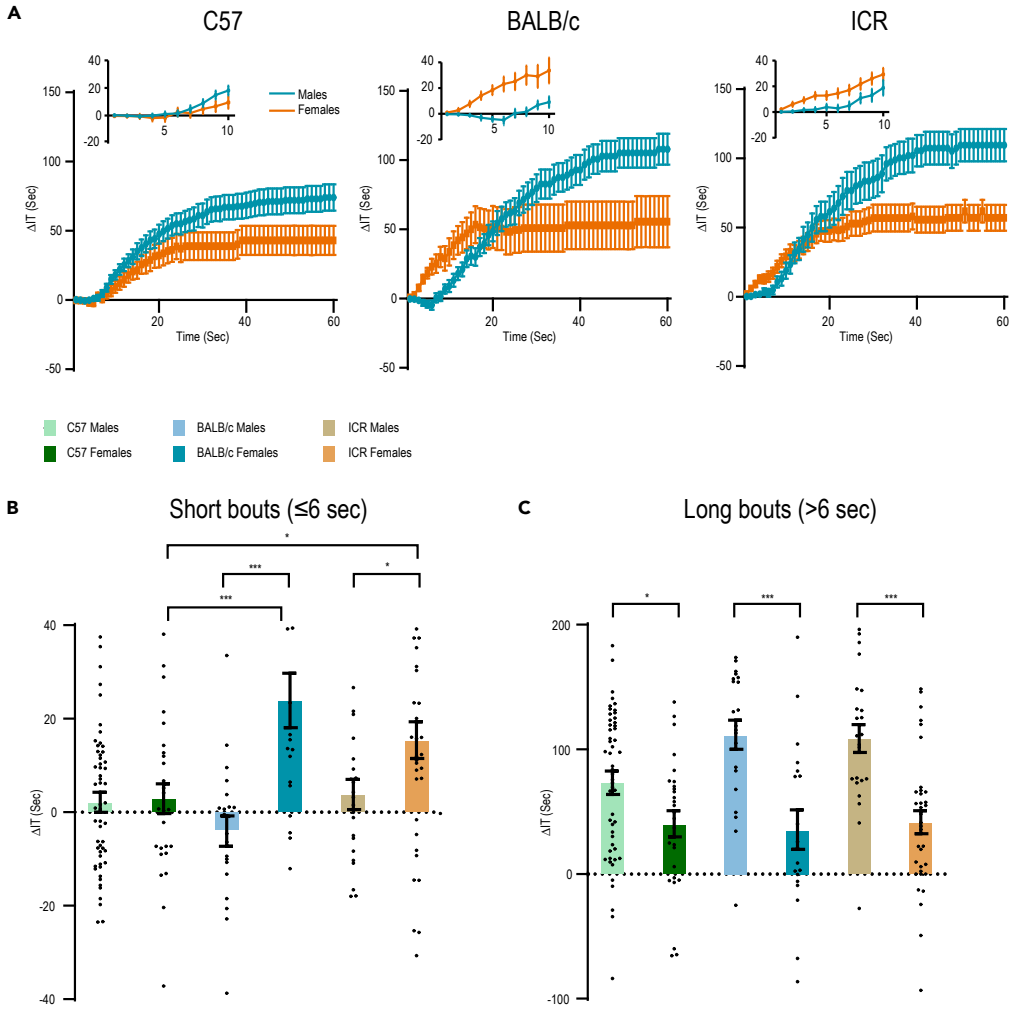

Figure 6. Sex-dependent differences in bout duration during the SP test

(A) Cumulative mean ΔIT (±SEM), plotted against bout duration during the SP test, separately for males (blue) and females (brown) of the three strains. Inset – same data on a larger scale for bout duration<10 s.

(B) Mean ΔIT (±SEM), calculated separately for short (≤6 s) investigation bouts made during the SP test by each animal group.

(C) As in (B), for long (>6 s) bouts. *p< 0.05, **p< 0.01, ***p< 0.001, ns – not significant, *post hoc* t test in cases of no multiple comparisons (C) and *post hoc* Holm-Sidak test, following main effect in two-way ANOVA test.

strains of a similar size and because they showed the strongest and most consistent difference in their pattern of transitions (Figure 4). We separately analyzed litters of C57BL/6J mothers and BALB/c fathers, termed by us as CB mice, and litters of BALB/c mothers and C57BL/6J fathers, termed BC mice (Figure S1B). Interestingly, we found that the BC and CB offspring showed different behavioral profiles, neither of which recapitulated the profiles of any of their parental strains. In the SP test (Figure 7A), we found that only CB mice showed a significant social preference (*BC:* $t_{35} = -0.298$, *n.s.; CB:* $t_{46} = 2.903$, p = *0.006, paired t test*). Nonetheless, the two crossbred strains were similar to each other in the dynamics of their behavior, with an initial preference at the beginning of the test and a loss of preference at a later stage (Figure 7B), a pattern not observed in any of the parental strains (Figure 5A). As for the transitions, both groups showed a pattern resembling their mothers, with BC mice showing a pattern similar to BALB/c mice and CB mice to C57BL/6J mice (Figure 7C). In the SNP test (Figure 7D), both BC and CB mice did not show any preference, similar to BALB/c males but in contrast to C57BL/6J mice (*BC:* $t_{18} = 0.531$, *n.s.; CB:* $t_{23} = 1.137$, *n.s., paired t test*). The behavioral similarity of both groups to BALB/c mice was also apparent in the dynamics of the investigation behavior (Figure 7E) and transitions (Figure 7F). In the case of the SxP test, while both CB and BC mice

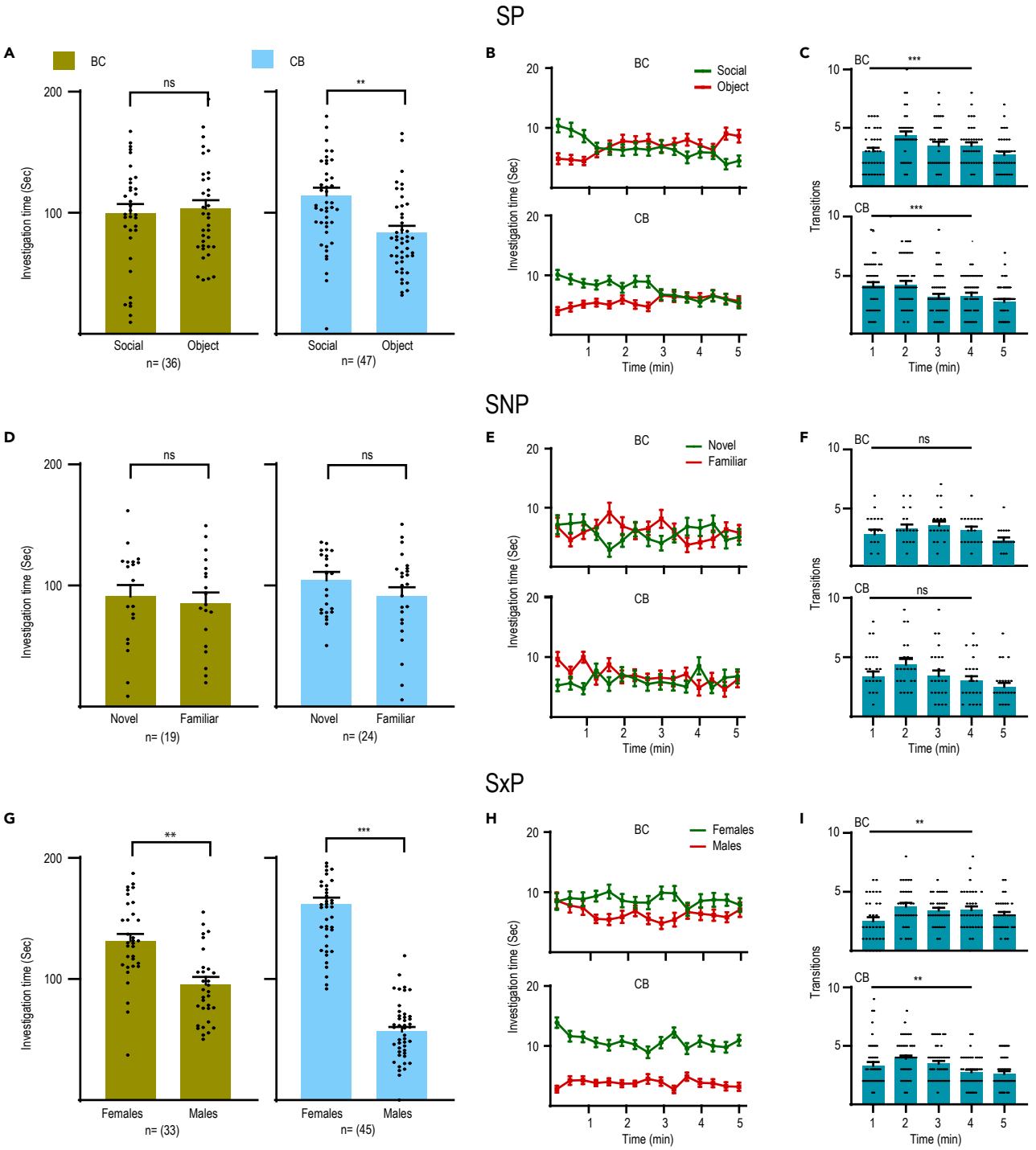

Figure 7. Offspring crossbreeding between C57BL/6J and BALB/c mice exhibit distinct social behavioral characteristic

(A) Mean investigation time (±SEM), for each stimulus during the SP test performed by BC (green) and CB (blue) mice. **p<0.01, paired t test.

(B) Mean investigation time (±SEM), measured separately for each stimulus along the time course of the SP test session (20-s bins) for BC (upper panel) and CB (lower panel) mice.

(C) Mean number of transitions (±SEM), between the two stimuli made during the SP test for BC (upper panel) and CB (lower panel) subjects. ***p< 0.001, one-way repeated ANOVA.

(D–F)As in (A–C), for the SNP test.

Figure 7. *Continued*
(G) As in (A), for the SxP test. **p<0.01, ***p<0.001, paired t test.
(H) As in (B), for the SxP test.
(I) As in (C), for the SxP test. **p< 0.01, one-way repeated ANOVA.

exhibited a significant preference for the female over the male stimulus (Figure 7G), this preference looked stronger in CB mice, similar to C57BL/6J males, and much weaker in BC mice, similar to BALB/c males, which did not show any sex preference (*BC:* t_{32} *= 3.430,* p *<0.002; CB:* t_{44} *= 12.926,* p *<0.0001, paired t test*). These differences were also apparent from the dynamics of investigation behavior (Figure 7H) and transitions (Figure 7I). Thus, in the SxP test, both types of cross-bred male offspring showed a behavioral pattern which resembles males of their maternal strain.

To make sure that the differences in the behavior of the F1 offspring (BC and CB mice) and their parental strains (C57BL/6J and BALB/c mice) does not emerge from the fact the parental strains were purchased while the F1 offspring were raised in our mouse facility, we examined the behavior of C57BL/6J male mice that were raised in our facility. We found that these animals behaved exactly like purchased C57BL/6J mice (Figure S5), thus excluding the possibility that the animal source affected our results.

For statistical comparison between the groups, we calculated the ΔIT (Figure 8A) and ΔTransitions (Figure 8B) and compared between BC and CB male mice separately for each test. For both parameters, we found that CB mice exhibited significantly higher values than BC mice in both the SP (ΔIT: t_{81}*= −2.049,* p *<0.044;* ΔTransitions: t_{81}*= −2.672,* p *<0.009, independent t test*) and SxP (ΔIT: t_{76}*= −5.308,* p *<0.0001;* ΔTransitions: t_{76}*= −2.944,* p *<0.004, independent t test*) tests, while no differences were found in the SNP test (ΔIT: t_{41}*= −0.466, n.s.;* ΔTransitions: t_{41}*= −1.041, n.s., independent t test*). Notably, although the difference in ΔTransitions was not significant in the SNP test, the trend was similar to the other two tests. Thus, regarding the dynamics of transitions, it seems as if in all cases the offspring behavior resembled their mothers' behaviors. We conclude that offspring of BALB/c and C57BL/6J mice exhibit a profile of social behavior which depends on the parental combination and is different from both parental strains, with a tendency to mimic the behavior of the maternal strain.

DISCUSSION

Sex-dependent differences in murine social behavior have been comprehensively explored in previous studies, but this was done mainly regarding hormonal-driven aspects of social behavior (Dulac and Kimchi, 2007; Yang and Shah, 2014), such as sexual (Shelley et al., 2006), anxiety/aggressive (Bredewold and Veenema, 2018), and parental behaviors (Zilkha et al., 2017). In contrast, evidence for differences in other aspects, such as social preference and social novelty preference have been reported only anecdotally, and in some cases with contradicting results (see for example Contestabile et al., 2021; Moy et al., 2004). Here, we systematically explored such differences across three types of social discrimination tasks and three commonly used laboratory mouse strains. We have used a relatively large number of animals and at least two independent cohorts for each animal group, to verify replicability (see Table S1). We found marked sex- and strain-specific differences in social investigation behavior. Interestingly, some differences were either sex- or strain-specific, but not both. Notably, we observed sex-specific differences especially in the SNP and SxP tests. While the SxP test may be related to sexual behavior (Kondo and Hayashi, 2021), the SNP test is not, and is well known to reflect social novelty seeking (Moy et al., 2004). Thus, our results demonstrate that even social behaviors which are not directly related to the sexual, aggressive, or parental aspects may be sex-dependent.

Our computerized experimental system enables analyzing the dynamics of the social discrimination behavior as well as categorizing each investigation bout according to its duration (Netser et al., 2017, 2019). Using these features, we found that even in the SP test, where all strains and sexes exhibit a significant preference for a social stimulus over an object, the dynamics of the behavior may markedly differ between strains and sexes. This was most profoundly demonstrated by the difference between ICR and BALB/c males, which exhibited a significant change over time in their social preference behavior, and all other groups. We have previously shown that this reduction over time in social preference reflects dynamic changes in the motivation for social interactions. Thus, ICR and BALB/c males seem to exhibit a very strong drive for social interactions at the beginning of the encounter, which gradually decreases over time, while the other groups show a lower level of social motivation, which is kept rather constant over time.

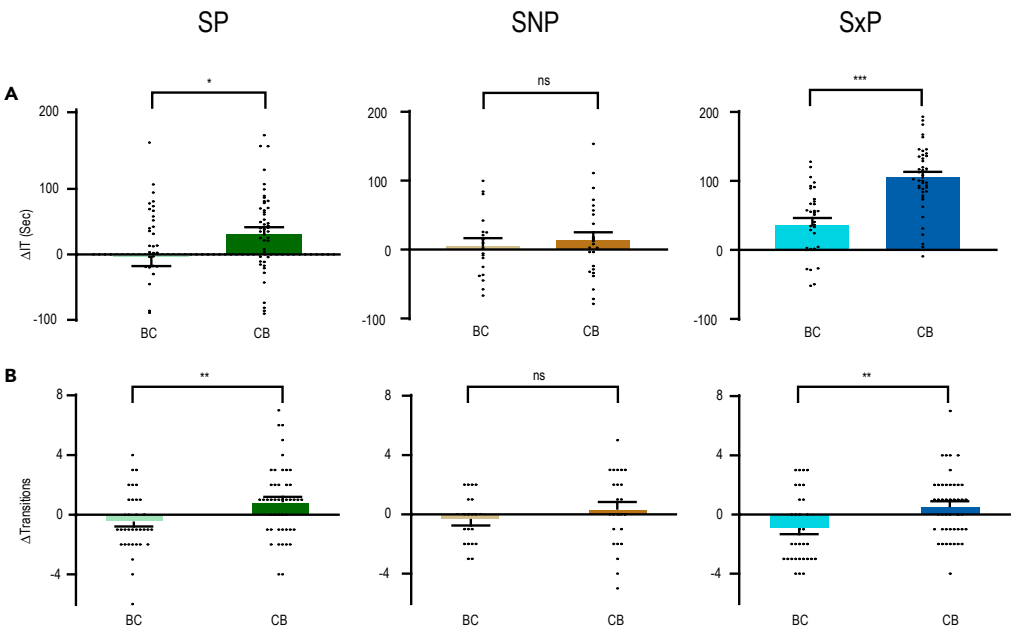

Figure 8. Comparing offspring of C57BL/6J mothers and BALB/c fathers with offspring of BALB/c mothers and C57BL/6J fathers

(A) Mean ΔIT (±SEM), for the SP, SNP, and SxP results of BC and CB mice, shown in Figures 7A, 7D, and 7G, respectively. (B) Mean ΔTransitions (±SEM), for the SP, SNP, and SxP results of BC and CB mice, shown in Figures 7C, 7F, and 7I, respectively. *p<0.01, **p<0.01, ***p<0.001, ns – not significant, unpaired t test.

Another dynamic aspect of social preference found to be sex-specific is the duration of investigation bouts. We have previously shown for male C57BL/6J mice that there is no difference in investigation using short bouts between the stimuli. In contrast, the preference for the social stimulus is specifically expressed by more investigation using long bouts (Netser et al., 2017). We thus suggested that long bouts reflect the interaction of the subject with the social stimulus while short bouts reflect curiosity per se (Netser et al., 2020). Here, we show for the first time that in females, specifically in ICR and BALB/c females, the picture is quite different. In contrast to males, females express their social preference by both long and short bouts, with no apparent difference between them. Moreover, in all strains, males exhibited longer investigation bouts toward the social stimulus than females. This sex-specific difference in the duration of investigation bouts may reflect a weaker motivation for interaction with a novel same-sex social stimulus exhibited by females, compared to males.

Another aspect of the behavioral dynamics we explored is the transitions between the two stimuli. Interestingly, while most differences in investigation behavior were between males and females, we found no sex-dependent differences in the pattern of transitions. Instead, this characteristic seems highly strain-dependent. For example, the C57BL/6J and BALB/c strains exhibited a rather uniform and specific pattern across both sexes and all tests, while ICR mice showed a pattern similar to BALB/c mice in the SP and SNP tests and a pattern similar to C57BL/6J mice in the SxP test. In a previous study (Netser et al., 2020), we suggested that similarly different patterns of transitions between SD rats and C57BL/6J mice reflect their distinct dynamics of social motivation. Accordingly, a recent study found a specifically high level of activity in dopaminergic neurons of the ventral tegmental area (VTA) during transition from object to social stimuli (Contestabile et al., 2021). Thus, the strain-dependent distinct patterns of transitions found by us may reflect distinct dynamics of social motivation between the strains.

The genetic basis of social behavior is well established throughout the animal kingdom. Significant differences between various mouse strains have been previously found using the three-chamber test (Moy et al., 2004, 2007, 2008). Yet, to our knowledge, our study is the first to examine the consequences of cross-breeding between distinct mouse strains which exhibit different social behavior. Interestingly, the behavior of F1 male offspring of the crossbreeding scheme (C57BL/6J x BALB/c) was different from both parental strains in a test-dependent manner. This was most strikingly demonstrated by the dynamics of the

social-preference behavior, where both BC and CB mice showed such preference only at the beginning of the test. Such a phenomenon, of F1 behavior which is weaker than both parental mouse strains, was previously shown for morphine analgesia (Miner et al., 1993). Even more surprising is the observation that F1 offspring that were born to C57BL/6J mothers and BALB/c fathers differ in their behavior compared to offspring of BALB/c mothers and C57BL/6J fathers. Interestingly, in several aspects of their behavior, the F1 offspring showed resemblance specifically to the strain of their mothers. The question whether this difference between BC and CB mice is caused by genetic, epigenetic, or environmental (i.e., the strain of the female taking care of the newborn animals) factors should be addressed by future studies.

Overall, we conclude that social behavior of laboratory mice, even if not related to sexual, aggressive, or parental aspects, is highly sex- and strain-dependent. Moreover, we show that the behavioral outcomes of a crossbreeding between mouse strains are unpredictable and may differ markedly between tests and breeding schemes. These conclusions should be taken into account in future studies exploring modified social behavior in genetic mouse models.

Limitations of the study

Our results should be interpreted while considering several limitations. First, social behavior is strongly influenced by the experimental system and design. For example, in the three-chamber test, the sociability of male ICR (CD-1) mice was found to be dependent on the type of habituation they experienced (Hsieh et al., 2017). Thus, our results, obtained using a unique experimental system, may differ from those obtained using the three-chamber (Moy et al., 2007) or free-interaction (An et al., 2011) tests. Second, we have used juvenile mice as social stimuli for the SP ad SNP tests, which is not the case for many other studies. Finally, we did not check the estrus cycle of either the subjects or the social stimuli. Previous studies showed that the estrus cycle of the stimuli does not influence their sex-preference behavior (Ago et al., 2015), and at least for rat females it was demonstrated that the estrus cycle does not change their social-preference behavior (Lukas and Neumann, 2014). Nevertheless, we cannot rule out the possibility that using female subjects at specific phase of their estrus cycle would yield slightly different results.

INSTITUTIONAL REVIEW BOARD STATEMENT

All experiments were performed according to the National Institutes of Health guide for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Haifa.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.103735.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.W. and S.N.; methodology, S.N.; software, S.N.; validation, S.W. and S.N.; formal analysis, S.N.; investigation, N.K.; resources, S.W.; data curation, S.N.; writing—original draft preparation, S.W; visualization, S.N and N.K.; supervision, S.W.; project administration, S. N.; funding acquisition, S.W. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Source data file	Medeley Data	https://data.mendeley.com/datasets/zf73c4dn95/1
Experimental models: Organisms/strains		
Adult and juvenile male and female mice	Envigo, Rehovot, Israel	C57BL/6J, BALB/c, ICR
Software and algorithms		
TrackRodent	Custom-made	https://github.com/shainetser/TrackRodent

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shlomo Wagner (shlomow@research.haifa.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All raw data presented in this study are deposited at https://data.mendeley.com/datasets/zf73c4dn95/1 and are publicly available as of the date of publication. All codes and datasets used for the current study are available on request from the corresponding author within a reasonable time. The code used for the video analysis is publicly available at the following link [https://github.com/shainetser/TrackRodent].
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

The research subjects were naive male and female adult (8–12 weeks old) mice of three distinct laboratory strains: C57BL/6J, BALB/c, and ICR (CD-1) and adult male mice generated by crossing C57BL/6J and BALB/c mice (BC and CB mice). The social stimuli were C57BL/6J, BALB/c, and ICR (CD-1) juvenile (21–30 day-old) naive male and female mice (SP, SNP), and C57BL/6J, BALB/c, and ICR (CD-1) adult (8–12 weeks old) naive male and female mice (SxP). For testing BC and CB mice, the stimuli were also BC and CB juvenile (SP and SNP) or adult (SxP) male and female mice, as described above. Adult and juvenile C57BL/6J, BALB/c, and ICR mice were commercially obtained (Envigo, Rehovot, Israel) and housed in Plexiglas cages in groups of 2–5 animals per cage. They were kept at 22 $\pm$ 2°C under a 12-h light/12-h dark cycle, with lights being turned on at 7 p.m. each night. All animals had *ad libitum* access to food (standard chow diet; Envigo, Rehovot, Israel) and water. Behavioral experiments were performed during the dark phase, under dim red light. All experiments were performed according to the National Institutes of Health guide for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Haifa.

METHOD DETAILS

Behavioral assays

All social discrimination tasks were conducted using our published automated experimental system (Netser et al., 2019). SP and SNP tests were conducted on the same day, as previously described (Netser et al., 2019). The SxP test was conducted two days later (see Figure S1A) as previously described (Jabarin et al., 2021). Briefly, it consisted of 15 min habituation to the arena with empty chambers, followed by exposing the subject for 5 min to both novel adult male and female social stimuli located in individual

chambers at opposite corners of the arena. The menstrual cycle was not determined, since it was previously shown to have no influence on murine sex preference behavior (Ago et al., 2015). All stimuli used in all three tests (SP, SNP, and SxP) were of the same strain as the subject, including the case of CB and BC mice.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis

Video data analysis was conducted by our published custom-made TrackRodent software, as previously described in detail (Netser et al., 2019).

Statistical analysis

All statistical tests were performed using SPSS 23.0 (IBM) or Graph Pad 9.4 statistics software. No animal was excluded from the analysis. Shapiro-Wilk test was used for examining the normal distribution of the dependent variables. A 2-tailed paired ttest was used to compare between parameters within a group, and a 2-tailed independent ttest was used to compare a single parameter between distinct groups. For examining the influence of one categorical independent variable on one continuous dependent variable, a one-way ANOVA model was applied to the data. This model assesses the main effect of the independent variable on the dependent variable. For examining the influence of two different categorical independent variables on one continuous dependent variable, a two-way ANOVA model was applied to the data. This model assesses the main effect of each independent variable and the interaction between them. For comparison between multiple groups and parameters, a mixed-model analysis of variance (ANOVA) model was applied to the data. This model contains one random effect (ID), one within effect, one between effect, and the interaction between them. All ANOVA tests were followed, if the main effect or interaction were significant, by a *post hoc* test. We used *post hoc* Student's t-test if no multiple comparisons were done and *post hoc* Holm-Sidak test in cases of multiple comparisons. Significance was set at p value < 0.05 but borderline significance of $p < 0.1$ was also considered in the *post hoc* tests. All results of the statistical tests are detailed in relation to the relevant figure in Table S2.