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# A large QTL for fear and anxiety mapped using an $F_2$ cross can be dissected into multiple smaller QTLs

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## Abstract

Using chromosome substitution strains (**CSS**), we previously identified a large quantitative trait locus (**QTL**) for conditioned fear (**CF**) on mouse chromosome 10. Here, we used an $F_2$ cross between CSS-10 and C57BL/6J (**B6**) to localize that QTL to distal chromosome 10. That QTL accounted for all of the difference between CSS-10 and B6. We then produced congenic strains to fine-map that interval. We identified two congenic strains that captured some or all of the QTL. The larger congenic strain (Line 1; 122.387121 – 129.068 Mb; build 37) appeared to account for all of the difference between CSS-10 and B6. The smaller congenic strain (Line 2; 127.277–129.068 Mb) was intermediate between CSS-10 and B6. We used haplotype mapping followed by qPCR to identify one gene that was differentially expressed in both lines relative to B6 (*Rnf41*) and one that was differentially expressed between only Line 1 and B6 (*Shmt2*). These cis-eQTLs may cause the behavioral QTLs; however, further studies are required to validate these candidate genes. More generally, our observation that a large QTL mapped using CSS and $F_2$ crosses can be dissected into multiple smaller QTLs demonstrates a weaknesses of two-stage approaches that seek to use coarse mapping to identify large regions followed by fine-mapping. Indeed, additional dissection of these congenic strains might result in further subdivision of these QTL regions. Despite these limitations we have successfully fine mapped two QTLs to small regions and identified putative candidate genes, demonstrating that the congenic approach can be effective for fine mapping QTLs.

### Keywords

Shmt2; Rnf41; congenic mice; conditioned fear; anxiety; QTL; chromosome substitution strains; $F_2$ intercrosses; quantitative trait locus mapping; mouse behavioral genetics

## Introduction

Excessive and/or inappropriate fear is one of the most prominent symptoms of anxiety disorders, in particular disorders of fear regulation such as posttraumatic stress disorder (**PTSD**; Mahan & Ressler 2012; Johnson *et al.* 2012). The importance of genetic influences on anxiety disorders is well known, yet identifying the underlying genetic mechanisms has proven difficult (Amstadter *et al.* 2009). Knowledge of the specific genes that confer susceptibility will aid in both diagnosis and the development of more efficacious treatments.

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While the full spectrum of any human psychiatric disorder cannot be fully recapitulated in a mouse model, there is substantial behavioral, genetic, and neuroanatomical conservation between humans and mice. Thus, translational mouse models can provide a powerful strategy for understanding the genetic and biological underpinnings of the acquisition of fear, as well as the etiologic processes related to anxiety.

Conditioned fear (**CF**) is a form of Pavlovian learning in which an aversive unconditioned stimulus (**US**) is paired with a previously neutral cue (conditional stimulus; **CS**). In rodents, the strength of the association between the tone and the shock is measured by freezing, a species-specific response to fear. Freezing is used to measure response to the tone or to the context in which the fearful memory was acquired (Dexter and Merrill 1969; Fendt & Fanselow 1999; LeDoux 2000; Phillips & LeDoux 1992). In contrast to most common tests of anxiety-like behaviors in mice, CF is highly conserved across species, is exhibited in both laboratory and natural environments, and can easily be measured in humans (Amstadter *et al.* 2009). In addition, CF is heritable in both mice and humans (Wehner *et al.* 1997; Hettema *et al.* 2003), is more quickly acquired and more difficult to extinguish in patients diagnosed with anxiety disorders (Lissek *et al.* 2005), and its neurological, anatomical, and pharmacological underpinnings are well established (Davis *et al.* 2010; Leuner & Shors 2012; Johnson *et al.* 2012).

We previously used a set of chromosome substitution strains (**CSS**), in which a single chromosome from the A/J (**AJ**) strain was introgressed onto a C57BL/6J (**B6**) background, to identify a number of alleles that influenced CF (Ponder *et al.* 2007a). One of our strongest findings was that CSS-10 had increased freezing to cue and freezing to context compared B6. In a separate study we selectively bred mice for high or low CF and examined loci that responded to selection as well gene expression in the hippocampus in mice from the $4^{th}$ selection generation. We identified a quantitative trait locus (**QTL**) on chromosome 10 and a gene on distal chromosome 10 (*Rnf41* and *Grip1*) that showed highly divergent expression in the selected lines (Ponder *et al.* 2008). Taken together, those prior studies strongly support the presence of QTL on chromosome 10 that influences fear and anxiety-like behaviors.

In the present study, we used an $F_2$ intercross between CSS-10 and B6 mice to map a QTL for conditioned fear on chromosome 10. We then created congenic and sub-congenic lines to fine map the QTLs in this region. We also examined gene expression differences between the congenic strains and B6 in an effort to identify expression QTLs (**eQTLs**) that might cause the behavioral QTLs. Thus, these studies replicated and extended on our prior investigations of QTLs for fear learning.

## Materials and Methods

### Animals and Housing

All experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals as approved by the University of Chicago's Institutional Animal Care and Use Committee. Mice were housed in standard vivarium cages of two to five same-sex littermates and were maintained in the animal colony at the University of Chicago. The colony was on a 12:12hr light-dark cycle (lights on: 0600h) with food and water available ad libitum. Experiments were conducted in the light phase. All mice were between 45–90 days of age at the time of testing. Given that no sex differences were observed in CSS10 mice (Ponder *et al.* 2007a), we used both male and female mice in the current study.

## Subjects

**CSS-10 $F_2$ mice—**To create the $F_2$ population, C57BL/6J-Chr 10AJ/NAJ (CSS-10) males were backcrossed to B6 females (Jackson Laboratories, Bar Harbor, ME) to create $F_1$ progeny. $F_1$ offspring were subsequently intercrossed to create $F_2$ mice. A total of 83 $F_2$ mice were used (M=45, F=38) for mapping QTLs associated with conditioned fear.

**CSS10 congenic mice—**Two congenic lines were created by backcrossing CSS-10 $F_2$ mice to B6 mice to generate heterozygous congenic founders which were then used to create heterozygous congenic breeder pairs. These breeders produced wild-type-, heterozygous- and homozygous-congenic offspring. Therefore, all congenic studies used littermate controls. Note that because we used CSS-10 rather than AJ mice as a source of AJ alleles, there is no possibility of residual AJ alleles on other chromosomes, which is an advantage relative to more conventional congenic strains.

DNA was extracted from congenic mice and genotyped to define the boundaries of the congenic segments. The first congenic line (Line 1), had a congenic region that spanned from 122.387 – 129.068 Mb (Build 37; rs29362176 – rs45982283). The second congenic line (Line 2), which was derived from Line 1, had a congenic region spanning 127.277–129.068 Mb (Build 37; rs3723970 – rs45982283). Genotyping was accomplished by a combination of Taqman custom assays (below) and Sanger sequencing across known SNPs that are polymorphic between B6 and AJ. For Line 1, the congenic region began between rs29380524 (122.384 Mb) and rs29362176 (122.387 Mb). For Line 2, the congenic region began between rs29382217 (127.264) and rs3723970 (127.277 Mb). Both congenic regions ended between rs13480830 (128.964 Mb) and rs45982283 (129.068 Mb). Thus, these congenic strains allowed us to assess a ~ 6.7Mb region (Line 1) and a ~1.8 Mb region (Line 2) using a method similar to the sequential analysis of congenic lines of Shao *et al.* (2010). In total, 59 mice from 23 litters were tested: 27 wild-type (M=14, F=13), 14 mice homozygous for the AJ allele from Line 1 (M=7, F=7), and 18 mice homozygous for the AJ allele from Line 2: (M=9, F=9). A separate cohort of 22 behaviorally naïve congenic male mice (B6: n=8; Line 1: n=7; Line 2: n=7) were used for gene expression analysis.

## Behavioral Testing

Prior to each test described below, mice were transported to the testing room in their home cages and were given a minimum of 30 min to acclimate to the testing room.

**Open Field (OF) Test—**Naïve congenic mice and wild-type littermates from both lines were tested on the OF and LD tests. The OF was a custom-made opaque Plexiglas box (39 × 39 × 39 cm). The center was 24 × 24 cm. Mice were tested in a well-lit room with illumination in the center of the field measuring approximately 300 lux. Mice were removed individually from their home cage and placed immediately in the center of the OF and allowed to explore the OF for 10 min. Total distance (cm) traveled in the OF, percent time spent in the center of the arena, latency to leave the center, and transitions between the center and periphery were measured using EthoVision XT software (v5, Noldus Information Technology, Wageningen, The Netherlands). After testing, mice were returned to their home cage. The OF was cleaned with 10% isopropyl alcohol after each animal was tested.

**Light Dark (LD) Test—**Twenty four hours after OF testing mice were tested in the LD test. To create the dark chamber, an insert (39 × 19.5 × 39 cm) constructed of black Plexiglas was placed in the OF arena. The dark chamber was accessible to the mice via a small door (4 × 4 cm). A black Plexiglas lid blocked the light from the room. Mice were then removed individually from the home cage and placed immediately on the light side of the arena facing away from the door to the dark chamber. Mice were allowed to explore the

LD arena for 10 min and latency to enter dark chamber, percent of time spent on the light side of the arena and transitions between the light and dark were measured using EthoVision XT software. After testing, mice were returned to the home cage. The LD chamber was cleaned with 10% isopropyl alcohol after each animal was tested.

**Conditioned fear (CF)—**Twenty four hours after the LD test, congenic mice were tested for CF using a 3-day protocol that has been described previously (see Ponder *et al.* 2007a). Mice were briefly placed into individual holding cages with clean bedding and transferred to the CF chambers. On day 1, baseline freezing was measured 30-s after mice were placed into the test chambers and ending 150-s later. Mice were then exposed to two 30-s tones (85 dB, 3 kHz) that coterminated with a 2-s, 0.5 mA foot shock delivered through the stainless steel floor grid. After each tone-shock presentation there was a 30-s period without any stimuli. On day 1, three measures were calculated: (1) baseline freezing, defined as average percent time freezing beginning 30 s after the mice were placed into the test chambers, and ending 150 s later (30–180 s; pre-training freezing), (2) time spent freezing to the first tone presentation (180–210 s, freezing to tone 1 day 1), and (3) time spent freezing during both tone presentations (180–210 s, 240–270 s; freezing to tones day 1). Note that freezing to tone 1, day 1 represents freezing in response to the novel tone but has no learned component because the tone has not yet been paired with the shock.

Test day 2 began exactly 24 h after the start of test day 1. On test day 2, the testing environment was identical to day 1; however, neither tones nor shocks were presented. On day 2 only one measure was analyzed: freezing to context. This was defined as the percent of time freezing during the same period of time as pre-training freezing (30–180 s; freezing to context). We chose this time period to allow for direct comparisons to the pre-training freezing scores on day 1, and to avoid measuring freezing behavior during the latter part of the trial in which the mice might have anticipated shocks based on the previous days test.

Test day 3 began exactly 24 h after the start of test day 2. On test day 3, the context was altered in several ways: (1) a different experimenter conducted the testing and wore a different style of gloves, (2) the transfer cages had no bedding, (3) the metal shock grid, chamber door and one wall were covered with hard white plastic, (4) yellow film was placed over the chamber lights, (5) chambers and plastic surfaces were cleaned with 0.1% acetic acid solution, and (6) the vent fan was partially obstructed to alter the background noise. On day 3, the tone was presented at the same times as on day 1, but there was no shock; two measures were calculated: (1) freezing to altered context, defined as average percent time freezing during the same period considered for days 1 and 2 (30–180 s; freezing to altered context), and (2) freezing to cue, defined as the average percent time spent freezing during the two 30 s tone presentations (180–210 s, 240–270 s; freezing to cue).

**QTL mapping—**DNA from the $F_2$ mice was extracted using a standard salting out protocol and genotyped using 12 single nucleotide polymorphism (SNP) markers that are polymorphic between B6 and AJ according to the manufacturer's instructions (rs4228126, rs13480566, rs13459120, rs13480612, rs13480621, rs13480632, rs13480679, rs13480719, rs13480762, rs13480781, rs13480796, rs13480832; Taqman, Applied Biosystems, Foster City, CA). We used the Scanone function in R/QTL (Broman *et al.* 2003) to calculate LOD scores at 1 cM intervals across chromosome 10 using the expectation maximization (EM) algorithm; 10,000 permutations were used to establish a threshold for significance for each trait ($p=<0.05$). We defined 95% Bayesian credible intervals and expanded to the nearest marker to define the QTL intervals. The average distance between markers was 10.64 Mb, range = 3.7 Mb to 16.91 Mb.

**Haplotype Association Mapping of expression QTL (eQTL)—**Haplotype association mapping focused on the interval from 122.387121 to 129.068183 Mb. Since there were 109 genes in this interval, we were interested in identifying cis-acting eQTL in these regions, as they may be the underlying molecular cause of the behavioral QTLs. We considered markers near the QTL as "cis" eQTLs, and note that this does not eliminate the possibility that something else linked to the region may be involved. Because there are no public databases of brain eQTL data for B6 and AJ mice, we performed haplotype association mapping of eQTL using an existing dataset of hippocampal and amygdala gene expression data taken from a panel of 28 inbred strains that included B6 and AJ (Mouse Diversity Panel; Loguercio *et al.* 2010). Haplotype association mapping takes advantage of the fact that laboratory inbred strains are derived from a small number of founder haplotypes that are identical by descent (Wiltshire *et al.* 2003). Haplotypes were constructed using a total of 10,990 SNPs spaced at approximately 300-kb intervals. A sliding 3-SNP window was used to assign each strain to a haplotype, with a minimum requirement of at least 5 strains per haplotype (McClurg *et al*. 2006; Pletcher *et al.* 2004). Using this approach we identified eQTLs across the congenic region, which we filtered so that only eQTLs in which B6 and AJ belonged to different haplotypes were considered. A marker association algorithm combined with family-wise error rate (gFWER) analysis was used to identify associations and to account for relatedness among strains and thus decrease the rate of false positive associations (McClurg *et al*. 2006). We report only eQTLs with $-\log(P) > 3.5$.

**Bioinformatic analyses—**We used high density sequence data provided by the Welcome Trust Sanger Institute (accessed on May 17, 2013; http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl; Keane *et al.* 2011; Yalcin *et al.* 2011) to compare genomic regions between B6 and AJ mice. These strains were sequenced to an average of 25X coverage on the Illumina GAII platform (Illumina, San Diego, CA, USA) with a mixture of 54, 76, and 108 bp paired reads. We used these data to search for genes within the congenic region that possessed "consequential" polymorphisms between B6 and AJ mice (such as nonsynonymous coding SNPs, stop-gain SNPs, stop-loss SNPs, SNPs resulting in frameshifts and SNPs located in essential splice sites).

**Quantitative PCR (qPCR)—**Each eQTL identified using haplotype association mapping was verified in our congenic mice by using qPCR of mRNA from the hippocampus. Hippocampal tissue was isolated from congenic and wild type mice and flash frozen; later it was homogenized and RNA was extracted with the RNeasy Mini Kit (Qiagen, Chatsworth, CA). RNA concentration was measured (NanoDrop 8000, Thermo Fisher Scientific, Waltham, MA) and all samples were brought to a concentration of 25 ng/μL and then reverse-transcribed to produce cDNA libraries. Primers for qPCR were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). Replicate samples were run for each primer pair and for β-Actin as a normalization control using Power SYBR® Green (Invitrogen, Grand Island, IL) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions.

**Statistical Analyses—**One-way ANOVAs were used to analyze CF data; post-hoc independent samples t-tests were then used when significant main effects were identified (SPSS Statistics 21; SPSS Inc., Chicago, IL, USA). Analysis of the quantitative PCR data was performed using the relative quantification method, as described by Livak & Schmittgen (2001). Briefly, average ΔCT was calculated for B6 and the ΔΔCT was calculated by subtracting the average ΔCT from the ΔCT value for each sample. Fold change was calculated using the equation: Fold change = $2^{-\Delta\Delta C_T}$. A Bonferroni correction was used to correct for the number of genes examined in each congenic line.

## Results

### CSS-10 $F_2$

As shown in Figure 1A and Table 1, QTL mapping of CF traits produced significant overlapping QTL for many traits related to FC on the distal portion of chromosome 10; permutation derived LOD thresholds were similar for all traits ($p < 0.05$; LOD=2.31–2.37). As expected based on our prior studies (Ponder *et al*. 2007a, 2008), the AJ allele resulted in increased freezing for all traits. The overlapping region on chromosome 10 for both contextual fear and cue-based fear was 10.1 Mb (117560107–127659097; Figure 1B). Neither pre-training freezing nor freezing to altered context resulted in significant QTL, emphasizing that these differences were specific to the presentation of stimuli; however, we did observe a significant QTL for freezing to tone 1. Because tone 1 occurs prior to the first shock, this indicates that this QTL influences non-learned behavior.

### Congenic mice

Wild type littermate (pure B6) mice generated from both congenic lines were pooled together after establishing with one-way ANOVAs that there were no significant differences in any of the anxiety or CF measures between wild type mice from Line 1 and Line 2. There was a significant effect of line on freezing to tone 1 day 1, freezing to both tones day 1, freezing to context day 2, and freezing to cue day 3 (tone 1 day 1: $F(2, 56) = 6.7$, $p = 0.002$; both tones day 1: $F(2,56)=16.0$, $p < 0.0001$; context day 2: $F(2,56)=12.8$, $p < 0.0001$; cue day 3: $F(2,56)=17.9$, $p < 0.001$). Line 1 displayed enhanced freezing to tone 1 day 1 as compared to both Line 2 ($p < 0.05$) and B6 mice ($p < 0.0001$; Figure 1C). Line 1 also displayed elevated freezing to both tones on day 1 as compared to B6 mice ($p < 0.0001$) and to Line 2 ($p < 0.05$; Figure 1D). Line 2 displayed elevated freezing to both tones on day 1 as compared to B6 mice ($p < 0.05$; Figure 1D). For freezing to context on day 2, only Line 1 displayed significantly increased freezing; this result was significant compared to both Line 2 ($p < 0.01$) and B6 ($p < 0.0001$) mice (Figure 1E). Finally, both lines displayed elevated freezing to cue on day 3 as compared to B6 mice (Line 1: B6 $p < 0.0001$; Line 2:B6 $p < 0.05$), with Line 1 also showing increased freezing to cue as compared to Line 2 ($p < 0.005$; Figure 1F). There were no significant differences in pre-training freezing or in freezing to altered context between congenic mice and their B6 littermates, which is in line with the results from the $F_2$ studies. Thus, both Lines 1 and 2 were different from B6 for some or all of the phenotypes, and Line 1 and 2 were also significantly different from each other in several cases.

Neither Line 1 nor Line 2 exhibited significant differences in time spent in the center of the OF or on the light side of the LD test. However, there were significant differences between lines in locomotor behavior on these tests. In the OF, there was a significant effect of line on distance traveled in the OF and center-periphery transitions in the OF (distance: $F(2,56)=11.0$, $p < 0.0001$; transitions: $F(2,56)=10.3$, $p < 0.0001$). Both Lines 1 and 2 showed less locomotion in the OF as compared to B6 littermates (Line 1 vs. B6 $p < 0.0001$; Line 2 vs. B6 $p < 0.05$; Figure 2A). For transitions from the center to the periphery in the OF (Figure 2B), Line 1 performed fewer transitions than either B6 ($p < 0.0001$) or Line 2 mice ($p < 0.05$). For the LD test, there was a significant effect of line on transitions between the dark box and the illuminated side of the test arena ($F(2,46)=6.0$, $p < 0.005$). As with the OF test, mice from Line 1 had significantly fewer transitions than either B6 ($p < 0.005$) or Line 2 mice ($p < 0.05$; Figure 2C).

### Bioinformatic analyses, haplotype association mapping and quantitative PCR

Even though congenic mice reduced the QTL confidence interval from 10.1 Mb to 6.7 Mb (Line 1) or 1.8 Mb (Line 2), there remained many candidate genes in the congenic region.

We used high density sequence data provided by the Welcome Trust Sanger Institute to search for genes within the congenic interval that possessed nonsynonymous coding polymorphisms. Of the 109 genes in the 6.7 Mb congenic region, only 14 contained nonsynonymous coding SNPs, one of which was Shmt2 (serine hydroxymethyltranferase 2). Supplemental table 1 lists the location, gene names, gene symbols, and number of coding SNPs per gene within the congenic region. Using haplotype association mapping, we identified 12 genes that were differentially expressed between B6 and AJ haplotypes in hippocampus and/or amygdala (see Table 2) for the whole 6.7 Mb congenic region; only one of these genes (*Rnf41*) has previously been implicated in anxiety-like behavior (Ponder *et al.* 2008; Kim *et al.* 2009). (Another gene (*Grip1*) which was also identified as being differentially expressed in Ponder *et al*. (2008), was outside of the congenic region and thus appears to be unlikely to cause this QTL.) We used qPCR to further evaluate 10 of these 12 genes identified by haplotype association mapping. The two other genes (D30004K10Rik and D10Ert610e) were not tested because we could not successfully design primers; their annotations also raised some suspicion about whether these are real genes. For the remaining ten genes that we did examine using qPCR, two genes were differentially expressed in hippocampal tissue of congenic mice (Table 2). *Shmt2* showed significantly higher expression in hippocampal tissue from mice with a B6 genotype (B6 and Line 2 mice) than mice with an AJ genotype (Line 1, $p < 0.01$; Table 2). *Rnf41* (ring finger protein 41) also showed significantly higher hippocampal expression in mice with a B6 genotype than mice with an AJ genotype (Lines 1 and 2, $p < 0.0001$; Table 2).

## Discussion

In the present study, we created an $F_2$ population derived from a cross between CSS-10 x B6 mice to replicate and fine map a highly significant QTL for CF on chromosome 10. Using this $F_2$ population we identified significant, overlapping QTLs on the distal end of chromosome 10 for increased freezing at multiple traits related to CF (Figure 1A). We then created two congenic lines to further dissect the QTL region (Figure 1B). The results from the congenic lines suggested that the QTL observed in the $F_2$ population was due to multiple smaller QTLs. Line 1 showed enhanced freezing to the first tone as compared to Line 2 and B6 mice (Figure 1C). This is interesting, given that the enhanced freezing to the first tone occurs before the tone has been paired with the shock. This suggests an unlearned fear of a mild, non-aversive stimulus. However, Line 1 congenics did not show anxiety-like behavior in the OF and LD tests (Figure 2). When considering both tones on day 1 (Figure 1D), Line 2 also showed significantly enhanced freezing as compared to B6 mice, but freezing behavior was still lower than Line 1. Freezing in response to both tones on day 1 may reflect rapid learning that presumably occurs before the consolidation of any fearful memories. Line 1 had greater freezing to context on day 2 than Line 2 or B6 and all three genotypes showed different freezing in response to the cues on day 3. In summary, Line 1 captured all of the QTL identified in the CSS-10 and $F_2$ mice, while Line 2 captured some but not all of the QTL alleles.

The primary difference in OF and LD presented here (Figures 2) was a reduction in locomotor behavior, which has been reported before for similar congenic strains (Kim *et al.* 2009; Zhang *et al.* 2005) and is not inconsistent with the findings of Singer *et al.* (2005). In a series of studies, interval specific congenic strains (ISCS) derived from AJ and B6 mice implicated the distal portion of chromosome 10 as being involved in significant differences in open field activity (Gershenfeld *et al.* 1999; Zhang *et al.* 2005; Kim *et al.* 2009). Line 1 did not differ in more commonly accepted measures of anxiety-like behavior such as the % time spent in the center of the OF or the % time spent on the light side of the LD box. However, decreased locomotor activity on these tasks may also reflect increased anxiety-like behavior. For example, Milner & Crabbe (2008) used a principal components analysis

to demonstrate that variables associated with both activity and anxiety-like behaviors loaded onto one factor. Along these lines, Kim *et al.* (2009) interpreted differences in open field behavior as reflecting anxiety-like behavior. This same region of chromosome 10 has also been associated with differences in obesity, liver triglycerides, blood glucose, and plasma cholesterol (Shao *et al.* 2008, 2010). The present findings indicate that this region also influences fear- and activity (possibly anxiety-like behavior) and further emphasizes the pleiotropic nature of this locus; although until causal genes are identified it remains unclear whether these behavioral differences are due to a single allele or more than one closely linked but different alleles.

In an effort to identify genes within the congenic regions that influenced conditioned fear, we sought to identify eQTLs in this interval by using haplotype mapping. We identified 12 putative genes differentially expressed in hippocampus or amygdala when comparing AJ and B6 haplotypes, two of which were confirmed by qPCR using mRNA from brains of the relevant congenic strains (Table 2). Consistent with previous expression results (Ponder *et al.* 2008; Kim *et al.* 2009; Ponder *et al.* 2008), *Rnf41* was identified as a significant eQTL in both brain regions ($-\log P > 5.7$). Both congenic lines carried the AJ allele for *Rnf41* which is associated with lower *Rnf41* expression compared to the B6 allele (Table 2). Hippocampal *Rnf41* gene expression levels have been significantly correlated with distance traveled in the open field in the Long Sleep × Short Sleep Recombinant Inbred Panel and *Rnf41* has been reported to have greater expression in postmortem human control brains as compared to patients diagnosed with bipolar disorder and major depressive disorder (Kim *et al.* 2009). However, because Line 1 and 2 have different CF phenotypes but share identical *Rnf41* alleles, it is clear that the eQTL for *Rnf41* cannot explain all of phenotype associated with the QTL on distal chromosome 10.

Another gene was differentially expressed only in the Line 1 congenic mice (*Shmt2;* Table 2). Serine hydroxymethyltransferases (SHMTs) are pyridoxalphosphate-dependent enzymes responsible for catalyzing, among other things, serine to glycine and are found throughout the brain (Dasgupta & Narayanaswami 1982; Stover *et al.* 1997). Although no role for *Shmt2* has previously been proposed for fear or anxiety, glycine has been suggested to play a role in fear extinction. For example, d-cycloserine and d-serine, partial agonists at the glycine site of the NMDA receptor, significantly increase extinction in fear-potentiated startle paradigms and auditory fear conditioning (Walker *et al.* 2002; Matsuda *et al.* 2010). In fact, d-cycloserine has also been shown to be efficacious in successful outcomes from exposure therapy in humans as well (see Norberg *et al.* 2008 for review). In our congenic mice, *Shmt2* exhibited significantly higher expression in mice with the B6 allele. The eQTL for *Shmt2* could explain the stronger phenotype observed in Line 1 since Line 1 but not Line 2 had the AJ allele of *Shmt2*.

Our study is not without limitations. For example, we focus only on annotated genes with known effects such as cis-regulated expression or non-synonymous SNPs. Clearly, there are other elements of the genome that could be the basis of the QTL(s). Overall, these results illustrate a common problem when using congenics to fine-map QTLs identified by coarse-mapping methods such as CSS or $F_2$ populations: the disintegration of large QTLs into multiple smaller QTLs. Note that we have carefully examined the boundary regions of Line 1 and 2 to eliminate the possibility that Line 2 showed a weaker phenotype because the boundary of the congenic segment was not the same in all mice such that some but not all mice from Line 2 captured a QTL allele. We defined the proximal boundary of Line 2 using two markers that are 14 Kb apart (the first indicated the segment was from B6, the second indicated the segment had transitioned to AJ). Thus, unless the QTL allele lies in this 14 Kb interval, we can confidently interpret these results to mean that Lines 2 captures at least one QTL but does not contain at least one other QTL that was captured by Line 1. While not

frequently reported with such precision, it is widely believed that this is a common outcome for congenic studies. We recently reported on another way in which congenic studies can sometimes fail: as we fine-mapped a small interval on chromosome 11 that was associated with the locomotor response to methamphetamine, we found that at least two loci were simultaneously required to observe any phenotypic difference from the pure background strain. After carefully eliminating other explanations, we concluded that two closely linked alleles were interacting epistatically, such that neither one produced any phenotype by itself (Bryant *et al*. 2012). Indeed, while there are some notable successes (Phelan *et al.* 2002; Shirley *et al.* 2004; Ferraro *et al.* 2004; Tomida *et al.* 2009), the congenic approach does not commonly lead to definitive gene identification in rodents, in part because of the limitations described here. As we have noted previously (Parker & Palmer 2011), it may be better to begin by fine mapping with a highly recombinant mapping populations such as an advanced intercross line, heterogeneous stock, or outbred mice. The strongest QTLs identified using those fine mapping populations are more likely to be tractable and can sometimes lead to definitive gene identification in a single step, thus avoiding time- and resource-intensive fine-mapping with congenics. Despite these considerations, in this paper we have successfully fine-mapped QTLs to small intervals and identified candidate eQTLs that might mediate these effects.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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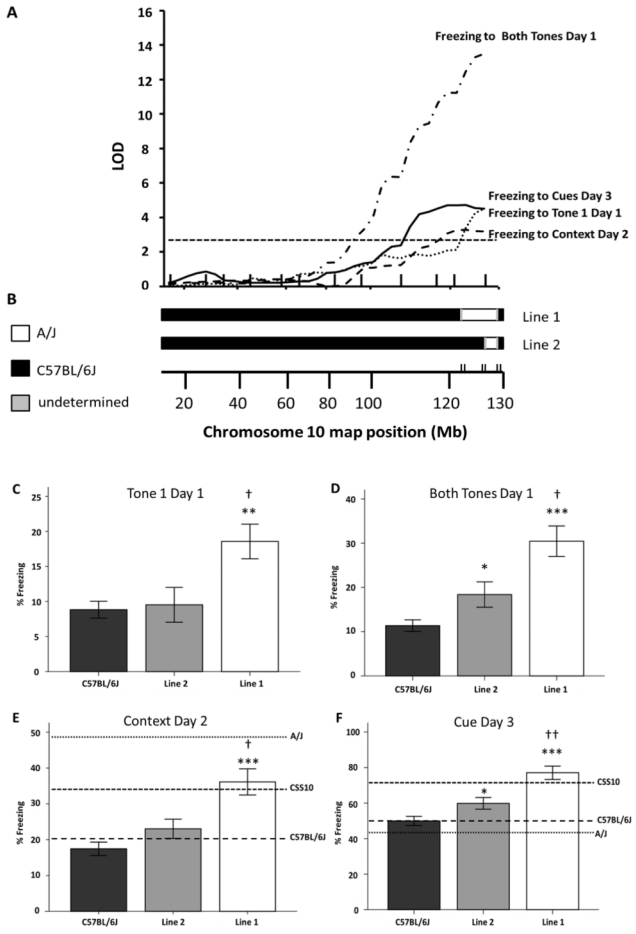


**Figure 1.**

**(A)** Significant and overlapping QTL results for freezing to tone 1 day 1 (LOD = 4.5), freezing to both tones day 1 (LOD = 13.5), freezing to context day 2 (LOD = 3.3) and freezing to cues day 3 (LOD = 4.7) in CSS10 $F_2$ mice. $P < 0.05$ significance threshold of LOD = 2.4 is indicated by the horizontal dotted line (LOD thresholds for each trait ranged from 2.31–2.37 LOD). **(B)** Congenic regions for Line 1 and Line 2. Regions on chromosome 10 inherited from AJ are indicated in white, and those from B6 are indicated in black, the gray region is the unresolved interval between two markers and is shown as being much larger than it really is for clarify; actual unresolved intervals are given in the text. Tick marks indicate the location of markers that defined the congenic boundaries and are also shown as being much farther apart than they really are. **(C–F)** Freezing behavior in Line 1 and 2 congenic mice and B6 littermates for freezing to tone 1 day 1 (**C**), freezing to both tones day 1 (**D**), freezing to context day 2 (**E**), and freezing to cue day 3 (**F**). Dashed lines indicate the values for freezing to context day 2 and freezing to cue day 3 values of AJ, B6 and CSS10 mice from Ponder *et al.* (2007a). **Significantly different from B6; $p < 0.005$, †Significantly di3erent from Line 2; $p < 0.05$, ††Significantly different from Line 2; $p < 0.005$.

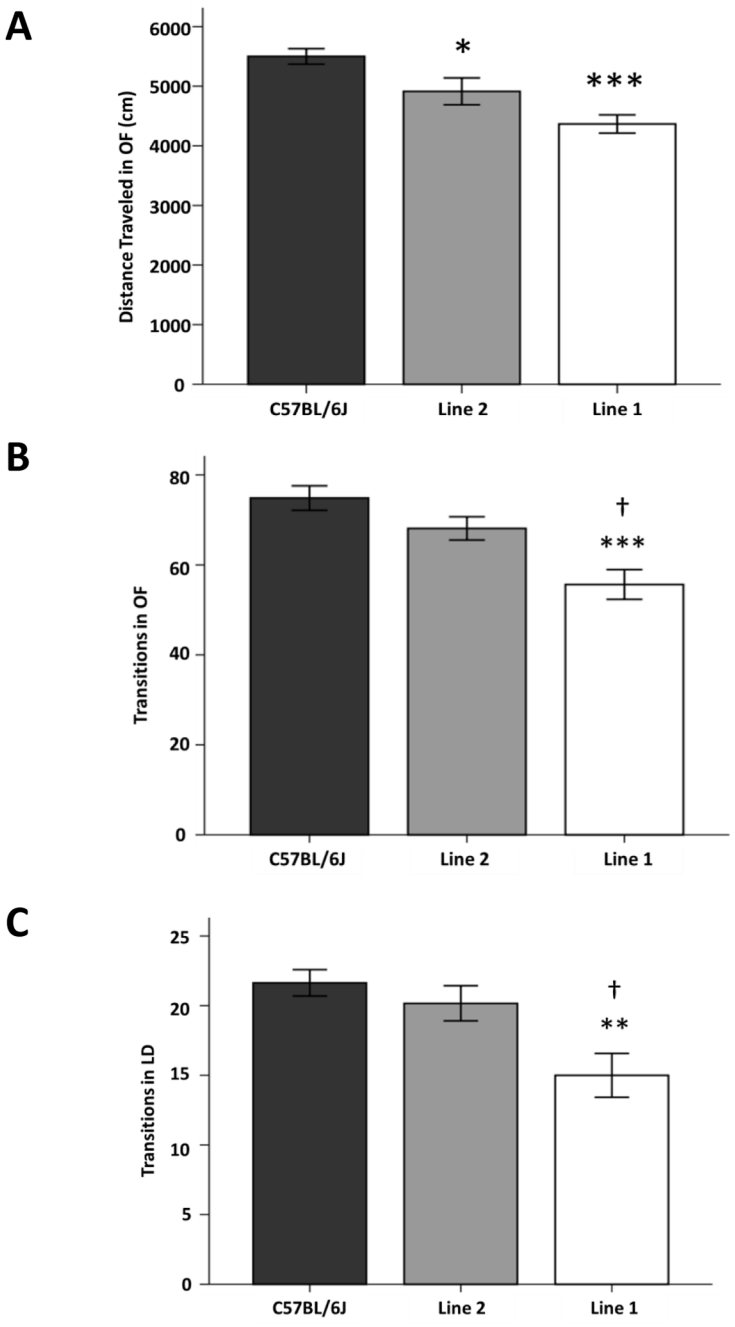


**Figure 2.**

Locomotor activity in the open field and light dark box in congenic mice and littermates. Mice from Line 1 showed less locomotor activity as measured by (**A**) distance traveled in the open field, (**B**) by a lower number of transitions between the center and periphery in the open field, and (**C**) by a lower number of transitions between the dark box and the illuminated side of the test arena. *Significantly different from B6; $p < 0.05$, **Significantly different from B6; $p < 0.005$, ***Significantly different from B6; $p < 0.0001$, †Significantly di3erent from Line 2; $p < 0.05$.

**Table 1**

QTL analysis of Conditioned Fear in CSS10$F_2$ mice

| Behavior | QTL Peak LOD | QTL Interval (Mb) |
|---|---|---|
| Tone 1 Day 1 | 3.99 | 121262479–127659097 |
| Avg. Tone Day 1 | 13.49 | 121262479–127659097 |
| Context | 3.29 | 117560107–127659097 |
| Avg. Tone Day 3 | 4.73 | 110233294–127659097 |

## Table 2

Haplotype Association Mapping eQTL Chromosome 10: 122387121–129068183Mb (Build 37)

| Gene ID | Probe ID | Position (Mb) | Hippocampus −log(p) | Amygdala* −log(p) |
|---|---|---|---|---|
| *Line 1 only* | | | | |
| Mon2 | 1440027_at | 122395119 | 4.60# | - |
| Usp15 | 1436891_at | 122552805 | 3.75 | - |
| D630004K10Rik | 1440840_at | 124429925 | 5.05 | - |
| Slc16a7 | 1448502_at | 124624199 | 6.00# | 4.21# |
| Xrcc6bp1 | 1453380_a_at | 126170029 | 5.52 | - |
| D10Ertd610e | 1419978_s_at | 126810840 | - | 3.67 |
| Tspan31 | 1416556_at | 126907352 | - | 3.90 |
| **Shmt2** | **1455084_x_at** | **126984762** | - | **4.85** |
| *Lines 1 and 2* | | | | |
| Pan2 | 1426700_a_at | 127686450 | 4.32 | - |
| Cnpy2 | 1416507_at | 127731442 | 4.74# | 4.15# |
| **Rnf41** | **1432003_a_at** | **127789526** | **5.70#** | **6.00#** |
| Myl6 | 1424269_a_at | 127882140 | - | 3.54 |

**Bold** indicates eQTLs verified using qPCR in hippocampal tissue of naïve congenic and wildtype mice. Genes are divided into those present in Lines 1 and 2 and those unique to Line 1 based on position.

# eQTL significant in both males and females according to the haplotype association mapping; the higher log(p) value is given.

* Only males showed significant eQTL according to the haplotype association mapping for the indicated tissue.