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ORIGINAL CONTRIBUTIONS

Genetic architecture of fear conditioning in chromosome substitution strains: relationship to measures of innate (unlearned) anxiety-like behavior

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Abstract We measured fear conditioning (FC) in a panel of chromosome substitution strains (CSS) created using the C57BL/6J (B6) and A/J (AJ) inbred strains. Mice were trained to associate a specific context and tone with a foot shock. FC was measured by observing freezing behavior during re-exposure to the context and tone. Freezing to context was more than twofold greater in the AJ strain relative to the B6 strain. Among the CSS we identified four strains with higher (CSS-6, -10, -11, and -18) and two strains with lower (CSS-7 and -14) freezing to context. CSS-10 and -18 also showed higher freezing to tone, while CSS-12 showed less freezing to tone. CSS-1 has been implicated in open-field (OF) and light-dark box (LDB); we observed significant activity differences prior to training but no differences in FC. Chromosomes 6 and 10 have been associated with differences in anxiety-like behaviors, suggesting the existence of pleiotropic alleles that influence both learned and innate fear. By utilizing a genetic reference population, we have identified chromosomes that pleiotropically influence multiple phenotypes hypothesized to reflect a common ethologic construct that has been termed emotionality. The CSS provide a straightforward means of isolating the underlying genetic factors.

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Introduction

Fear conditioning (FC) is a classic measure of emotional behavior in which a previously neutral cue (conditioned stimulus; CS) is associated with an aversive event (unconditioned stimulus; US). In rodents, the strength of the resulting fearful memory can be measured by observation of freezing behavior in response to the CS (Dexter and Merrill 1969; Fendt and Fanselow 1999; LeDoux 2000; Phillips and LeDoux 1992). The magnitude of the response to FC is a heritable trait in mice (Gershenfeld and Paul 1997; Wehner et al. 1997) and humans (Hettema et al. 2003). Previous studies have identified multiple quantitative trait loci (QTLs) for FC in mice (Caldarone et al. 1997; Owen et al. 1997; Radcliffe et al. 2000; Talbot et al. 2003), suggesting there are many genes making small contributions to the phenotypic differences among individuals.

Genetic studies in both rats (Fernandez-Teruel et al. 2002) and mice (Ponder et al. 2007) have indicated that learned fear and anxiety are controlled by some of the same genes. In patients diagnosed with anxiety disorders, acquisition of FC is increased and extinction is slower compared to normal controls (Lissek et al. 2005). In mice, FC is reduced by drugs that have anxiolytic properties in humans (Davis 1992; Risbrough et al. 2003; Santos et al. 2005). The neuroanatomical substrates of FC and anxiety are similar in both rodents (Davis 1992; LeDoux 2000; McNish et al. 1997; Phillips and LeDoux 1992) and humans (LaBar et al. 1995, 1998; Richardson et al. 2004).

Based on these data, we hypothesize that some genes affecting fear conditioning also affect anxiety-like behavior in mice and possibly pathologic anxiety in humans.

A panel of chromosome substitution strains (CSS) has been created in which each strain contains a single chromosome from the A/J (AJ) strain on an otherwise uniform C57BL/6J (B6) genetic background (Singer et al. 2004). By comparing each CSS to the pure B6 strain, each chromosome can be evaluated for the presence of a QTL; QTLs discovered in this way do not require epistatic interactions with AJ alleles on other chromosomes (Stylianou et al. 2006). Subsequent fine mapping steps are straightforward and have a number of practical and statistical advantages (Belknap 2003; Stylianou et al. 2006).

To identify QTLs for FC in the CSS and to permit comparison of individual CSS phenotypes for FC and anxiety-like behavioral phenotypes that have been measured before (Singer et al. 2005), we evaluated FC in B6, AJ, and the CSS. We analyzed the results in terms of freezing to context and freezing to tone and also examined pretraining freezing behavior and freezing in the altered context (in the absence of the tone) to identify nonspecific behavioral differences that might otherwise confuse the interpretation of the data.

Materials and methods

Environment and housing

All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the University of Chicago's Institutional Animal Care and Use Committees. Mouse colony rooms were maintained on a 12/12 light/dark cycle with lights on at 06:00 hours. Two to five same-sex littermates were housed in clear plastic cages with standard corn cob-type bedding. All mice were maintained with food and water *ad libitum*, except during testing. Testing was conducted during the light phase between 08:00 and 16:00; mice were brought into the testing room in their home cages and allowed to adapt for a minimum of 30 min before testing.

Animals

Mice were purchased as breeders from The Jackson Laboratory and bred at the University of Chicago. Inbred B6 and AJ mice were bred concurrently to CSS so all mice were exposed to the same environment. CSS breeders were purchased for 19 autosomes and the X chromosome, but due to differential breeding success, CSS-2 and CSS-8 were unavailable for study. Thus, 18 CSS strains were tested for fear conditioning. A total of 485 male and female mice were tested: 82 B6, 17 AJ, and 386 CSS mice. For each CSS strain the number of mice tested was between 8 and 14 mice per sex per strain, except for CSS-13, which had only four females and five males available, and CSS 16, which had only three males available. An average of 22 mice (male and female combined) were tested for the other CSS. Mice were between 48 and 80 days old on the first day of testing.

Fear-conditioning procedure

Fear-conditioning phenotyping was conducted over a period of three months at the University of Chicago using a procedure that has been previously described (Ponder et al 2007). Fear-conditioning chambers obtained from Med Associates (St. Albans, VT) had inside dimensions of 29 cm × 19 cm × 25 cm with metal walls on each side, clear plastic front and back walls and ceilings, and stainless steel bars on the floor. A fluorescent light provided dim illumination (~3 lux) and a fan provided a low level of masking background noise. Behavior was recorded with digital video and analyzed with FreezeFrame software from Actimetrics (Evanston, IL).

Fear conditioning was tested with a three-day protocol (Fig. 1) that was identical to that used in our previous studies (Ponder et al. 2007). On day 1 (training day)

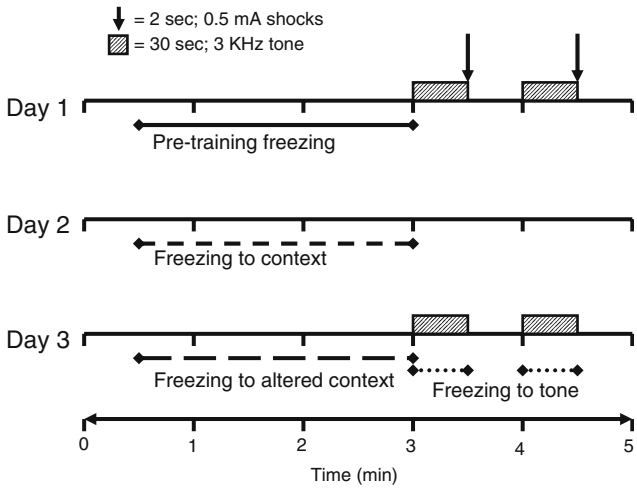


Fig. 1 A three-day procedure was used to phenotype each subject. Each test lasted 5 min. On day 1 pretraining freezing was measured from 30 to 180 sec after which mice received two 30-sec shocks paired with a 2-sec, 0.5-mA foot shock. On day 2 freezing to context was measured from 30 to 180 sec; the pretraining freezing was then subtracted from this value to obtain the variable that was analyzed in this study. On day 3 freezing to the altered context was measured from 30 to 180 sec after which freezing to the each tone was measured (180-210 + 240-270 sec); the time spent freezing to both tones was combined to obtain the variable called freezing to tone that was analyzed in this study

baseline activity (pretraining freezing) was measured from 30 to 180 sec, then mice were trained with two pairings of a 30-sec tone (CS) with a 2 sec, 0.5-mA foot shock (US), separated by a 30-sec intertrial interval (ITI). On day 2 the chamber was identical to that on day 1, however, no tones or shocks were presented and freezing in response to the test chamber (freezing to context) was measured from 30 to 180 sec. Freezing to context was a corrected value obtained by subtracting day 1 freezing from day 2 freezing during the 30-180-sec interval on both days. On day 3 the context was altered in several ways: a different experimenter wore a different style of glove, the transfer cages had no bedding, the metal shock grid was covered with a white plastic floor, a bent white plastic wall was inserted into the test chamber, a yellow light filter was placed over the chamber lights, chambers were cleaned with 0.1% acetic acid solution, and the vent fan was partially obstructed to change the background noise. The CS was again presented twice in this altered context; however, no foot shock was administered. Freezing to the altered context was defined as freezing that occurred between 30 and 180 sec on day 3. Freezing to tone was defined as the percentage of time spent freezing during the two 30-sec CS presentations (180-210 and 240-270 sec). Thus, there were four phenotypes measured in this study: freezing to context (day 2 – day 1), freezing to tone (day 3), pretraining freezing (day 1), and freezing in the altered context (day 3).

Statistical analysis

First, a two-way analysis of variance (ANOVA) with strain (B6 and AJ) and sex (male and female) as factors was used to examine freezing to context, freezing to tone, pretraining freezing, and freezing to the altered context in the inbred progenitor strains. Next, a two-way ANOVA for sex (male and female) and strain (all CSS and B6) was used to examine all four phenotypes. To follow-up on the main effects of strain, each phenotype for each CSS was compared to the B6 strain using equation (3) from Belknap (2003), which was used to assign Z scores. Scores greater than 2.9 were considered significant, while scores greater than 1.96 were considered suggestive. The effect size (proportion of the variance explained, v^2_{CvsB}) for each significant chromosome was estimated from this Z score as well [equation (4), Belknap 2003]. For the traits that showed a significant main effect of sex, we used a series of planned comparisons for sex (within each CSS) to identify CSS that showed different responses between males and females. Finally, to determine if there was additive genetic variance for each phenotype, the sum of squares (ss) between strains was divided by the total ss (between strain ss + residual ss) to determine the proportion of the trait variance due to additive genetic influences (narrow sense heritability; h^2).

Results

A two-way ANOVA for sex and strain (B6, AJ) for freezing to context revealed a significant main effect of strain for freezing to context ($F_{[1,98]} = 64.97$; $p < 0.0001$) but no main effect or interaction with sex (Fig. 2a). For freezing to tone there were no significant main effects or interactions between sex and strain (Fig. 2b). For pre-

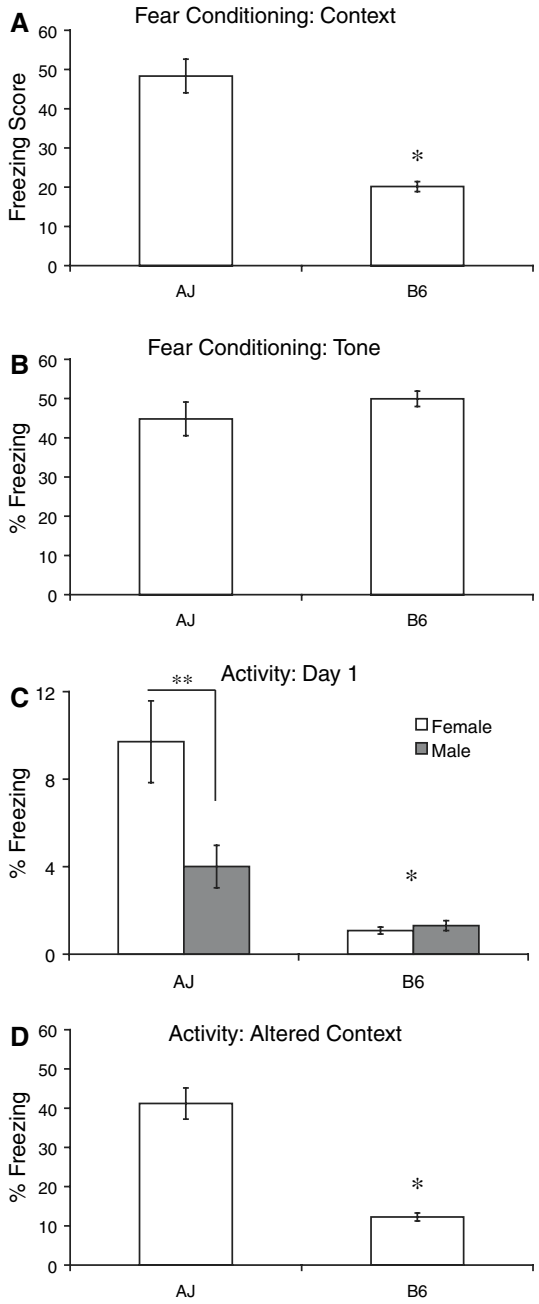


Fig. 2 Comparisons of freezing behavior in B6 and AJ strains. **A** Freezing to context. **B** Freezing to tone. **C** Pretraining freezing. **D** Freezing to the altered context. Bars represent mean ± SE. $^{*}p < 0.0001$ compared to B6; $^{**}p < 0.0001$ for strain × sex interaction

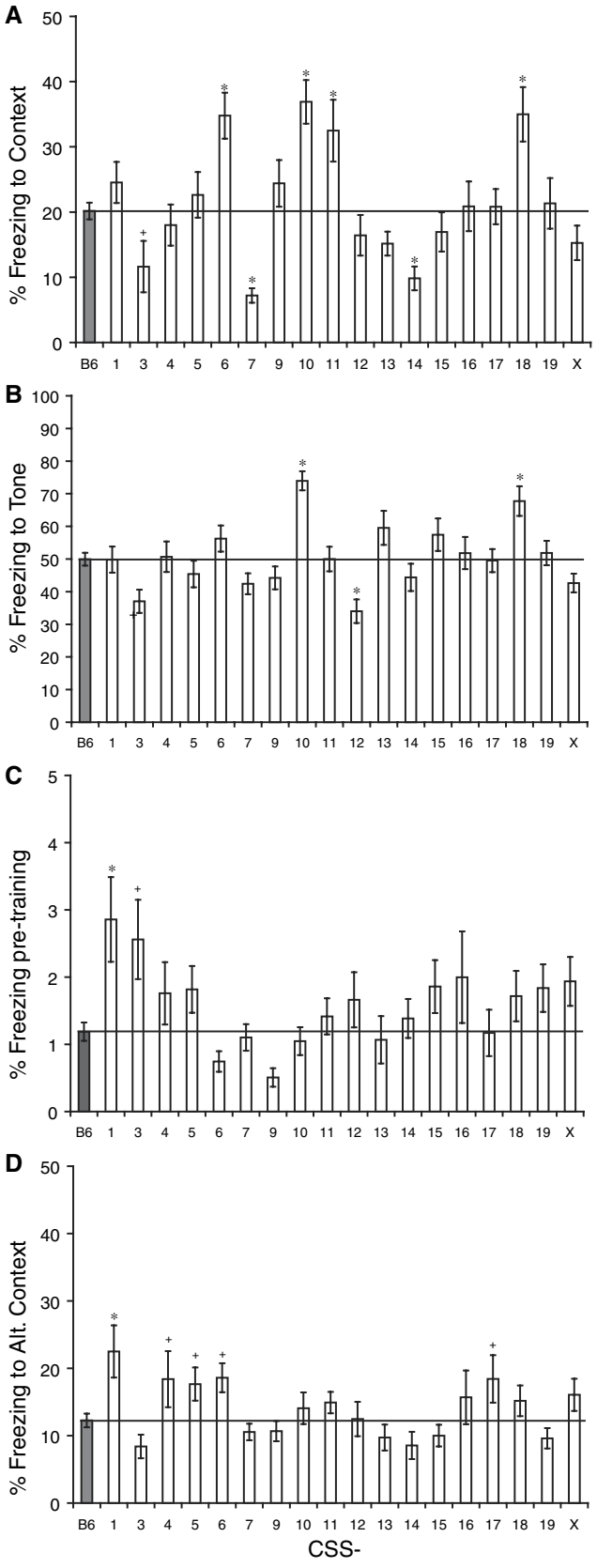
training freezing there was a significant main effect of sex ($F_{[1,98]} = 28.02$; $p < 0.0001$), for strain ($F_{[1,98]} = 119.86$; $p < 0.0001$), and for their interaction ($F_{[1,98]} = 64.97$; $p < 0.0001$) (Fig. 2c). For freezing to the altered context there was an effect of strain ($F_{[1,97]} = 97.14$; $p < 0.0001$) but no main effect or interaction with sex (Fig. 2d). These results demonstrate that the inbred recipient (B6) and donor (AJ) strains differ for all the measured phenotypes except freezing to tone (Table 1).

None of the two-way ANOVAs for sex and strain (all CSS and B6) identified significant interactions between sex and strain. There were significant main effects of sex ($F_{[1,427]} = 6.11$, $p < 0.05$) and strain ($F_{[18,427]} = 7.73$; $p < 0.0001$) for freezing to context. Similarly, there were significant main effects of sex ($F_{[1,427]} = 6.10$, $p < 0.05$) and strain ($F_{[18,427]} = 6.49$; $p < 0.0001$) for freezing to tone. For pretraining freezing and freezing to the altered context, the only significant results were main effects of strain ($F_{[18,427]} = 2.75$; $p < 0.001$; $F_{[18,427]} = 2.71$; $p < 0.001$, respectively). To further investigate the main effect of strain for each phenotype, we used a one-way ANOVA to examine the effect of strain (all CSS and B6) for each phenotype. This test revealed significant main effects of strain on every measure: freezing to context ($F_{[18,446]} = 7.47$; $p < 0.0001$), freezing to tone ($F_{[18,446]} = 6.23$; $p < 0.0001$), pretraining freezing ($F_{[18,446]} = 2.91$; $p < 0.0001$), and freezing in the altered context ($F_{[18,446]} = 2.78$; $p < 0.0001$). We then used equation (3) from Belknap (2003) to calculate Z scores for the comparison of each CSS with B6 because this was the relevant experimental question (pairwise comparisons among the CSS are not meaningful or interesting). For freezing to context (Fig. 3a), four CSS had significantly higher ($Z > 2.9$) freezing than B6 (CSS-6, -10, -11, and -18). Two CSS had significantly lower freezing (CSS-7 and -14) and one CSS had suggestively lower freezing (CSS-3) compared with B6. Freezing to tone (Fig. 3b) was significantly higher than the B6 strain for two CSS (CSS-10 and -18), lower for one CSS (CSS-12), and suggestively lower for another (CSS-3). We also examined baseline activity levels on day 1 by measuring pretraining freezing (Fig. 3c). Because this behavior is measured before training, it reflects innate differences in locomotor activity rather than fear learning. Two CSS showed significantly higher

Table 1 Heritability (h^2) of each phenotype and the result of a t test to compare AJ and B6 for each indicated phenotype and the Z score for the comparison between B6 and each indicated CSS strain

	Context		Tone		Pretraining		Altered context	
h^2	0.23		0.21		0.10		0.10	
B6 vs. AJ	<0.001		0.304		<0.001		<0.001	
B6 vs. CSS-	Z score	v^2_{CvsB}	Z score	v^2_{CvsB}	Z score	v^2_{CvsB}	Z score	v^2_{CvsB}
1					**3.9**	**0.13**	**3.7**	**0.12**
3	–2.2	0.05	–2.8	0.07	**3.1**	**0.09**		
4							2.2	0.04
5							2.1	0.04
6	**4.1**	**0.14**					2.4	0.05
7	**–3.9**	**0.12**						
9								
10	**4.9**	**0.18**	**5.9**	**0.24**				
11	**3.6**	**0.11**						
12			**–3.6**	**0.11**				
13								
14	**–2.9**	**0.07**						
15								
16								
17							2.4	0.05
18	**4.2**	**0.14**	**4.2**	**0.14**				
19								
X								

Z scores are considered significant when greater than 2.9 (shown in bold) and are suggestive when greater than 1.96 (see Materials and methods for details). The sign of the Z score reflects the direction of the effect; a positive score indicates increased freezing, a negative score indicates reduced freezing relative to B6. The amount of phenotypic variance explained by a particular chromosome (v^2_{CvsB}) is also shown.



◀ **Fig. 3** Comparisons of freezing behavior in B6 and CSS strains. **A** Freezing to context. **B** Freezing to tone. **C** Pretraining freezing. **D** Freezing to the altered context. The horizontal line indicates the mean of B6 for each phenotype. Bars represent mean ± SE. * indicates significantly different Z scores ($Z > 2.9$), + indicates suggestively different Z scores ($Z > 1.96$)

freezing (or lower activity) than B6 (CSS-1 and CSS-3). Finally, we examined freezing in the altered context on day 3 (Fig. 3d). One showed significantly more freezing than B6 (CSS-1) and four showed suggestive increases (CSS-4, -5, -6, and -17). All significant and suggestive Z scores and their corresponding v^2_{CvsB} are presented Table 1.

Despite the lack of a significant interaction between sex and strain, we used planned comparisons between the males and females within each CSS to identify the source of the main effect of sex. We identified higher freezing to context in females compared with males in CSS-3 ($F_{[1,427]} = 4.82$, $p < 0.05$), CSS-5 ($F_{[1,427]} = 6.41$, $p < 0.05$), and CSS-9 ($F_{[1,427]} = 6.58$, $p < 0.05$) (Fig. 4a). Similarly, planned comparisons revealed that females had higher freezing to tone in CSS-5 ($F_{[1,427]} = 9.03$, $p = 0.003$) and CSS-15 ($F_{[1,427]} = 3.85$, $p < 0.05$) (Fig. 4b). None of these results would be considered significant if subjected to a Bonferroni correction; therefore, the effects of sex may be considered merely suggestive.

The narrow sense heritability of each phenotype was found to be within the expected range of behavioral phenotypes in mice. For fear conditioning to context, $h^2 = 0.23$, and to tone, $h^2 = 0.21$. For pretraining freezing, $h^2 = 0.10$, and for freezing to the altered context, $h^2 = 0.10$ (Table 1).

Discussion

We have identified QTLs for fear-conditioning behavior by using a panel of B6.AJ CSS mice. Inbred AJ mice show more than twice the level of freezing to context compared eith inbred B6 mice (Table 1, Fig. 2a). Our data identify individual AJ chromosomes that affect freezing to context both positively (CSS-6, -10, -11, and -18) and negatively (CSS-7 and -14) when introgressed onto a B6 background (Fig. 3; Table 1). Freezing to tone was similar between the inbred AJ and B6 strains (Fig. 2b); however, when we used the CSS panel to genetically dissect this phenotype, we revealed AJ chromosomes that increased (CSS-10 and -18) and decreased (CSS-12) freezing to tone when placed on the B6 background (Table 1, Fig. 3b). Freezing to tone appears to be affected by some of the same chromosomes (and possibly loci) as freezing to context. Specifically, CSS-10 and -18 had greater freezing to context and freezing to tone relative to B6, and CSS-3 showed suggestive decreases in freezing to context and freezing to tone relative to B6.

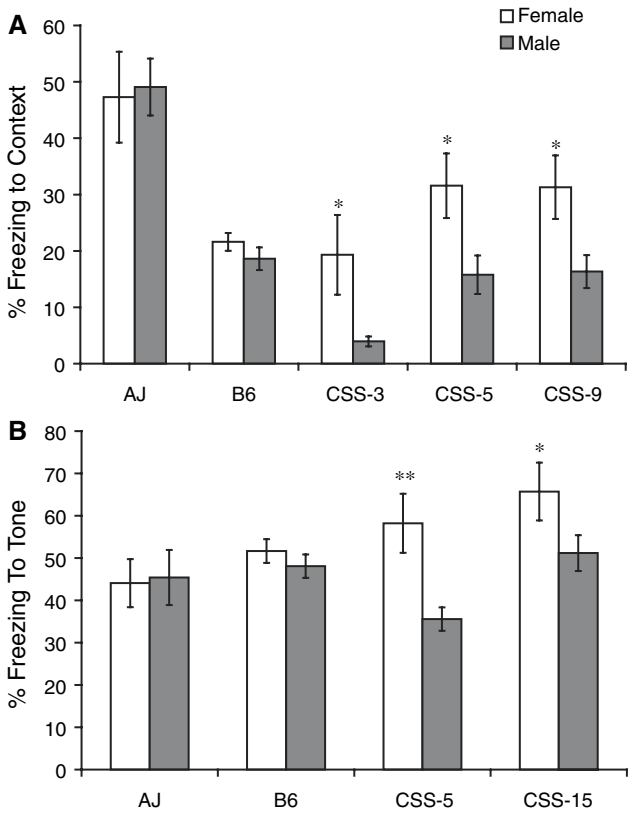


Fig. 4 Sex-specific effects for (**A**) freezing to context and (**B**) freezing to tone. Bars represent mean ± SE. $^{*}p < 0.05$; $^{**}p < 0.003$

We also examined pretraining freezing in all CSS to detect behavioral differences associated with hyper- or hypoactivity that might otherwise be interpreted as differences in FC. Pretraining freezing (hypoactivity) was higher in the inbred AJ and in CSS-1 and -3 relative to pure B6 (Table 1, Figs. 2c, 3c). As described in the Materials and methods section, we subtracted pretraining freezing from the freezing to context score to avoid confusing hypoactivity with freezing. In addition, we measured freezing in the altered context (before administration of the tone; Table 1, Figs. 2d, 3d). Freezing in the altered context was higher in the inbred AJ and in CSS-1, -4, -5, -6, and -17 relative to B6.

The CSS-10 and -18 demonstrated the most compelling evidence for increased fear learning compared with B6, because both freezing to context and freezing to tone was higher (more AJ-like) and did not appear confounded by differences in pretraining freezing or freezing in the altered context (Table 1, Fig. 3). In addition, CSS-3 showed lower levels of freezing to both the context and the tone (Fig. 3); CSS-3 also showed higher pretraining freezing, which makes the decrease in freezing to context and tone more striking. These results identify several alleles that influence the strength of the association between the CS (context or tone) and US (foot shock).

Sex-specific effects of several chromosomes were observed on FC in this study (Fig. 4). In all such cases female mice froze more than male mice. Statistically significant sex-specific QTLs are often hard to identify because the number of subjects is reduced and the number of comparisons is increased. Three CSS showed significant sex differences in freezing to context, CSS-3, -5, and -9, with females freezing more in all cases. Two CSS showed significant sex differences in freezing to tone, CSS-5 and -15. These chromosomes may contain sex-specific QTLs for fear conditioning. These results highlight the benefit of testing and reporting phenotypes for both sexes in a genetic reference panel.

Panels of CSS are useful for identifying QTLs for phenotypes that are controlled by multiple different alleles, such as FC. Because CSS provide a nonsegregating genetic background, they are more efficient for detecting small QTLs with moderate to small effects (Stylianou et al. 2006). The effect size of each chromosome (Table 1; v^2_{CvsB}) indicates the portion of the heritability of the phenotype that is explained by a particular chromosome (Belknap 2003). For example, chromosome 10 accounts for 24% of the genetic variance in freezing to tone (Table 1). According to equation (2) from Belknap (2003), this same QTL would have accounted for only 6% of the genetic variance in an F_2 cross (assuming no dominance), a four-fold difference.

In addition to their advantages for QTL detection, a panel of CSS may be considered a genetic reference population because the same strains may be studied by many different laboratories. This allows for the examination of correlations among multiple phenotypes. Because we believe that both learned fear and anxiety-like behaviors have a common genetic basis, we examined correlations between our measurements of fear learning and data from a previous study of anxiety-like behaviors (innate fear). Singer et al. (2005) examined anxiety-like behaviors in the open field (OF) and light-dark box (LDB) and reported increased anxiety-like behavior in CSS-1, -6, -11, -15, and -17. Our study identified an increase in contextual FC in CSS-6 as well. This may indicate the presence of a pleiotropic allele on CSS-6, which affects both learned fear and innate anxiety. Given the number of chromosomes implicated by each study, it is also possible that CSS-6 reflects the chance co-occurrence of two separate alleles on the same chromosome, one controlling anxiety-like behavior and one controlling FC.

In the Singer et al. (2005) study, CSS-1 showed a robust difference in several anxiety-like behaviors relative to B6. We found that CSS-1 had more pretraining freezing (hypoactivity) and more freezing in the altered context. However, we did not identify differences in freezing in response to either the context or the tone. Taken together,

these data indicate that CSS-1 has a difference in activity levels but not fear learning. It is less clear whether the difference in activity causes the differences in anxiety-like behavior; activity differences alone may be seen as either a necessary component of anxiety-like behavior or a confounding factor with an independent genetic basis. Numerous previous studies have identified QTLs on chromosome 1 for both anxiety-like behaviors (Gershenfeld and Paul 1997; Henderson et al. 2004) and fear learning (Caldarone et al. 1997; Owen et al. 1997; Wehner et al. 1997) using a variety of different inbred strains.

A difference in freezing to the altered context was observed in CSS-1, -3, -4, -6, and -17. High freezing to the altered context could indicate a tendency to overgeneralize a fearful association to a greater variety of similar contexts and cues, similar to a phenotype observed in 5-HT1A receptor knockout mice (Klemenhagen et al. 2006). A tendency to overgeneralize fearful associates might be related to the development of pathologic anxiety (Klemenhagen et al. 2006), and thus of significant importance. Three of these strains, CSS-1, -6, and -17, showed increased anxiety-like behaviors in the study by Singer et al. (2005), perhaps suggesting that freezing in the altered context is controlled by some of the same genes that control anxiety-like behaviors.

Anxiety-like behavior has been studied using a B6 × AJ F_2 cross (Gershenfeld and Paul 1997; Gershenfeld et al. 1997) and an advanced intercross line (AIL) produced by crossing B6 and AJ (Zhang et al. 2005). Although chromosome 10 did not show differences in anxiety-like behaviors in the previous study of CSS-10 by Singer et al. (2005), a significant QTL for anxiety-like behavior was mapped to chromosome 10 in the F_2, and two separate QTLs for anxiety-like behavior were mapped on chromosome 10 in the AIL (Zhang et al. 2005). It is not clear how to interpret the discrepancy between the Singer et al. (2005) results from CSS-10 and the results from the F_2 and AIL studies. The F_2 studies by Gershenfeld et al. (1997) also identified a suggestive QTL for light-dark transitions and center time in the OF on chromosome 6, which is consistent with data from Singer et al. (2005) for CSS-6.

We have identified several statistically significant QTLs for fear learning. QTLs in CSS-10 and -18 are particularly robust and appear to influence freezing to both context and tone. Chromosomes 6 and 10 appear to have pleiotropic effects on both fear learning and anxiety-like behaviors. All of these alleles can be fine mapped by intercrossing and backcrossing the relevant CSS with B6. Further fine mapping will be required to determine whether these findings actually reflect pleiotropy or whether they simply reflect linkage of two alleles, similar to the case identified by Talbot et al. (2003). If these alleles truly have pleotropic influence on learned and innate fear, they may identify genes or gene networks that underlie the enhanced acquisition of fear learning that has been reported in anxiety patients (Lissek et al. 2005).

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