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Genome-Wide Association for Fear Conditioning in an Advanced Intercross Mouse Line

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Abstract

Fear conditioning (FC) may provide a useful model for some components of post-traumatic stress disorder (PTSD). We used a C57BL/6J × DBA/2J F_2 intercross (n = 620) and a C57BL/6J × DBA/2J F_8 advanced inter-cross line (n = 567) to fine-map quantitative trait loci (QTL) associated with FC. We conducted an integrated genome-wide association analysis in QTLRel and identified five highly significant QTL affecting freezing to context as well as four highly significant QTL associated with freezing to cue. The average percent decrease in QTL width between the F_2 and the integrated analysis was 59.2%. Next, we exploited bioinformatic sequence and expression data to identify candidate genes based on the existence of non-synonymous coding polymorphisms and/or expression QTLs. We identified numerous candidate genes that have been previously implicated in either fear learning in animal models (*Bcl2*, *Btg2*, *Dbi*, *Gabr1b*, *Lypd1*, *Pam* and *Rgs14*) or PTSD in humans (*Gabra2*, *Oprm1* and *Trkb*); other identified genes may represent novel findings. The integration of F_2 and AIL data maintains the advantages of studying FC in model organisms while significantly improving resolution over previous approaches.

Keywords

Advanced intercross lines; Fear conditioning; Fear learning; Genome-wide association; Post-traumatic stress disorder; Quantitative trait loci

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Introduction

Translational mouse models have provided a useful strategy for understanding the genetic and biological underpinnings of the acquisition of fear and phobias (Carey 1990), as well as the etiologic processes related to fear and anxiety. Despite some limitations (Layton and Krikorian 2002), the classical conditioning paradigm known as fear conditioning (FC) has been commonly used to model various components of post-traumatic stress disorder (PTSD; Fanselow and LeDoux 1999; Amstadter et al. 2009; Jovanovic and Ressler 2010; Johnson et al. 2011). FC is a form of Pavlovian learning in which an aversive unconditioned stimulus (US) is paired with a previously neutral conditioned cue (CS). Following training, recall of the fearful memory is measured by observation of freezing, a species-specific response to fear. Freezing is used to measure fear of the CS or fear of the context in which the fearful memory was acquired. In contrast to most common tests of anxiety-like behaviors in mice, FC is highly conserved across species, is exhibited in both laboratory and natural environments, and can easily be measured in humans; and may thus be a useful intermediate phenotype for aspects of PTSD (LaBar et al. 1995; Amstadter et al. 2009). For example, the sight, sound, and smell of traumatic events become potent memories through which Pavlovian fear is acquired in both PTSD and FC (Johnson and LeDoux 2004). Additionally, PTSD patients have been shown to be more “conditionable” than individuals without PTSD and take longer to extinguish fear (Peri et al. 2000; Blechert et al. 2005; Orr et al. 2000). Lastly, Pavlovian fear is heritable in both mice and humans (Wehner et al. 1997; Grillon et al. 1998; Hettema et al. 2003) and its neurological underpinnings have been well established (LeDoux 2000; Richardson et al. 2004).

Genetic mapping studies in mice have traditionally used recombinant inbred lines (RI), backcrosses (BC), F_2 intercrosses, short-term selected lines (STSL), consomic and congenic mice to identify quantitative trait loci (QTLs) for FC (Caldarone et al. 1997; Owen et al. 1997a, b; Wehner et al. 1997; Radcliffe et al. 2000; Ponder et al. 2007a; Brigman et al. 2009; Wilson et al. 2011; Sokoloff et al. 2011). Due to limited recombination, these techniques are only able to identify large genomic regions and are therefore sub-optimal for identifying the genes that underlie QTLs (Peters et al. 2007; Flint 2011; Parker and Palmer 2011). We have recently begun to address this limitation by using populations with greater numbers of accumulated recombinations such as advanced intercross lines (AILs; Cheng et al. 2010; Samocha et al. 2010; Lionikas et al. 2010; Parker et al. 2011a, b; Parker and Palmer 2011). AILs are created by successive generations of pseudo-random mating after the F_2 generation. Each additional generation leads to the accumulation of new recombinations, which allows for more precise mapping due to a breakdown in linkage disequilibrium. Because AILs are derived from two inbred founders, they maintain the simplicity of more traditional crosses, possess no rare alleles, and every marker perfectly discriminates between the two founder strains.

In the present experiment, we created an F_2 intercross and an F_8 AIL derived from C57BL/6J (B6) × DBA/2J (D2) mice. We chose B6 and D2 inbred mice as our progenitor strains in order to take advantage of the vast amount of bioinformatics resources associated with these strains and because these strains display robust differences in fear conditioning (Owen et al. 1997b; Ponder et al. 2007a). By combining GWAS with complementary bioinformatics resources available for B6 and D2 mice, we utilized sequence data to identify coding single nucleotide polymorphisms (SNPs), and gene expression data to identify putatively causal expression polymorphisms.

Materials and methods

Animals and housing

All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC) in accordance with NIH guidelines for the care and use of laboratory animals. Inbred female C57BL/6J (B6) and male DBA/2J (D2) mice were obtained from Jackson Labs (Bar Harbor, ME). These mice were used to produce the B6 × D2 F_1 mice, which were then bred to create the subsequent F_2 (308 male, 317 female) and F_8 (284 male, 283 female) generations. We have previously reported a genetic analysis of anxiety-like behavior and FC in the F_2 but not the F_8 mice (Sokoloff et al. 2011). Colony rooms were maintained on a 12:12 h light–dark cycle (lights on at 0630) in same-sex groups of two to five mice with standard lab chow and water available ad libitum. Mice were approximately 2–3 months of age at the start of testing (F_2 mean age = 86.6 days, SD = 7.8, range = 70–102; F_8 mean age = 74.0 days, SD = 6.4, range = 60–85).

Fear conditioning (FC)

FC procedures are identical to those described in Ponder et al. (2007a). Briefly, FC chambers were obtained from Med Associates (St. Albans, VT, USA). Chambers had inside dimensions of 29 cm × 19 cm × 25 cm with metal walls on each side, clear plastic front and back walls, clear plastic 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. Chambers were cleaned with 10% isopropanol between animals. Behavior was recorded with digital video and analyzed with FreezeFrame software from Actimetrics (Evanston, IL, USA).

Testing for FC consisted of a 5 min test that occurred three times over consecutive days during the light phase, between 0800 and 1,700 h (Fig. 1). Mice were transported from the adjacent vivarium and allowed to habituate to the procedure room for 30 min in their home cages. Mice were then transferred to the FC chambers in individual holding cages with clean bedding. On test day 1, mice were placed into the chamber. 180 s later, mice were exposed twice to the conditioned stimulus (CS), which consisted of an 85 dB, 3 kHz tone that persisted for 30 s and co-terminated with the unconditioned stimulus (US), which was a 2 s, 0.5 mA foot shock delivered through the stainless steel floor. After each CS-US pairing, there was a 30 s inter-trial interval (ITI). On day 1, two measures were calculated for QTL mapping: (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), and (2) time spent freezing to each CS presentation, calculated by averaging the percent time spent freezing to the tone presentations (180–210 s, 240–270 s; freezing to tone day 1).

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 (CS) nor shocks (US) were presented. On day 2, freezing to context was used for QTL mapping; this was defined as average percentage of time freezing in response to the test chamber during the same period of time as pre-training freezing (30–180 s; freezing to context). We chose this time period for QTL mapping to allow for direct comparisons to the freezing scores to tone day 1 and altered context day 3, and to avoid measuring freezing behavior during the latter part of the trial in which the mice might have predicted 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 (CS) was presented at the same times as on day one, but no foot shocks (US) were paired with it. On day 3, freezing to cue was defined as the average percent time spent freezing during the two 30 s CS presentations (180–210 s, 240–270 s; freezing to cue) and used for QTL mapping.

Data analysis

Initial analyses began with an independent samples *t* test to assess percent freezing differences between the F_2 and F_8 AIL populations. All analyses were conducted in PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

Genotyping

DNA from the F_2 generation was extracted and genotyped by KBiosciences (Hoddesdon, Hertfordshire, UK) using KASPar, a fluorescence-based PCR assay. Markers consisted of 164 polymorphic SNPs selected from Petkov et al. (2004). The average distance between informative markers inthis panel was 15.78 Mb, range = 2.68–44.34 Mb. DNA from the F_8 AIL was extracted using a salting out protocol and was genotyped using the Illumina Mouse Medium Density Linkage Panel (Illumina, San Diego, CA, USA) at the Genomics Core Facility at Northwestern University (http://web.cgm.northwestern.edu/cgm/Core-Facilities/Genomics-Core). The SNP panel consists of 1,449 SNPs, 1,060 of which were polymorphic between B6 and D2 mice. In our population, the average distance between markers was 2.77 Mb, range = 0.13 Mb to 23.27 Mb.

QTL mapping

Freezing data was converted to z-scores prior to genome-wide analysis. *G*enome-wide association analysis was performed in the combined population of the F_2 and F_8 AIL using the R package QTLRel (http://cran.r-project.org/web/packages/QTLRel/index.html). This software accounted for the complex relationships (e.g. sibling, half-sibling, cousins) among the F_8 mice by using a mixed model as described previously (Cheng et al. 2010, 2011). For each analysis, $P < 0.05$ genome-wide significance thresholds were estimated using 1,000 permutations. Sex was included as an interactive covariate.

Bioinformatic analyses

The GeneNetwork mapping module (www.genenetwork.org; Wang et al. 2003; Chesler et al. 2004) was used to identify expression QTLs (eQTLs) in whole brain, amygdala, and hippocampal mRNA from B6 × D2 F_2 and B6 × D2 RI mice that co-mapped to our behavioral QTLs (whole brain accessed on April 27, 2011, database: OHSU/VA B6D2F2 Brain mRNA Affymetrix M430 (Aug 05) RMA; Hitzemann et al. 2004, Hofstetter et al. 2008; amygdala accessed on June 28, 2011, database: INIA Amygdala Cohort Affymetrix Mouse Gene 1.0ST (Mar11) RMA; Mozhui and Williams unpublished data, hippocampus accessed on June 28, 2011, database: Hipp Consortium Affymetrix M430 (June 06) PDNN; Overall et al. 2009). Next, in order to narrow the list of candidate genes within the QTL intervals, we used high density sequence data provided by the Welcome Trust Sanger Institute (accessed on October 25th, 2011; 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 D2 mice. These strains were sequenced to an average of 25× coverage on the Illumina GAII platform (Illumina, San Diego, CA, USA) with a mixture of 54, 76, and 108 bp paired reads. We used this data to search for genes within the QTL intervals that possessed “consequential” polymorphisms

between B6 and D2 mice (such as nonsynonymous coding SNPs, stop-gain SNPs, stop-loss SNPs, SNPs resulting in frameshifts and SNPs located in essential splice sites).

Results

Phenotypic analysis

The F_2 and F_8 AIL differed from each other for pre-training freezing (Fig. 1a, F_2 mean = 2.6%, SD = 2.6%; Fig. 1b, F_8 mean = 6.4%, SD = 7.2%; $F_{1,\ 1,172} = 146.22$; $P < 0.0001$). The two populations did not differ significantly in their freezing to context (Fig. 1c, F_2 mean = 29.88%, SD = 18.6%; Fig. 1d, F_8 mean = 23.68%, SD = 19.39%) or in freezing to cue (Fig. 1e, F_2 mean = 52.16%, SD = 20.84%; Fig. 1f, F_8 mean = 52.53%, SD = 21.36%). The slight disparity in pre-training freezing between populations may be due to the segregation of alleles associated with freezing behavior during the creation of the F_8 AIL mice, or the result of handling effects of different testers across the ~2 year period between the F_2 and F_8 generations. As a result of these differences, both the F_2 and the F_8 AIL data were converted to *z* scores prior to genome-wide analysis in order to control for pre-training freezing differences that might otherwise be interpreted as differences in FC. Figure 1 displays the percent freezing across all 3 days in both the F_2 and F_8 AIL mice.

QTL mapping

We performed genome-wide analysis on the integrated B6 × D2 F_2 and B6 × D2 F_8 populations for pre-training freezing, freezing to tone/shock day 1, freezing to context, and freezing to cue. Using 1,000 permutations, significance thresholds for these traits were determined to range from 3.92 to 4.03 LOD. No QTLs reached genome-wide significance for pre-training freezing. We identified two QTLs associated with freezing to tone day 1 (on chromosomes 1 and 13), five QTLs associated with freezing to context (on chromosomes 1, 2, 5, 10, and 13) and four QTLs associated with freezing to cue (on chromosomes 1, 2, 5 and 13). Figures 2, 3, 4 and 5 display the results of the integrated analysis for pre-training freezing, freezing to tone day 1, freezing to context and freezing to cue, respectively. The 1.5-LOD support intervals for these QTL ranged from 6.2 to 39.4 Mb, with an average 1.5-LOD support interval of 26.3 Mb (Table 1).

Bioinformatic analyses

Numerous genes were identified whose mRNA expression co-mapped to the behavioral QTLs for freezing to tone day 1 (Supplementary Table 1), freezing to context (Supplementary Table 2) or freezing to cue (Supplementary Table 3). Many of these differentially expressed genes have been previously reported in mice selectively bred for differences in contextual fear conditioning (Ponder et al. 2007a, b, 2008) and are listed in italics in the supplementary tables. Others have been implicated in fear learning in other animal models and/or PTSD in human subjects: B-cell leukemia/lymphoma2, *Bcl2*, (Ding et al. 2010; Li et al. 2010; Liu et al. 2011); B-cell translocation gene 2, anti-proliferative, *Btg2*, (Farioli-Vecchioli et al. 2009; Kurumaji et al. 2008); diazepam bindinding inhibitor, *Dbi*, (Katsura et al. 2002; Sherrin et al. 2009); gamma-aminobutyric acid (GABA) A receptor, subunit α-2, *Gabra2*, (Nelson et al. 2009); Ly6/Plaur domain containing 1, *Lypd1*, (Tekinay et al. 2009); μ-opioid receptor 1, *Oprm1*, (Pitman et al. 1990; Glover 1993; Good and Westbrook 1995; Liberzon et al. 2007); peptidylglycine alpha-amidating monooxygenase, *Pam*, (Gaier et al. 2010); neurotrophic tyrosine kinase receptor type 2, *Trkb*, (Takei et al. 2011). We then examined our 1.5-LOD support interval for the presence of “consequential” SNPs that had the potential to directly alter proteins (i.e. nonsynonymous coding, stop-gain, stop-lost, frameshift, splice sites). Numerous SNPs were identified in genes with known relevance to fear learning and/or PTSD: *Cdh7*, (Ponder et al. 2007a); *Trkb*, (Givalois et al. 2001; Kozlovsky et al. 2007); gamma-aminobutyric acid (GABA) A receptor, subunit β-1,

Gabrb1, (Ciocchi et al. 2010); regulator of G-protein signaling 14, *Rgs14*, (Lee et al. 2010). Supplemental Tables 4, 5, and 6 list the location, gene names, gene symbols, and number of coding SNPs per gene for freezing to tone day 1 (Supplemental Table 4), freezing to context (Supplemental Table 5) and freezing to cue (Supplemental Table 6).

Discussion

We performed genome-wide mapping of QTL underlying fear conditioning in an F_2 and F_8 AIL mouse population. We identified two QTLs associated with freezing to tone on day 1 (on chromosomes 1 and 13), five QTLs associated with freezing to context (on chromosomes 1, 2, 5, 10, and 13) and four QTLs associated with freezing to cue (on chromosomes 1, 2, 5, and 13). No QTLs reached significance for baseline freezing measures. Some of the QTLs we identified were concordant with QTLs implicated in previous studies, although the direction of the allelic effect was not always replicated. For example, both we and Wehner et al. (1997) identified a QTL on chromosome 10 for freezing to context, and the B6 allele was consistently associated with increased freezing. This effect was not observed in the chromosome 10 QTL reported by Ponder et al. (2007a) in which the A/J, not the B6 strain, was responsible for increased freezing. The discrepancies may be due to extremely high freezing observed in A/Js (such that A/J > B6 > D2 freezing) or there maybe be two different alleles in this region of chromosome 10, such that B6 ≠ D2 at locus 1 and B6 ≠ AJ at locus 2. Two other QTLs we observed for freezing to context (on chromosomes 2 and 13) were also described in STSL mice derived from B6 × A/J (Ponder et al. 2008), but the direction of the effect was not reported. Importantly, the AIL provided significantly greater resolution and narrower support intervals as compared to the F_2, CSS, and STSL mice.

Both contextual and cued fear mapped to similar chromosomal regions, despite known differences in their neuroanatomical substrates (Fanselow and LeDoux 1999; Jovanovic and Ressler 2010). This may indicate the presence of alleles that influence both traits; alternatively it could be due to different alleles that are located closely to each other in the genome. In support of the former explanation, we recently performed QTL mapping and factor analysis on numerous anxiety and FC traits in the same F_2 B6 × D2 mice used in this study (Sokoloff et al. 2011). While factor analysis suggested that contextual and cued fear learning loaded onto separate factors, QTL mapping tended to identify the same QTLs. Talbot et al. (2003) reported that contextual and cued fear were highly correlated (r = 0.63) in HS mice, and others (Radcliffe et al. 2000; Ponder et al. 2007b) have observed that selection for freezing to context caused coincident changes in freezing to cue. Thus, it is likely that contextual and cued fear are modulated by some of the same alleles, but only gene identification can definitively show this to be the case. The chromosome 1 QTL for freezing to the tone/shock on day 1 also overlapped with the cued fear QTLs and the chromosome 13 QTL for freezing to tone/shock on day 1 overlapped with both contextual and cued QTLs. These QTLs may reflect an acute fear response that is distinct from the learned, conditioned response. In order to more closely examine this, we compared QTL mapping results from freezing to the first tone/shock pairing versus freezing to the second tone/shock pairing on day 1. The chromosome 1 QTL for freezing behavior to the tone/shock pairing on day 1 was observed in both the first and second tone/shock pairing (Tone 1 LOD score = 6.09, Tone 2 LOD score = 11.36), but the chromosome 13 QTL for freezing behavior was only seen for the second tone/shock pairing (Tone 1 LOD score = 2.65, Tone 2 LOD score = 4.98). In addition, we observed a QTL on chromosome 11 that was specific to freezing to Tone 1 and not Tone 2. Because freezing to tone 1 occurs before any shocks have been presented, the QTLs on chromosome 1 and 11 represent a response to the tone itself, whereas the chromosome 13 QTL detected during tone 2 reflects a conditioned response. Earlier work in our lab (Sokoloff et al. 2011) has shown a dissociation of the day 1

freezing response from the day 2 and day 3 freezing responses through factor analysis, but not through QTL mapping in the B6 × D2 F_2 mice. Bush et al. (2007) examined the dissociation of fear reactivity (initial fear response) from fear recovery (conditioned fear response) in outbred rats. They reported that rats displaying the highest levels of freezing behavior during fear conditioning continued to have significantly increased levels of CS-elicited fear in subsequent tests, but that it did not predict fear recovery as measured by freezing during extinction. Studies of fear conditioning in humans have also begun to investigate the genetics of individual differences in the acquisition of fear memories (Hettema et al. 2003).

To further narrow our list of candidate genes we used a series of bioinformatic approaches. First, we identified eQTLs that co-mapped with our QTLs. eQTLs are genomic loci that regulate gene transcription and expression on a genome-wide scale, and are believed to underlie many QTLs for complex traits (Nicolae et al. 2010; Li and Deng 2010). While co-mapping of a QTL and an eQTL does not constitute proof that the latter causes the former, it does suggests a clear and testable hypothesis—the candidate gene can be directly manipulated using a variety of molecular or pharmacological approaches. We used an existing database (http://www.GeneNetwork.org) of mRNA expression from amygdala, hippocampus and whole brain of untreated B6 × D2 F_2 or RI mice. This identified a number of genes whose expression co-mapped within the 1.5-LOD intervals of our QTLs (Supplemental Tables 1, 2, and 3). Many of these genes (*C1ql2*, *Cd59a*, *Cdh7*, *Fryl*, *Hdac4*, *Kit*, *Lypd1*, *Mcm6*, *Rab3gap1*, *Stk25*, *Slc35f5*, *Ubxd*, *Zfp71rs1*) have previously been shown to have differential expression in the hippocampus and/or the amygdala of B6 × D2 or B6 × A/J derived mice selectively bred for differences in contextual freezing (Ponder et al. 2007a, b, 2008).

We also identified differential expression of numerous genes that have been implicated in fear learning in other rodent models (Table 2). For example, expression of *Trkb* mapped to the QTL on chromosome 13 for freezing to context. Takei et al. 2011 recently demonstrated that *Trkb* signaling in the hippocampus is enhanced in response to fear conditioning and Musumeci et al. (2009) report that *Trkb* receptors modulate specific phases of fear learning and amygdalar synaptic plasticity. Another gene we identified in our eQTL region was *Bcl2*. Expression of *Bcl2* mapped to the QTL on chromosome 1 for freezing to cue, and hippocampal *Bcl2* expression is significantly upregulated in a rat model of PTSD (Li et al. 2010). We also found expression differences in *Btg2* within the QTL on chromosome 1 for freezing to cue. Farioli-Vecchioli et al. (2009) have reported impaired contextual fear conditioning in a *Btg2* null mouse model. Additionally, expression of *Dbi* mapped to the QTL on chromosome 1 for freezing to cue. *Dbi* is an anxiogenic neuropeptide whose expression increases following conditioned psychological stress and is functionally involved in hippocampal-dependent enhancement of contextual fear (Sherrin et al. 2009; Katsura et al. 2002). We also observed that expression of *Lypd1* mapped to the QTL on chromosome 1 for freezing to cue, and *Lypd1* knockout mice display increased cued fear conditioning (Tekinay et al. 2009). Lastly, expression of *Pam* mapped to the QTL on chromosome 1 for freezing to cue and mice heterozygous for the *Pam* gene are deficient in short- and long-term contextual and cued fear conditioning (Gaier et al. 2010).

Next, we detected expression differences in genes that have known relevance to PTSD in humans (Table 2). Expression of *Gabra2* co-mapped to the QTL for freezing to cue on chromosome 5. Nelson et al. (2009) reported three SNPs in *Gabra2* interacted with childhood trauma exposure to predict PTSD in adulthood. The expression of *Oprm1* co-mapped to the QTL on chromosome 10 for freezing to context. The opioid system has been implicated in the modulation of fear (Good and Westbrook 1995), and endogenous opioid abnormalities have been reported in patients with PTSD (Glover 1993; Pitman et al. 1990).

Furthermore, treatment with opioid antagonists reduces PTSD symptoms and central μ-opioid receptor binding is altered after psychological trauma (Liberzon et al. 2007). Lastly, *Trkb* (known to be important for fear learning in rodents) may also be functionally relevant to PTSD in humans. Induction of brain derived neurotrophic factor (BDNF) and activation of its intracellular receptor *Trkb* increases neural survival, synaptic transmission, long term potentiation and long term depression (Lipsky and Marini 2007). This has significant implications for memory formation in individuals with PTSD yet few studies have been conducted examining BDNF/Trkb in human populations. Dell'osso et al. (2009) reported significantly lower levels of plasma BDNF in patients with PTSD as compared with healthy individuals and human carriers of the $BDNF_{Met}$ allele displayed slower suppression of a learned fear response (Frielingsdorf et al. 2010), impaired fear extinction (Soliman et al. 2010), and abnormal fronto-amygdala activity (Soliman et al. 2010). However, Zhang et al. (2006) reported no association between three BDNF gene variants and PTSD in a sample of 96 cases and 250 control subjects.

Finally, we identified coding SNPs within each of our 1.5-LOD QTL intervals (Supplemental Tables 4, 5, and 6). Some of the genes within these regions had coding SNPs known to be involved in fear learning and/or PTSD (Table 2). *Cdh7* has two non-synonymous coding SNPs within the freezing to cue QTL on chromosome 1, and is thought to play a role in acquisition of fear memories, fear learning and regulation of the Pavlovian fear neural network through calcium mediated cell adhesion (Irvine et al. 2005; Ponder et al. 2007a, b; Johnson et al. 2011). *Trkb* has one non-synonymous coding SNP within the freezing to context QTL on chromosome 13. Trkb antagonists have anxiolytic properties in mice (Cazorla et al. 2011a, b), and *Trkb* has been implicated in the neuro-biological mechanisms underlying behaviorally induced stress associated with PTSD (Givalois et al. 2001; Kozlovsky et al. 2007). The gamma-aminobutyric acid (GABA) A receptor, subunit β-1 (*Gabrb1*) within the chromosome 5 QTL for freezing to cue contains a non-synonymous coding SNP. GABAergic neurotransmission in the amygdala have been shown vital in encoding of conditioned fear (Ciocchi et al. 2010) and alterations in GABA receptor levels are evident following fear conditioning (Chhatwal et al. 2005; Stork et al. 2002). We also identified non-synonymous coding SNPs in the regulator of G-protein signaling 14 (*Rgs14*) for both freezing to cue and freezing to context QTLs on chromosome 13. *Rgs14* is a natural suppressor of synaptic plasticity in hippocampal neurons as well as a suppressor of hippocampal-based learning and memory (Lee et al. 2010).

Our study has several important limitations. First of all, because we have used a cross between two inbred strains, we are studying the alleles that segregate between them and not the total number of alleles that segregate among all laboratory strains or wild mice. However, we did observe significant overlap in the QTLs we identified in our population with QTLs identified in other populations of mice, which is consistent with the idea that laboratory mice are segregating a relatively limited number of alleles (Yang et al. 2007, 2011). Additionally, our gene expression data was from brains of untreated mice. Thus, examining gene expression differences in mice that underwent the FC paradigm may provide useful information regarding gene expression levels following exposure to traumatic events. However, we have not taken that approach, in part because it is difficult to determine at what time(s) after treatment gene expression should be considered. Furthermore, we did not control for the effect of SNPs located within probes. Many groups have noted the highly significant overrepresentation of transcripts for which the additive allele effect is greater for the B6 than for the D2 strain (Chesler et al. 2005; Ciobanu et al. 2010; Radcliffe et al. 2006). This is most likely due to the fact that both the Affymetrix M430 and 1.0 ST probes were designed using sequence from B6 mice, and some of the *cis*-QTLs are artifacts of D2 polymorphisms occurring in probe-complimentary sequence (Radcliffe et al. 2006). Thus, a systematic imbalance exists in which some fraction of our cis-QTLs is likely caused by

SNPs that overlap probes rather than by genuine quantitative differences in mRNA levels. This is a particular concern for eQTLs in which the B6 allele shows higher expression. Unfortunately, even if one removes these probes from analysis, the bias in favor of B6 alleles remains; this may be due to the presence of unknown SNPs, isoform variation, and differences in alternative splicing, initiation, and termination of transcription (Ciobanu et al. 2010). Bias associated with array-based measures of expression may ultimately be resolved by a transition to next-generation sequencing of RNA samples, which allows for a more direct way to measure mRNA abundance (Ciobanu et al. 2010). In addition, a key component of PTSD is the failure to extinguish fearful associations (Amstadter et al. 2009; Johnson et al. 2011). Our FC paradigm focused on *acquisition* of fearful associations rather than *extinction*. Furthermore, the observed FC phenotype is the combined result of many other genetically influenced factors including anxiety, sensory modalities (nociception, auditory, visual, olfaction, tactile), learning and memory. Any number of these elements may be driving the association we have reported between the identified QTLs and their phenotypes. Finally, while the use of an AIL produced smaller QTL intervals, we did not obtain single gene resolution, which would clearly provide the most specific and actionable information.

In conclusion, we have mapped numerous QTLs associated with fear conditioning in an AIL. Some of the QTLs we identified correspond to QTLs identified by other researchers, and in the majority of cases we have narrowed the confidence intervals quite significantly as compared to those previous studies. The combination of high resolution mapping with sequence and expression data offers a powerful approach and permits identification of several candidate genes that may underlie differences in these phenotypes. This has allowed us to integrate multiple lines of complementary evidence from both the mouse and human literature to provide further support for particular candidates. In summary, by using an AIL we performed a GWAS in a situation where all alleles were common, and where uniform environmental conditions were maintained, which limited the interactions between genes and environment. These advantages allowed us to map QTL with a modest sample size and identify small regions that warrant further molecular evaluation.

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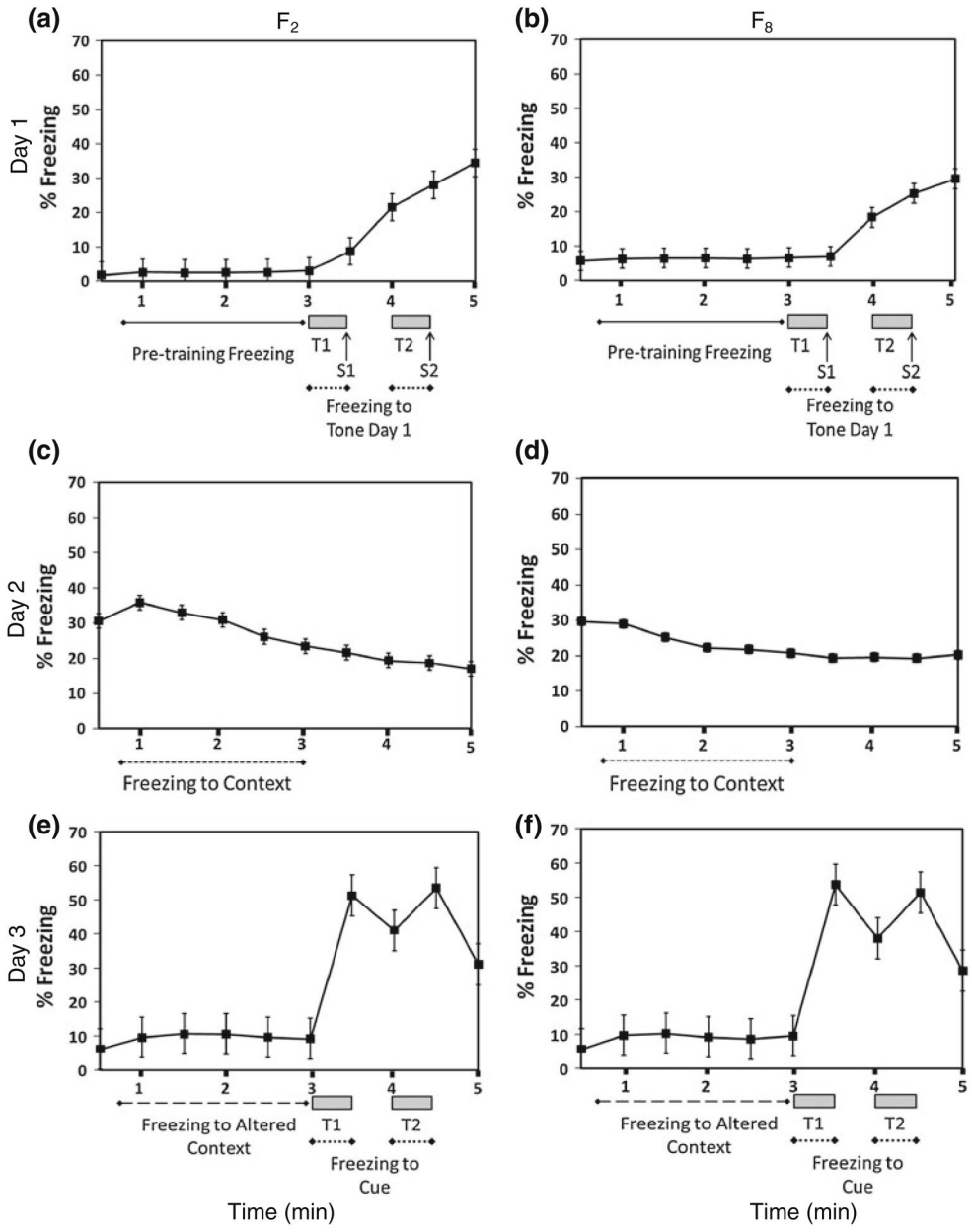

Fig. 1.

Fear conditioning in F_2 and F_8 mice. A three-day procedure was used to phenotype each subject. Each test lasted 5 min. On day 1, pre-training freezing was measured in F_2 (**a**) and F_8 (**b**) mice from 30 to 180 s, after which mice were exposed to two 30-s tones (indicated by hatched *bars*, labeled T1 and T2) that co-terminated with a 2-s, 0.5-mA foot shock (indicated by *arrows*, labeled S1 and S2). On day 2, freezing to context was measured in F_2 (c) and F_8 (d) mice from 30 to 180 s. On day 3, freezing to the altered context was measured in F_2 (e) and F_8 (f) mice from 30 to 180 s after which freezing to cue was measured (180–210 + 240–270 s); the time spent freezing to each tone was averaged to obtain the freezing to tone/cue variable. *Each data* point represents the average % freezing calculated across the 30 s time bin. *Error bars* represent the standard error of the mean

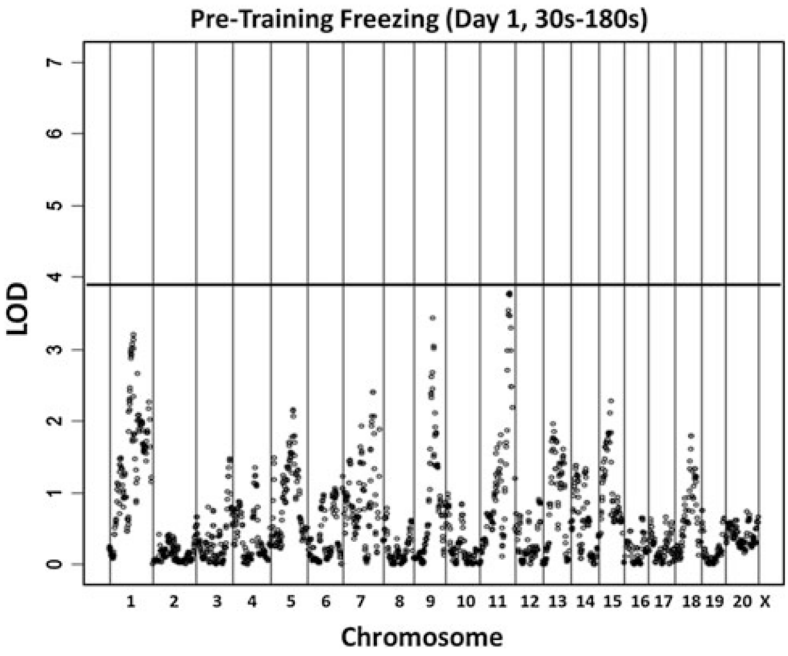

Fig. 2.
Integrated genome-wide results for percent freezing during pre-training (Day 1, 30–180 s; $P < 0.05$ significance threshold LOD = 3.92)

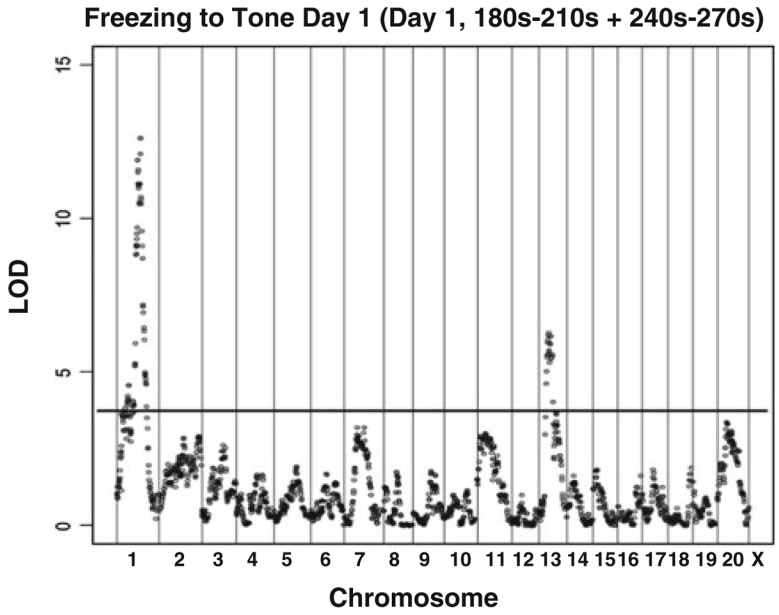

Fig. 3.
Integrated genome-wide results for percent freezing to tone day 1 (Day 1, 180–210 s, 240–270 s; $P < 0.05$ significance threshold LOD = 4.01)

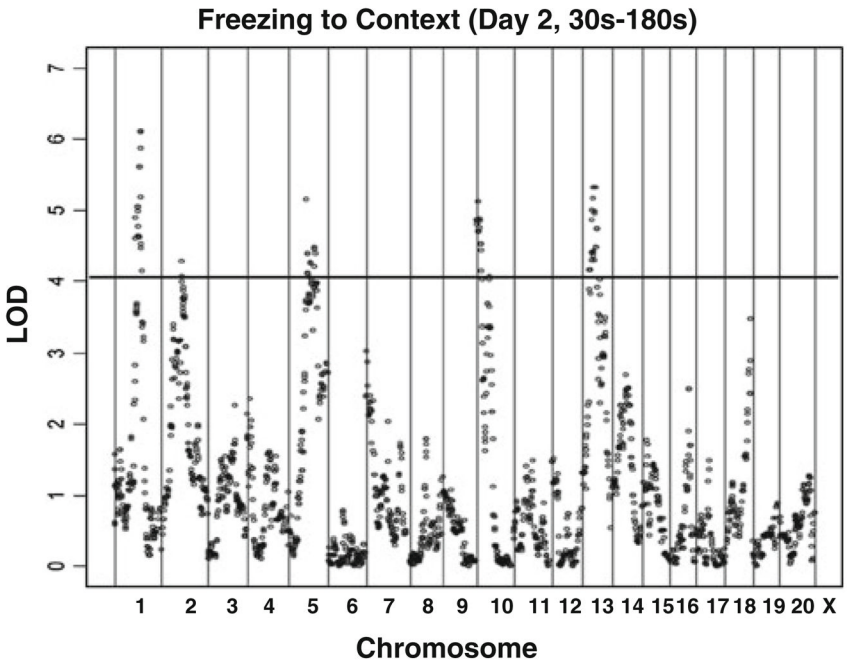

Fig. 4.

Integrated genome-wide results for percent freezing to context (Day 2, 30–180 s; $P < 0.05$ significance threshold LOD = 4.01)

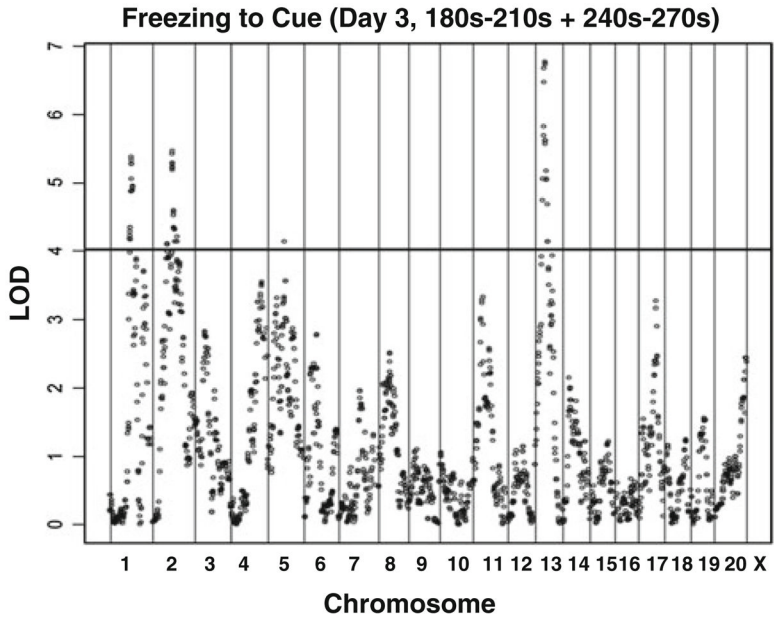

Fig. 5.

Integrated genome-wide results for percent freezing to cue (Day 3, 180–210 s + 240–270 s; $P < 0.05$ significance threshold LOD = 4.03)

Table 1

QTLs for freezing to tone day 1, freezing to context and freezing to cue

Behavior	Chr.	LOD	Peak LOD location (Mb)	1.5-LOD start (Mb)	1.5-LOD end (Mb)	Width (Mb)	SNP genotype with increased freezing
Freezing to tone day 1	1	14.0	109.515	104.264	127.206	22.94	D2
	13	6.4	55.240	46.459	73.083	26.62	D2
Freezing to context	1	6.9	132.415	129.953	136.107	6.15	D2
	2	4.6	91.483	73.315	107.506	34.19	B6
	5	5.3	76.761	73.058	112.437	39.38	D2
	10	4.8	12.931	3.470	27.855	24.39	B6
	13	5.2	67.311	40.134	73.083	32.95	D2
Freezing to cue	1	5.5	111.832	93.792	129.953	36.16	D2
	2	5.4	84.709	80.456	108.521	28.07	B6
	5	4.12	76.761	64.285	91.490	27.21	D2
	13	6.9	55.240	47.380	58.826	11.45	D2

Table includes chromosome, peak LOD score, peak SNP, Mb location and width of the QTLs as well as the SNP genotype with increased freezing

Table 2

Candidate genes

Gene	eQTL	Coding SNP	eQTL in selected lines	KO with abnormal FC phenotype	Implicated in rodent FC	Implicated in human PTSD
Bcl2	X				Li et al. (2010)	
Btg2	X			Farioli-Vecchioli et al. (2009)		
Cdh7		X	Ponder et al. (2007b)		Irvine et al. (2005)	
Dbi	X				Katsura et al. (2002) Sherrin et al. (2009)	
Gabra2	X					Nelson et al. (2009)
Gabrb1		X			Stork et al. (2002) Chhatwal et al. (2005) Ciocchi et al. (2010)	
Lypd1	X		Ponder et al. (2007b)	Tekinay et al. (2009)		
Oprm1	X				Good and Westbrook (1995)	Pitman et al. (1990) Glover (1993) Liberzon et al. (2007)
Pam	X			Gaier et al. (2010)		
Rgs14		X			Lee et al. (2010)	
Trkb	X	X			Musumeci et al. (2009) Takei et al. (2011)	Frielingsdorf et al. (2010) Soliman et al. (2010)

The gene symbol is listed, along with an “X” to indicate if it was an eQTL or a non-synonymous coding SNP. Citations are provided for genes that have been previously reported in selectively bred mice, if a knockout mouse exists with an abnormal FC phenotype, or if additional evidence from rodent or human studies provides support