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Anxiety and fear in a cross of C57BL/6J and DBA/2J mice: mapping overlapping and independent QTL for related traits

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Anxiety, like other psychiatric disorders, is a complex neurobehavioral trait, making identification of causal genes difficult. In this study, we examined anxiety-like behavior and fear conditioning (FC) in an F_2 intercross of C57BL/6J and DBA/2J mice. We identified numerous quantitative trait loci (QTL) influencing anxiety-like behavior in both open field (OF) and FC tests. Many of these QTL were mapped back to the same chromosomal regions, regardless of behavior or test. For example, highly significant overlapping QTL on chromosome 1 were found in all FC measures as well as in center time measures in the OF. Other QTL exhibited strong temporal profiles over testing, highlighting dynamic relationship between genotype, test and changes in behavior. Next, we implemented a factor analysis design to account for the correlated nature of the behaviors measured. OF and FC behaviors loaded onto four main factors representing both anxiety and fear behaviors. Using multiple QTL modeling, we calculated the percentage variance in anxiety and fear explained by multiple QTL using both additive and interactive terms. Quantitative trait loci modeling resulted in a broad description of the genetic architecture underlying anxiety and fear accounting for 14–37% of trait variance. Factor analysis and multiple QTL modeling showed both unique and shared QTL for anxiety and fear; suggesting a partially overlapping genetic architecture for these two different models of anxiety.

Keywords: Anxiety, fear, fear conditioning, mice, open field, QTL, QTL modeling

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Anxiety disorders affect 18% of people in the United States each year (Kessler *et al*. 2005). These disorders are prevalent worldwide and debilitating to the people who suffer from them (Demyttenaere *et al*. 2004). Anxiety disorders are variable in behavioral and psychological expression and therefore are likely to be affected by a diverse architecture of genetic factors. Some anxiety disorders are elicited by ambiguous stimuli, whereas others are associated with distinct stimuli and thus more akin to an exaggerated fear response (i.e. general anxiety disorder vs. phobia; Davis *et al*. 2010). Both anxiety and fear are well-defined psychological phenomena controlled by homologous brain regions in humans and experimental animal models (Davis 1992; LeDoux 2000). Importantly, although both are modulated by amygdala activity, anxiety and fear have independent neural pathways (Davis *et al*. 2010). Therefore, it is likely that the genetic substrates of anxiety and fear will share some common and some unique genes. To date, few genes have been consistently identified by forward genetic studies that contribute to our understanding of the etiology of anxiety disorders in either human or animal studies (Hovatta & Barlow 2008; Williams *et al*. 2009; Yalcin *et al*. 2004).

Rodent models have been successfully used to measure anxiety and fear for decades. Classic paradigms such as the open field test (OF) and fear conditioning (FC) have been used to study innate anxiety and the development and maintenance of fear and anxiety through associative learning (Brown *et al*. 1951; Hall 1934). Common to most studies of anxiety and fear in rodents is the intercorrelated nature of the behaviors measured within tasks. For example, one frequent result from OF studies is a robust negative correlation between activity level (i.e. distance traveled) and anxiety (i.e. reduced center time). Using principal component analysis, Milner and Crabbe (2008) showed that activity and anxiety were significantly negatively correlated in OF, light–dark and elevated plus-maze tests, but activity and anxiety were not dissociable as independent factors. In another example, we selectively bred mice for high or low levels of contextual fear, which resulted in elevated anxiety and decreased activity across multiple paradigms (i.e. OF, elevated plus maze, etc.; Ponder *et al*. 2007). Our findings and those of others suggest that anxiety and activity are related traits and tests of anxiety (OF) and learned fear (FC) share a common genetic architecture (López-Aumatell *et al*. 2009; Milner & Crabbe 2008).

In this study, we looked at OF activity following saline injection and freezing behavior in a standard FC paradigm in an F_2 cross of C57BL/6J (B6) and DBA/2J (D2) mice. Most

of the behaviors measured were significantly correlated and we were able to identify many significant QTL. In order to reduce the number of independent tests and to explore the correlation structure in greater depth, we performed a factor analysis on all behavioral data with significant QTL. Anxiety and fear behaviors loaded onto four main factors which could be categorized based on loading strength, contextual fear, altered context/OF, cue-based fear and cued fear training. We also used a multiple QTL modeling approach on summary behavioral data to identify QTL of small effect obscured in our QTL analysis, as well as any potential interactions between QTL. Using both factor analysis and QTL modeling, we were able to identify unique as well as QTL common for both anxiety and fear.

Materials and methods

Animals and housing

Subjects were 612 F_2 mice (305 males and 307 females) derived from an F_1 cross between C57BL/6J (B6) female and DBA/2J (D2) male mice. Colony rooms were maintained on a 12:12 h light–dark cycle with lights on at 0630 h. Mice were housed in clear plastic cages with standard corn-cob bedding in same sex groups of two to five mice with food and water available *ad libitum*. Testing was conducted during the light phase, between 0800 and 1700 h, and mice acclimated to the testing room for a minimum of 30 min prior to testing for all tests. Mice were approximately 2–3 months of age (75.6 ± 0.3 days old, range: 62–90 days) on the first day of testing and all mice went through the identical testing sequence. First, OF behavior was measured as part of a locomotor testing paradigm. One week after locomotor testing, mice began FC. All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the University of Chicago's Institutional Animal Care and Use Committee.

Behavioral testing

Open field

The first day of locomotor testing provided OF behavior. The procedures for locomotor testing have previously been described in detail (Bryant *et al*. 2009; Palmer *et al*. 2005). Briefly, after acclimation mice were removed from the home cage and placed into individual holding cages with clean bedding for 5 min. After 5 min, mice were weighed, injected i.p. with physiological saline (0.01 ml/g body weight) and immediately placed in the center of the OF (AccuScan Instruments, Columbus, OH, USA). Each OF chamber consisted of a clear acrylic arena ($40 \times 40 \times 30$ cm) placed inside a frame containing evenly spaced photocells and receptors. Each activity chamber was housed within a sound-attenuating environmental chamber (AccuScan Instruments) with overhead lighting providing illumination (~80 lux) and a fan in the rear wall that provided ventilation and masking of background noise. The mouse's behavior was then measured by infrared beam break and converted into distance traveled (Versamax; AccuScan Instruments). Mice were allowed to explore the OF arena for 30 min, the first 10 min of which was used for OF behavior. Behaviors measured were distance traveled (cm) in the periphery (width: 10 cm) and center (20×20 cm) of the arena as well as time spent in the center (%) of the arena. After 30 min, mice were placed back in their home cage and the OF was cleaned with 10% isopropanol between tests. At the end of testing, mice were returned to the vivarium.

Fear conditioning

Fear conditioning procedures are identical to those described previously in Ponder *et al*. (2007). Mice were tested in standard FC chambers ($29 \times 19 \times 25$ cm with a stainless steel floor grid). A light on the top of the chamber provided dim illumination (~3 lux) and a fan provided a low level of masking background noise. Each chamber was housed within a sound-attenuating chamber (Med Associates, St. Albans, VT, USA). Chambers were cleaned with 10% isopropanol between animals. Behavior was recorded with digital video, acquired to computer and analyzed with FreezeFrame (Actimetrics, Evanston, IL, USA). Freezing behavior was exported in 30-second blocks and also averaged into summary measures.

Fear conditioning was conducted 10 days after OF, and took place over 3 days. Each day, after acclimation, mice were placed individually into holding cages with clean bedding and transferred to the FC chambers for a 5-min trial. On day 1, baseline freezing was measured beginning 30 seconds after mice were placed into the test chambers and ending 150 seconds later (30–180 seconds; pre-training freezing). Mice were then exposed twice to the conditioned stimulus (CS), a 30-second tone (85 dB, 3 kHz) that co-terminated with the unconditioned stimulus (US), a 2-second, 0.5 mA foot shock delivered through the stainless steel floor grid. After each CS–US pairing, there was a 30-second intertrial interval (ITI) and freezing to tone on day 1 was measured by averaging the percentage time spent on freezing to each CS presentation (%freezing tone day 1). On day 2, the testing environment was identical to day 1 but no stimuli were presented. Percentage of time freezing in response to the test chamber (%freezing context) was measured during the same period of time as pre-training freezing. On day 3, testing was altered in several ways: a different experimenter tested mice wearing different gloves, holding cages containing no bedding, the shock grid, chamber door and one wall were covered with white plastic, yellow film was placed over the top of the chamber, chambers and plastic surfaces were scented and cleaned with a 0.1% acetic acid solution, and the vent fan was partially obstructed to change the background noise. From 30 to 180 seconds, the percentage of time freezing to the altered context (%freezing to altered context) was measured and then the tone CS was presented as on day 1 with no US presentation. Average freezing to tone was calculated for day 3 by taking the average percentage time freezing of each tone presentation (%freezing tone day 3).

QTL mapping

DNA from the F_2 generation was extracted and genotyped by KBiosciences (Hoddesdon, Hertfordshire, UK) using KASPar, a fluorescence-based PCR assay. One hundred sixty-four evenly spaced, informative markers selected from Petkov *et al*. (2004) were used.

Phenotypic and genotypic data were imported into R/qtl for QTL mapping (Broman *et al*. 2003, Appendix S1). The 'scanone' command was used to identify QTL for OF and FC data using the expectation maximization (EM) algorithm. For each analysis, $P < 0.05$ significance levels were estimated using 1000 permutations and 95% Bayesian credible intervals (CIs) (Mb; build 37) expanded to the nearest marker were calculated when significant QTL were found. Sex and age were also examined as both additive and/or interactive covariates.

For single QTL scans, OF data consisted of data for the entire 10 min of testing for all analyses: distance traveled periphery (cm), distance traveled center (cm) and center time (%). The 10-min duration of the OF paradigm was chosen as locomotor activity habituates in the OF during the first 5 min [See Wahlsten1; Mouse Phenome Database (MPD), JAX]. For single QTL scans of freezing behavior in FC, data were exported in 30-second bins for the entire 5-min test period. This time bin duration corresponds to the duration of the tone CS presentation as the CS presentations on days 1 and 3 are discrete and punctate cues that differ greatly in salience from general exposure to the conditioning chamber.

Factor analysis

Behavioral data exhibiting significant QTL (LOD $P < 0.05$, 1000 permutations) in the single QTL analysis were used in the factor analysis (SPSS 17.0; SPSS, Chicago, IL, USA). Raw behavioral data were used in the factor analysis and were not normalized, and average behavioral data were used to replace missing data points

(<1.5% of all data cells). A preliminary dimension reduction of the data resulted in six factors (Fig. S1b). Using a cutoff for initial eigenvalues (~1.5 or larger), the factor analysis was suppressed to four factors and rerun. Behaviors and behavior time points with factor loadings ≤|0.1| were suppressed and varimax rotation was used.

QTL modeling

For QTL modeling, the 'scantwo' command was used to test QTL under both full and additive models. Next, QTL and QTL interactions were assessed by building stepwise models ('stepwiseqtl' command) for multiple QTL which used forward selection and backward elimination to identify the best QTL model of each behavior.

Stepwise modeling was conducted on summary data across tasks: distance traveled OF periphery, distance traveled OF center, %center time, %freezing tone day 1, %freezing context, %freezing altered context and %freezing tone day 3 (as described in FC methods above). In order to include multiple QTL within each model, it was necessary to apply a penalty term for the addition of each new QTL to the model as well as a penalty for any QTL interactions. Penalties used in this analysis were determined by Broman and Sen (2009) with a simulated mouse genome, genotyped with evenly spaced markers: additive penalty, 3.52; heavy interaction penalty, 4.28 and light interaction penalty, 2.69. Each penalty term was subtracted from the LOD score of the model during the selection process.

Statistical analyses

Correlations between behavioral measures were calculated using standard regression analyses. Percentage variance for summary data and factor analysis QTL were computed using the following equation:

$$(1 - 10\text{^}(-2 \times \text{LOD}/n))$$

where LOD equals the peak LOD score and n the number of subjects for which genotype and phenotype data were available. Percentage variance and significance of stepwise models were obtained by using the 'fitqtl' command (Broman & Sen 2009).

Results

Single QTL mapping of multiple traits at several time points showed numerous QTL at a genome-wide significance level of 0.05 (LOD threshold range 3.3–3.8). Specifically, over 20 QTL were identified on 12 chromosomes. Many of these QTL exhibited a temporal specificity. Figure 1a presents a three-dimensional (3-D) LOD score plot of a QTL on chromosome 1 for day 1 FC in 30-second time bins. These QTL are not significant until the first-tone CS presentation (LOD peak: 4.7, genome-wide $P < 0.006$). Figure 1b shows the relationship between %freezing and chromosome 1 LOD scores on day 1; illustrating the tight coupling between the change in freezing behavior and the emergence of the QTL. Figure 1c shows the relationship between freezing behavior and LOD scores on day 3. Here, the QTL on chromosome 1 are significant by the end of acclimation (LOD peak: 5.5, $P < 0.0001$) and stay elevated during the altered context exposure when %freezing is low. Peak LOD scores remain relatively stable even when %freezing increases dramatically with the presentation of the first CS. Therefore, utilizing the temporal profile of the behavior in the QTL analysis shows important information about which aspects of the behavior influence QTL.

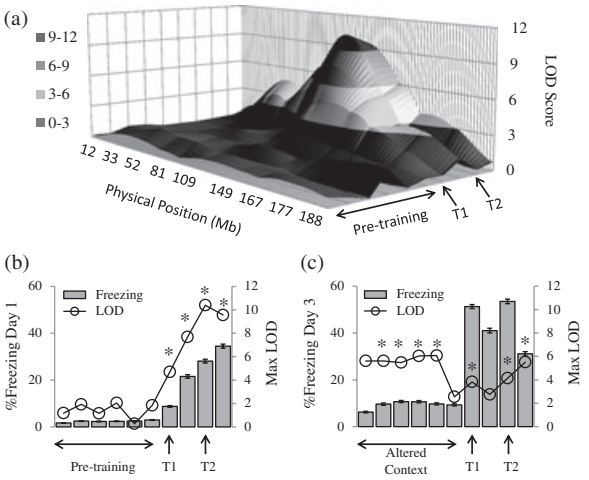

Figure 1: Three-dimensional LOD score plot for significant QTL on chromosome 1 exhibiting a temporal relationship with changes in freezing on day 1. (a) Three-dimensional representation of LOD scores for day 1 FC in 30-second time bins. The QTL is not significant until the first-tone CS presentation (T1). During each subsequent 30-second time bin (z-axis) the LOD score increases, peaking at the second-CS presentation (T2). (b) Relationship between %freezing (bars) and LOD scores (open symbols) on day 1 illustrates the relationship between changes in behavior and the emergence of the QTL. (c) Relationship between %freezing (bars) and LOD scores (open symbols) on day 3. In contrast to day 1, the QTL precedes changes in behavior. The QTL is significant when freezing is low and does not increase during CS presentation when %freezing increases. *Significant LOD score, $P < 0.05$ (LOD score > 3.8).

Factor analysis

Single QTL scans produced a multitude of significant QTL with varying temporal profiles (see Figs S2–S5 for examples). However, for the analysis of 33 behaviors a Bonferonni adjusted P-value for significance ($P < 0.0015$) would have resulted in far fewer significant QTL and thereby make it difficult to identify small-effect QTL. In order to better identify QTL unique to particular behavioral parameters, such as anxiety or fear, we used factor analysis to reduce the number of behaviors analyzed. All 33 behaviors with significant QTL identified in single QTL scans were used in the factor analysis. On the basis of the initial Eigenvalues, dimension reduction was suppressed to four factors (see *Materials and methods*). Loading weights for each of the factors are presented in Table 1. After rotation, each factor explained approximately 11% of the variance, in total explaining almost 44% of the total variance in behavior. Factors were ascribed the following descriptors based on loading strength: contextual fear (Factor 1), altered context/OF (Factor 2), cue-based fear (Factor 3) and cued fear training (Factor 4).

Figure 2 presents single QTL analyses of each of the four factors. LOD peak and QTL interval values are presented in Table 2. Each factor had multiple significant QTL and while some QTL overlapped, some factors had unique, nonoverlapping QTL. For contextual fear (Factor 1), a novel

Table 1: Rotated factor matrix for behavioral data from the OF test and FC

	Factor			
	1	2	3	4
Distance periphery	−0.141	**−0.288**	—	−0.139
%Center time	—	**−0.214**	−0.102	−0.132
Day 1: 90–120 seconds	—	—	—	**0.222**
Day 1: tone1	0.187	0.264	0.250	**0.336**
Day 1: ITI	—	0.281	0.408	**0.611**
Day 1: tone2	—	0.221	0.474	**0.549**
Day 1: 270–300 seconds	—	0.175	0.458	**0.463**
Day 2: acclimation	0.463	0.176	0.165	**0.496**
Day 2: 30–60 seconds	0.488	0.168	0.152	**0.555**
Day 2: 60–90 seconds	**0.517**	0.206	0.134	0.503
Day 2: 90–120 seconds	**0.589**	0.180	0.189	0.458
Day 2: 120–150 seconds	**0.553**	0.180	0.169	0.366
Day 2: 150–180 seconds	**0.567**	0.193	0.158	0.331
Day 2: tone1 time	**0.526**	0.225	0.120	0.234
Day 2: ITI time	**0.530**	0.188	0.134	—
Day 2: tone 2 time	**0.572**	0.211	0.137	—
Day 2: 270–300 seconds	**0.474**	0.242	0.165	—
Day 3: acclimation	0.184	**0.540**	0.144	0.317
Day 3: 30–60 seconds	0.207	**0.565**	—	0.287
Day 3: 60–90 seconds	0.288	**0.597**	0.113	0.268
Day 3: 90–120 seconds	0.193	**0.650**	0.104	0.121
Day 3: 120–150 seconds	0.192	**0.590**	0.105	—
Day 3: 150–180 seconds	0.200	**0.575**	0.155	—
Day 3: tone1	0.157	—	**0.640**	—
Day 3: ITI	0.245	0.222	**0.705**	0.130
Day 3: tone2	0.215	—	**0.755**	—
Day 3: 270–300 seconds	0.276	0.361	**0.589**	0.113
%Variance = 44.189	12.423	11.186	10.485	10.094
Initial eigenvalue	8.98	1.92	1.64	1.46

Bold font indicates where data load most heavily. Percentage variance from the rotation sums of squared loadings and initial eigenvalues are presented at the bottom of each factor.

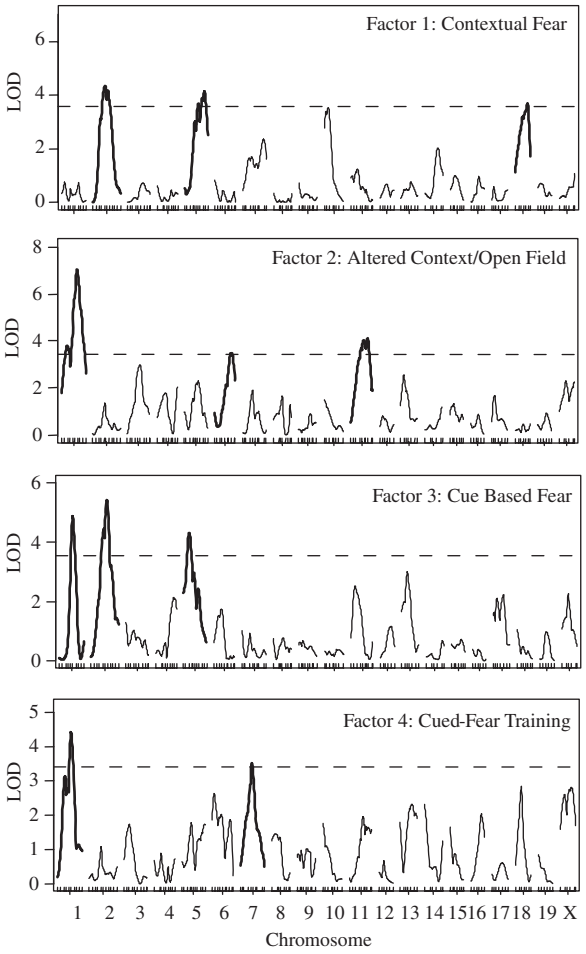

Figure 2: Genome-wide LOD scores for factors derived from the factor analysis of OF and FC data. Dashed line represents the genome-wide significance threshold ($P < 0.05$).

QTL on chromosome 18 (95% Bayesian CI: 46.0–76.3 Mb) was identified that was not identified in the original QTL scans of raw data (Fig. S2). Altered context/OF (Factor 2) was the only factor with a significant QTL on chromosome 6 (CI: 118.4–144.0 Mb). Overlapping QTL on chromosomes 1 and 2 were found for two or more factors. A significant QTL on chromosome 1 was found for altered context/OF, cue-based fear (Factor 3) and cued fear training (Factor 4). Similarly, overlapping QTL on chromosome 2 were found for contextual and cue-based fear. Contextual and cue-based fear both had significant QTL on chromosome 5 but CI, when expanded to the nearest marker, did not overlap suggesting two distinct QTL on this chromosome (CI: 86.9–148.5 Mb and 25.2–86.9 Mb, respectively).

Factor analysis showed a QTL on chromosome 1 common to both anxiety and fear, whereas the QTL on chromosome 6 was specific to anxiety and the QTL on chromosome 2 specific to fear. In general, factor analysis resulted in a reduced number of QTL due in part to reduced LOD scores resulting from the universal loading of this correlated behavioral dataset onto multiple factors (Table 1). Factor analysis may have highlighted the QTL accounting for the highest %variance for behavioral data comprising each factor but CI was still large (range: 25.6–96.7 MB) and the %variance of the anxiety-like behaviors explained by any given QTL was still small (range: 2.5–5.1%; Table 2).

Summary measures

Factor analysis results ultimately identified summary measures that we have typically analyzed (i.e. %freezing to context; Ponder *et al*. 2007). In fact, correlations between behaviors were always strongest within tasks and days and between adjacent time points (Table S1). For FC, correlations were highest within a day of testing but correlated most strongly within the context and tone periods. Therefore, we returned to summary measures of both OF and FC for multiple QTL modeling. Figure 3 presents LOD scores from QTL scans of OF behaviors and Fig. 4 presents LOD scores from QTL analyses for FC (for direct comparison of summary measures to factor analysis results see Fig. S6).

Table 2: Summary of significant QTL from factor analysis and summary behavioral data

Behavior*	Chr†	LOD peak‡	95% CI (Mb)§	%Variance¶	Anxiety/fear allele**
Factor 1-Contextual fear	2	4.3	58.7 (57–116)	3.2	B6
	5	4.1	61.5 (87–149)	3.0	B6
	18	3.7	30.2 (46–76)	2.7	D2
Factor 2-Altered context/OF	1	7.0	57.8 (109–167)	5.1	D2
	6	3.5	25.6 (118–144)	2.5	D2
	11	5.9	32.2 (73–105)	4.3	B6
Factor 3-Cue-based fear	1	4.9	68 (81–149)	5.1	D2
	2	5.4	65.7 (74–140)	2.5	B6
	5	4.3	61.8 (25–87)	4.3	D2
Factor 4-Cued fear training	1	4.4	96.7 (52–149)	3.2	D2
	7	3.5	34.2 (68–102)	2.6	D2
Distance traveled – periphery	1	9.2	9.9 (167–177)	5.6	D2
	4	4.3	23.4 (125–148)	3.1	B6
	11	7.6	33.6 (63–96)	5.6	B6
	X	5.3	76.6 (50–127)	3.7	B6
Distance traveled – center	1	8.0	39.4 (109–149)	5.8	D2
	5††	5.0	42.3 (64–107)	3.6	D2-F
	6	7.7	55.4 (88–144)	5.7	D2
	13	3.5	71.8 (40–111)	2.6	D2
	17††	5.0	49.9 (14–64)	3.7	B6-M
	X	4.2	80.3 (10–90)	3.1	B6
Center time	1	4.6	39.4 (81–149)	3.4	D2
(%)	5	5.5	42.3 (64–107)	4.0	D2
	6	5.1	99.4 (32–132)	3.7	D2
%Freezing tone day 1	1	11.2	68 (81–149)	8.0	D2
	2	5.1	82.4 (58–140)	3.7	B6
	X	4.7	117.3 (96–127)	3.4	B6
%Freezing context	1	4.4	68 (81–149)	3.2	D2
	2	5.6	58.7 (58–117)	4.1	B6
	5	5.3	19.4 (121–140)	3.9	D2
	10	4.6	30.8 (16–47)	3.4	B6
%Freezing altered context	1	7.6	68 (81–149)	5.5	D2
	2	4.3	58.7 (58–117)	3.1	B6
	5	3.9	68.5 (64–133)	2.8	D2
	10	3.8	30.8 (16–47)	2.8	B6
	11††	4.8	43 (63–106)	3.6	B6-F
%Freezing tone day 3	1	5.2	68 (81–149)	3.8	D2
	2	6.1	58.7 (81–149)	4.4	B6
	5	5.0	95.3 (25–121)	3.7	D2
	13	3.8	45 (40–85)	2.8	D2

Significant QTL from genome-wide scans (rQTL; $P < 0.05$). F or M indicates the sex with more anxiety-like behavior for a given allele.
*See *Materials and methods* for description.
†Chromosome.
‡Peak LOD score of QTL
§95% Bayesian CI (Mb; Build 37) expanded to the nearest single nucleotide polymorphism (SNP) marker.
¶Percentage of variance in behavior explained by QTL.
**Allele associated with higher anxiety or elevated fear.
††QTL significant if sex was included as an interactive covariate.

Similar to results from the factor analysis, QTL scans of summary measures resulted in the identification of numerous QTL, many of which overlapped and some of which were unique to a particular measure. On average, however, using summary measures resulted in higher peak LOD scores, 5.5 vs. 4.6 (Table 2). Significant QTL on chromosome 1 as well as a QTL on chromosome 5 were again identified in both anxiety and fear paradigms (Table 2). As in the factor analysis, the QTL on chromosome 6 only mapped to anxiety behaviors and the chromosome 2 QTL was specific to fear.

Multiple QTL modeling

In an attempt to identify epistatic interactions between QTL, we used the 'scantwo' command to test for epistatic

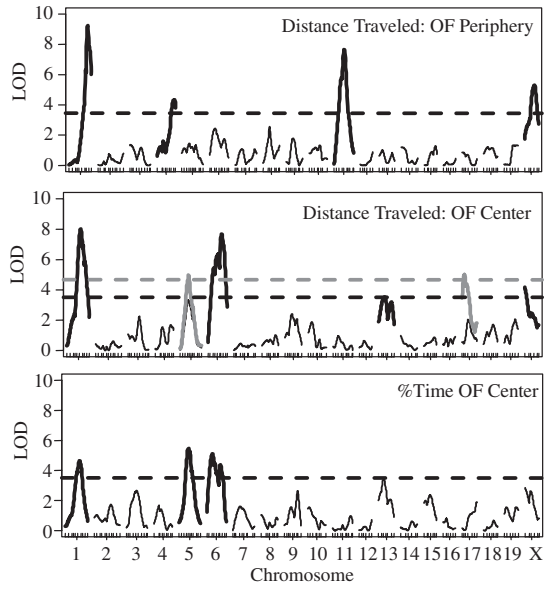

Figure 3: Genome-wide LOD scores for OF behavior. Single QTL analysis results for OF measures: distance traveled in the periphery (cm/10 min; top chart), distance traveled in the center (cm/10 min; middle chart) and %time in the center of the arena (bottom chart). Gray QTL traces indicate LOD scores when sex is included as interactive covariate. Black (no covariate) and gray (sex as interactive covariate) LOD thresholds represent significance at $P < 0.05$.

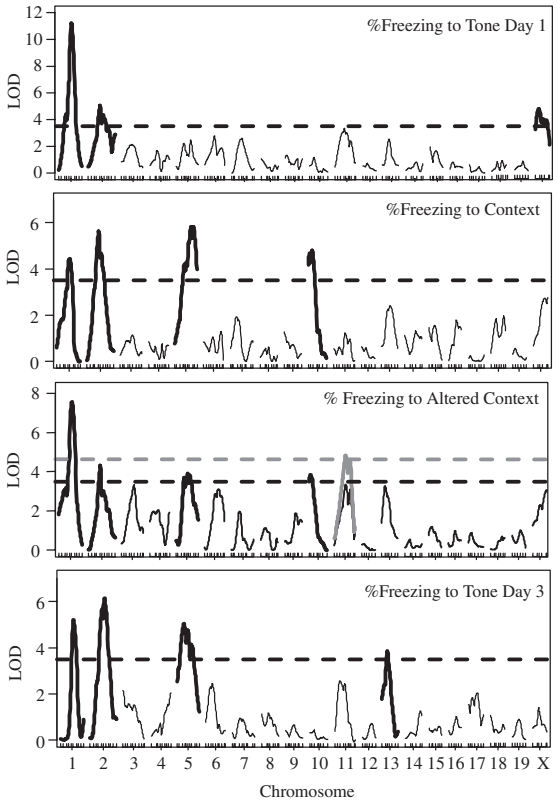

Figure 4: Genome-wide LOD scores for FC freezing behavior. Single QTL analysis results for FC measures: %freezing to tone day 1, %freezing to context, %freezing to altered context and %freezing to tone day 3. Gray QTL traces indicate LOD scores when sex is included as interactive covariate. Black (no covariate) and gray (sex as interactive covariate) LOD thresholds represent significance at $P < 0.05$.

interactions genome wide (Table S2). The 'scantwo' analyses did not identify any significant epistatic interactions from pairwise QTL tests, although a number of suggestive interactions for FC measures, %freezing altered context and %freezing to tone day 3 were found (full vs. additive LOD >3.8). Phenotypes and genotypes were subsequently analyzed using the 'stepwiseqtl' QTL model search algorithm.

Using forward selection and backward elimination, stepwise modeling indicated QTL interactions and QTL for most summary measures. Figure 5 presents multiple QTL models of distance traveled in the periphery and center of the OF. The equation above each figure represents the model with the best penalized LOD score following forward selection and backward elimination. Three new QTL were identified for distance traveled in the periphery on chromosomes 6, 9 and 18. The model search algorithm also identified two QTL interactions, one between chromosomes 1 and 9 and the other between chromosomes 6 and 18. Similarly, for distance traveled in the center of OF, new QTL were identified on chromosomes 4, 9 and 19. For distance traveled in the center, four interactions were identified.

Figure 6 presents multiple QTL models of %freezing to context, %freezing to altered context and %freezing to tone day 3 from FC. For %freezing to context, like distance traveled in the center of the OF, an interaction between chromosomes 1 and 5 was identified. Three new QTL were found for %freezing to altered context on chromosomes 3, 6 and X as well as two interactions; both interactions were identified in the 'scantwo' analysis but LOD scores were only suggestive ($LOD_i \sim 4.0$). One new QTL on chromosome 8 was selected for the model of %freezing to tone day 3 as well as a second locus on chromosome 1 (Fig. 6), and interactions were selected between QTL on chromosomes 1 and 8 and chromosomes 5 and 13. This interaction between QTL on chromosomes 5 and 13 was also identified in the 'scantwo' analysis but again the LOD score was only suggestive ($LOD_i = 3.88$).

Table 3 presents the QTL results of the model search algorithm. Both additive models and models with interacting QTL were highly significant and explained 14–37% of the phenotypic variance in OF and FC measures. Overall, stepwise modeling of QTL resulted in the identification of novel QTL, replication of previously identified QTL and a substantial increase in the %variance of each summary measure explained by QTL. Quantitative trait loci modeling, like factor analysis and single QTL scans of summary measures, not only identified QTL on chromosomes 1 and 5 for both anxiety and fear measures but also suggested an

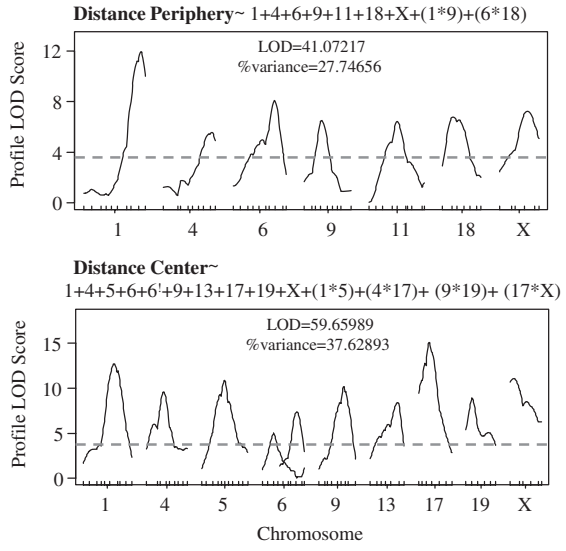

Figure 5: Stepwise QTL models of OF behavior with QTL interactions. Models selection results for distance traveled in the periphery and center and corresponding profile LOD score plots. Interactions are represented as QTL products (i.e. (1 × 2)). !Second QTL position on a previously identified chromosome.

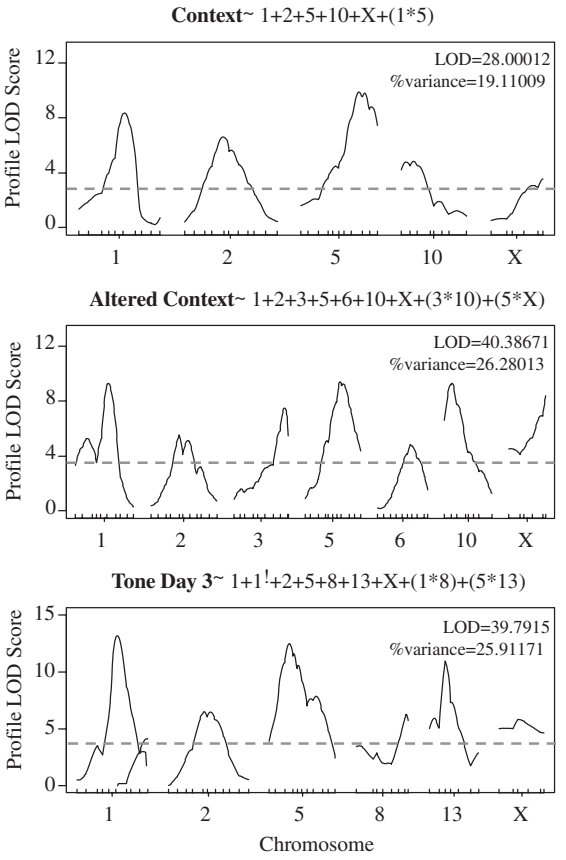

Figure 6: Stepwise QTL models of FC behavior with QTL interactions. Models selection results for %freezing to context, %freezing to altered context and %freezing to tone day 3. Interactions are represented as QTL products (i.e. (1 × 5)). !Second QTL position on a previously identified chromosome.

interaction between these two loci. A more proximal QTL on chromosome 6 still mapped exclusively to anxiety measures from the center of the OF and QTL on chromosome 2 only mapped to FC measures.

Discussion

In this study, we used factor analysis and QTL modeling in an attempt to delineate the genetic architecture underlying anxiety and fear in an F_2 cross of B6 and D2 mice. In preliminary single QTL scans the temporal nature of QTL, especially for fear measures, was highlighted (Fig. 1). Both factor analysis and QTL modeling provided additional information about the relationship between anxiety and fear but had opposite effects on QTL discovery. Anxiety and fear behavioral measures were significantly correlated to each other and the loading of an activity measure (distance traveled) and an anxiety measure (center time) onto a single factor supports previous findings that the two measures are not independent in most tests of anxiety (Brigman *et al*. 2009; Henderson *et al*. 2004; Milner & Crabbe 2008). Furthermore, overlapping QTL for anxiety and fear from

Table 3: Stepwise QTL models of OF and FC behaviors

Behavior	Model – $chromosome^{pos\ (cM)}$	Model LOD	% Variance model
Distance – periphery	$1^{79} + 4^{67} + 6^{57} + 9^{24} + 11^{38.6} + 18^{14} + X^{39} + (1^{79} \times 9^{24}) + (6^{57} \times 18^{14})$	41.07	27.75
Distance – center	$1^{54} + 4^{30} + 5^{39.2} + 6^{19.5} + 6^{60} + 9^{43} + 13^{48} + 17^{18} + 19^{10} + X^{6} + (1^{54} \times 5^{39.2}) + (4^{30} \times 17^{18}) + (9^{43} \times 19^{10}) + (17^{18} \times X^{6})$	59.66	37.63
Center time (%)	$1^{53} + 5^{37} + 6^{22} + 9^{50}$	19.62	14.38
Tone day 1	$1^{46.6} + 2^{41.8} + X^{31}$	21.46	15
Context	$1^{48} + 2^{40} + 5^{61} + 10^{13} + X^{54.3} + (1^{48} \times 5^{61})$	28	19.11
Altered context	$1^{48} + 2^{41.8} + 3^{74} + 5^{51} + 6^{48} + 10^{11} + X^{54.3} + (3^{74} \times 10^{11}) + (5^{51} \times X^{54.3})$	40.39	26.28
Tone day 3	$1^{49} + 1^{84} + 2^{44} + 5^{24} + 8^{61} + 13^{19.1} + X^{23} + (1^{48} \times 8^{61}) + (5^{24} \times 13^{19.1})$	39.79	25.91

Significant additive and interactive models (all $P < 6.6^{-16}$) created using a stepwise modeling approach.

factor analyses support our previous finding that selection based on %freezing to context results in selection for anxiety measures as well (Ponder *et al*. 2007).

Factor analysis identified QTL not originally seen in the single QTL scans but ultimately identified 'test session factors' (Henderson *et al*. 2004) and did not result in more resolved QTL identification (i.e. small-effect QTL or narrow intervals). Henderson *et al*. (2004) used factor analysis in a QTL study of anxiety (but not fear) using multiple behavioral tasks. Similar to this study, the authors concluded that the use of summary measures corresponding to test or test session was more appropriate for identifying genetic loci, especially those loci accounting for a small amount of the trait variance (Henderson *et al*. 2004). In this study, QTL analysis of summary measures (Figs 3 and 4) did result in higher LOD peak scores and comparable Bayesian CIs with a marginal improvement in the percentage variance of the phenotype that was captured by each QTL (Table 2).

Using summary data from OF and FC measures, we performed a QTL model search algorithm to identify additive or interactive QTL models accounting for a greater percentage of trait variance in this F_2 population. Stepwise modeling resulted in an improvement in the amount of variance in behavior explained by each model, accounting for 14–37% of trait variance depending on the measure (Table 3). In general, OF activity measures had more significant QTL and stronger models (27–37% of variance) than center time (14%); a result consistent with previous studies in which activity measures produce much stronger LOD scores than time measures in anxiety tests (Henderson *et al*. 2004). Quantitative trait loci modeling did identify QTL of small effect not found in single QTL scans or QTL analysis of factors, e.g. a QTL on chromosome 9 for OF (Fig. 5). Similarly, a QTL was identified on chromosome 17 for distance traveled in the center of the OF but only if sex was included as an interactive covariate (i.e. B6 males' low activity in OF center). This QTL was also identified with QTL modeling and overlaps with a QTL previously identified in an F_2 cross of LG/J and SM/J mice for startle response where males had a greater startle response than females (Samocha *et al*. 2010). The overlap of these two QTL in different strains of mice for different measures of anxiety-like behavior provides strong evidence for a sex-specific effect of this contributing locus on chromosome 17. Finally, the CI for the QTL on chromosome 17 includes *Glo1*, a gene which we and other investigators have found to be involved in differences in anxiety-like behavior (Hovatta *et al*. 2005; Williams *et al*. 2009).

Many of the QTL identified replicate QTL identified in previous studies using B6 and D2 mice. Significant QTL on chromosomes 1, 2 and 10 were previously identified for contextual fear, freezing in the altered context and cue-based fear in a B6XD2 F_2 intercross (Wehner *et al*. 1997). These three chromosomal regions were further validated in a line of B6XD2 mice selected for contextual fear (Radcliffe *et al*. 2000) with similar allele effects to those found in this study (Table 2). Specifically, on chromosome 1 the D2 allele is the high anxiety/fear allele, whereas the B6 allele is the high anxiety/fear allele for chromosomes 2 and 10 (Radcliffe *et al*. 2000). Furthermore, consistent with our previous analysis of a selected line of B6XD2 mice selected for freezing to context, QTL identified in this study spanned intervals including significantly associated SNPs and differential gene expression in hippocampal tissue on chromosomes 1, 4, 5 and 13 (Ponder *et al*. 2007).

In almost every QTL analysis conducted in this study, a significant QTL was identified on chromosome 1. All chromosome 1 QTL had overlapping CI (80.6–148.8 Mb) with the exception of a nonoverlapping QTL for distance traveled in the periphery of the OF (167.1–177.1 Mb; Fig. S5). Significant QTL on chromosome 1 have been identified in numerous studies of anxiety-like behavior in multiple crosses of mouse strains as well as heterogeneous stock (HS) mice (Caldarone *et al*. 1997; Henderson *et al*. 2004; Talbot *et al*. 2003; Valdar *et al*. 2006; Wehner *et al*. 1997). The overlapping CI of the QTL on chromosome 1 for anxiety and fear measures includes the gene, *Rgs2*, which has been shown to affect anxiety-like behavior in tests of unlearned fear (OF; Yalcin *et al*. 2004). This region also includes *Mcm6*, a gene which we found to be differentially expressed in the hippocampus of mice selected for elevated freezing to context (Ponder *et al*. 2007). The more distal QTL on chromosome 1 found for distance traveled in the periphery was recently identified for a OF distance traveled in an F_2 cross of B6 and C58/J mice (Eisener-Dorman *et al*. 2010). This distal QTL has a CI overlapping with *Qrr1* (172.5–177.5 Mb), a region with a high proportion of *trans*-expression QTL (eQTL) in neural tissue associated with numerous physiological and behavioral traits (Mozhui *et al*. 2008).

Another QTL that has previously been reported for anxiety and fear-related traits (Ponder *et al*. 2007; Valdar *et al*. 2006) was also identified on chromosome 5 for both OF and FC measures. The model search algorithm identified an interaction between chromosomes 5 and 1 for both distance traveled in the center of the OF as well as freezing to context in FC. Using the overlapping CI for the QTL on chromosome 1 for both behaviors (CI: 109.3–148.7 Mb; Table 2), we ran a search on WebQTL, an extensive database of QTL, mRNA expression and trait data for B6 and D2 mice (www.genenetwork.org; Chesler *et al*. 2004). We identified 133 differentially expressed genes (LOD $\geq$ 3.26) in whole brain tissue of B6XD2 F_2 mice and 137 differentially expressed genes in hippocampal tissue of B6XD2 recombinant inbred lines (RILs) within our CI interval on chromosome 1. With QTL modeling results indicating an interaction between QTL on chromosomes 1 and 5, we took those differentially expressed genes on chromosome 1 and looked for significant *trans*-eQTL on chromosome 5. Of those differentially expressed genes, only 11 had significant *trans*-eQTL within the CI on chromosome 5, for freezing to context (CI: 120.5–140 Mb), *Trip12*, *Farp2*, *Phlpp*, *Lpgat1*, *Ccnt2*, *Ubxd2*,*Pfkfb2*, *Mdm4*, *Rnpep*, *Camsap1l1* and *Kif14*. If the interaction between QTL on chromosomes 1 and 5 is validated, QTL modeling in combination with bioinformatic tools may allow for the identification of candidate genes, even in low-resolution QTL analyses like the F_2 intercross.

Although many of the QTL presented here replicate results of previous studies, some novel QTL were also discovered. In contrast to well-documented QTL, like the

Table 4: Quantitative trait loci in B6 × D2 RILs for phenotypes related to OF and FC

Chr	QTL peak (Mb)	Trait IDs	Experimental assay	LOD	Publication
7	64	11390	Light–dark	2.80	Philip *et al*. (2010)
	35	10898	Plus maze	2.76	Yang *et al*. (2008)
8	72	10675	Amygdala volume	3.59	Yang *et al*. (2008)
9	52	12362	Zero maze	3.30	Cook *et al*. (2009)
	35	11917	FC	2.96	Philip *et al*. (2010)
	57	10075	OF	3.02	Crabbe *et al*. (1983)
	57	10505	OF	4.59	Plomin *et al*. (1991)
	73	11868	OF	3.80	Philip *et al*. (2010)
17	65	11724	OF	3.04	Philip *et al*. (2009)
	57	10446	FC	3.61	Owen *et al*. (1997)
18	84	11620	OF	3.63	Philip *et al*. (2010)
19	15	12341	Zero maze	4.09	Cook *et al*. (2009)
	17	11659	FC	2.56	Philip *et al*. (2010)

Suggestive and significant QTL (LOD $\geq$ 2.5) for traits in the GeneNetwork database related to anxiety and fear. Keyword search: fear, anxiety, OF and FC. For novel QTL found in this study, this table presents corresponding QTL found in other studies utilizing both similar and different experimental assays.
Chr, Chromosome; QTL peak, Mb position of peak LOD score; Trait IDs, GeneNetwork trait ID number.

QTL on chromosome 1, these results must be interpreted with caution. Specifically, because multiple QTL analyses were performed on multiple behaviors, some or all of these novel QTL may in fact be false discoveries. For example, QTL on chromosomes 7, 8, 9, 17, 18 and 19 were identified only in the factor analysis and QTL modeling steps. Using the GeneNetwork database, we conducted a search of phenotypes for B6XD2 RIL related to those measured in this study and specifically looked for suggestive or significant LOD scores ($\geq$2.5) for QTL on these chromosomes (Table 4). For all six chromosomes, we found evidence to support the QTL findings of this study. We are currently using a B6 × D2 advanced intercross line to replicate and fine-map these QTL.

As in most F_2 studies, the primary limitation in this experiment is QTL mapping resolution. The majority of QTL identified had 95% CI >40 Mb and even the smallest interval of 10 Mb on chromosome 1 for distance traveled in the periphery contains hundreds of genes. In an attempt to overcome mapping resolution issues, we used factor analysis to reduce the number of behaviors measured and in parallel QTL modeling to identify small-effect QTL but each approach has limitations with respect to interpretation of the results. Quantitative trait loci analysis of behavioral factors was greatly affected by the universal loading of most behavioral data onto each of the four factors (Table 1). In fact, if a more stringent eigenvalue cutoff had been employed (i.e. eigenvalues $\geq$2) (Henderson *et al*. 2004; Milner & Crabbe 2008), only one factor would have been obtained. Results from QTL modeling must also be interpreted with care and considered exploratory in nature as *P*-values for each QTL do not account for other QTL included in the model (Broman & Sen 2009). Current methods of QTL mapping in HS, outbred populations and advanced intercross lines have the potential to provide single-gene resolution (Cheng *et al*. 2010; Flint 2011; Flint *et al*. 2005; Valdar *et al*. 2006; Yalcin *et al*. 2010) and subsequently these populations should be more robust for identifying precise epistatic interactions between significant loci when multiple QTL modeling is employed.

In summary, we identified, and in some cases replicated, multiple QTL for OF and FC with multiple analysis approaches. Anxiety and fear had both overlapping and nonoverlapping QTL suggesting some portion of shared genetic architecture which is consistent with anatomical (Davis 1992; Davis *et al*. 2010; LeDoux 2000) and genetic data (López-Aumatell *et al*. 2009; Ponder *et al*. 2007). The relationship between anxiety and fear was further supported by our factor analysis results showing that all behavioral measures from OF and FC are significantly correlated. Quantitative trait loci modeling results improved the amount of phenotypic variance explained by QTL with the identification of small-effect QTL as well as QTL interactions. Quantitative trait loci modeling, especially in high-resolution mapping populations like advanced intercross lines and outbred populations, may prove to be useful for identifying the dynamic gene interactions and large gene networks that underlie complex and variable traits like anxiety and fear and ultimately replicate and refine the results presented here. Further delineation of both the shared and nonoverlapping genetic architecture of anxiety and fear will provide a clearer understanding of the molecular mechanisms underlying both traits and hopefully lead to the identification of potential therapeutic targets ideally suited for specific anxiety disorders in humans.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1: Raw behavioral data and genotypes for F2 mice. Raw data used in rQTL analysis for all phenotypes including individual factor analysis scores and genotype data. For genotype data 1 = B6, 2 = F1 and 3 = D2. All other units for phenotype data are described in the Methods.

Figure S1: (a) Single QTL analysis results for %freezing to tone 1 day 1 (top QTL plot) and %freezing to tone 2 day 3 (bottom QTL plot). To the right of each QTL plot is the frequency histogram of raw behavioral data used in the factor analysis. Some behaviors were skewed, whereas other behaviors were normally distributed. (b) Screen plot for first pass factor analysis with no suppression. Eigenvalues $<\sim 1.5$ were suppressed and the factor analysis was recalculated with four factors.

Figure S2: Single QTL analysis results for %freezing to the tone and post-tone periods on day 1. Purple QTL traces indicate significant LOD scores when sex is included as interactive covariate. Red (no covariate) and orange (sex as interactive covariate) LOD thresholds represent significance at $P < 0.05$.

Figure S3: Single QTL analysis results for %freezing to Context on day 2 in 30-second intervals from top to bottom. Red LOD thresholds represent significance at $P < 0.05$.

Figure S4: Single QTL analysis results for %freezing to altered context on day 3 in 30-second intervals from top to bottom. Red (no covariate) and orange (sex as interactive covariate) LOD thresholds represent significance at $P < 0.05$.

Figure S5: Single QTL analysis results for %freezing to the tone and post-tone periods on day 3 in the altered context. Red LOD thresholds represent significance at $P < 0.05$.

Figure S6: QTL analysis results for factor analysis and corresponding summary data. Gray trace indicates significant LOD scores when sex is included as interactive covariate (c). Red (no covariate) and orange (sex as interactive covariate) LOD thresholds represent significance at $P < 0.05$.

Figure S7: LOD score plot for significant QTL on chromosome 1 for OF and FC measures. Red and orange shading represent the 95% Bayesian CIs expanded to the nearest marker (shown in Mb). The Bayesian interval for the QTL for distance traveled in the periphery appears to be adjacent to, but not overlapping, the combined interval for the other QTL suggesting two loci on chromosome 1.

Table S1: Bivariate correlation table for OF and FC measures used in the factor analysis. Correlations for various behavioral measures and time points during testing. $**P < 0.01$ and $*P < 0.05$. Bold font highlights correlations where $r > 0.5$ and italic font $0.5 > r > 0.3$ (correlations <0.3 are de-emphasized). Gray shaded boxes represent regions where blocks of adjacent time points have correlations >0.5.

Table S2: Significant results from 'scantwo' analysis. Pairwise interactions between chromosomes were tested for the entire genome. Only chromosome pairs with significant LOD scores (see threshold values below; Broman & Sen 2009) for either full, allowing for epistatic interactions, or additive models are presented (bold font). Only additive relationships between chromosomes were indicated in this pairwise scan (i.e. no interactions between QTL) but unique QTL, not found in the single QTL scan, were identified for center time (chromosome 13) and %freezing to altered context (chromosomes 3, 6 and 13). The rest of the significant QTL pairs replicated QTL identified in the 'scanone' analysis.

Table S3: Stepwise QTL modeling results using Haley–Knott regression. Chromosome and cM locations (chr@cM) of QTL peaks identified from either forward selection of backward elimination of QTL by the modeling algorithm.

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