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Identification of quantitative trait loci and candidate genes for an anxiolytic-like response to ethanol in BXD recombinant inbred strains

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Genetic differences in acute behavioral responses to ethanol contribute to the susceptibility to alcohol use disorder and the reduction of anxiety is a commonly reported motive underlying ethanol consumption among alcoholics. Therefore, we studied the genetic variance in anxiolytic-like responses to ethanol across the BXD recombinant inbred (RI) mouse panel using the light–dark transition model of anxiety. Strain-mean genetic mapping and a mixed-model quantitative trait loci (QTL) analysis replicated several previously published QTL for locomotor activity and identified several novel anxiety-related loci. Significant loci included a chromosome 11 saline anxiety-like QTL (*Salanq1*) and a chromosome 12 locus (*Etanq1*) influencing the anxiolytic-like response to ethanol. *Etanq1* was successfully validated by studies with BXD advanced intercross strains and fine-mapped to a region comprising less than 3.5 Mb. Through integration of genome-wide mRNA expression profiles of the mesocorticolimbic reward circuit (prefrontal cortex, nucleus accumbens and ventral midbrain) across the BXD RI panel, we identified high priority candidate genes within *Etanq1*, the strongest of which was *Ninein* (*Nin*), a *Gsk3β*-interacting protein that is highly expressed in the brain.

Keywords: Anxiety, BXD recombinant inbred, ethanol, microarray, Ninein, QTL

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Individual differences in acute behavioral responses to ethanol are reported to contribute to the susceptibility of ethanol abuse and alcoholism (Schuckit & Smith 1997), now collectively referred to as alcohol use disorder (AUD). Among the various acute ethanol behavioral responses, ethanol is a well-documented anxiolytic. Ethanol-induced anxiolysis has been observed using multiple rodent models of anxiety-like behavior, including the light–dark box (Bilkei-Gorzo *et al.* 1998; Boehm *et al.* 2002; Costall *et al.* 1988), elevated plus-maze (Boehm *et al.* 2002; LaBuda & Fuchs 2000), social interaction (Varlinskaya & Spear 2002) and mirrored chamber (Cao *et al.* 1993; Kliethermes *et al.* 2003). Human studies have shown that in addition to a high comorbidity between ethanol and anxiety (Bibb & Chambless 1986; Cornelius *et al.* 2003), the reduction of anxiety is a commonly reported motive among alcoholics (Conger 1956; Newlin & Thomson 1990; Pohorecky 1981). Therefore, anxiety is hypothesized as a risk factor in the initiation of ethanol abuse and recidivism. While much is known about neural pathways and signaling mechanisms involved in anxiety, the molecular mechanisms underlying the anxiolytic-like response to ethanol are still poorly understood.

Acute behavioral responses to ethanol are modified by environmental and genetic factors. Although the genetic component of numerous acute ethanol behaviors has been extensively studied, minimal genetic research has been conducted on ethanol-induced anxiolysis. A common approach to explore genetic contributions to complex polygenic traits in animal models has been through mapping quantitative trait loci (QTL) in recombinant inbred (RI) mouse strains (Crabbe *et al.* 1994; Peirce *et al.* 2004). This method has been widely used to identify anxiety-related QTL (Henderson *et al.* 2004) and loci involved in various acute behavioral responses to ethanol (Browman & Crabbe 2000; Buck *et al.* 1997; Cook *et al.* 2015; Crabbe 1998; Demarest *et al.* 1999, 2001; DuBose *et al.* 2013; Phillips *et al.* 1998). The BXD RI strains are of particular interest because of contrasting behavioral responses to ethanol between the C57BL/6J (B6) and DBA/2J (D2) inbred progenitor mouse strains. For example, while D2 mice show larger locomotor responses to acute ethanol, B6 mice consume more ethanol (Belknap *et al.* 1993; Lessov *et al.* 2001; Phillips *et al.* 1995). Additionally, because the genome of the B6 and D2 progenitor strains have been completely sequenced, the utilization of a full genome polymorphism database increases the precision with which one can identify candidate genes and polymorphisms within QTL.

Here, we initiated a genetic analysis of mechanisms underlying the anxiolytic effects of ethanol using the light–dark transition model to measure anxiety- and anxiolytic-like

behavior in response to saline or ethanol, respectively. The light–dark transition model was chosen because it shows predictive validity, particularly in regard to clinically used benzodiazepines, allows the simultaneous recording of locomotor activity and is potentially less influenced by activity differences and investigator bias (Crawley 1985). Furthermore, because the light–dark transition model of anxiety is based on an unconditioned fear paradigm, animal training was not required, thereby enabling high-throughput behavioral testing. Mounting evidence suggests that even similar behavioral models of anxiety identify different genetic aspects of anxiety-like behavior (Belzung & Le Pape 1994; Brigman *et al.* 2009; File 1995; Flint 2003; Milner & Crabbe 2008; Ramos *et al.* 1997; Turri *et al.* 2001). Thus, while employing only the light–dark transition model does not completely sample the genetic variation in anxiolytic-like responses to ethanol, the advantages of the model make it useful for an initial study on ethanol-induced anxiolysis.

Strain-mean genetic mapping and a mixed-model QTL analysis to identify genotype-by-treatment interactions enabled the detection of novel provisional QTL influencing anxiety-related behavioral responses, distinct from loci influencing locomotor activity. In particular, a significant QTL (*Etanq1*) modifying the anxiolytic-like response to ethanol was identified on mouse chromosome 12 and verified using BXD advanced recombinant inbred (ARI) strains (Peirce *et al.* 2004). Through integration of the B6 and D2 progenitor genome sequences and genome-wide transcription profiles for the prefrontal cortex (PFC), nucleus accumbens (NAc) and ventral midbrain (VMB), we identified candidate genes underlying *Etanq1*, the strongest of which was Ninein (*Nin*), a Gsk3β-interacting protein that is highly expressed in the brain.

Materials and methods

Experimental subjects

A total of 597 male mice across 38 strains (see Table S2, Supporting Information) were studied in three phases: provisional mapping, confirmation and fine-mapping. While sex-specific QTL are of significant interest to anxiety and behavioral responses to ethanol, only male mice were used for this initial assessment to reduce phenotypic variation of anxiety-related behaviors and to make the number of animals tractable in terms of manpower and expense (see *Discussion*). For the provisional phase, 448 mice from 27 BXD RI strains and their B6 and D2 progenitor strains were purchased at 8–9 weeks of age from the Jackson Laboratory (Bar Harbor, ME, USA). All animals were habituated to the animal facility for a minimum of 2 weeks prior to testing and housed four per cage with *ad libitum* access to standard rodent chow (catalog #7912, Harlan Teklad, Madison, WI, USA) and water in a 12 h dark/light cycle with lights on at 0600 h. Each BXD RI strain consisted of 6–8 mice per treatment and each progenitor strain had 13–16 mice per treatment. For the replication and fine-mapping analyses, we acquired, respectively, six and three novel BXD ARI strains ($N = 5–10$ per strain/treatment) and additional B6 and D2 progenitor strains ($N = 8$ per strain/treatment) from Oak Ridge National Laboratory (Oak Ridge, TN, USA) at 6–9 weeks of age. These novel BXD ARI strains were derived from two independent advanced intercrosses between the B6 and D2 progenitor strains (Peirce *et al.* 2004). All Oak Ridge animals were housed two to five mice per cage and kept in a colony room until their age range corresponded with previously tested Jackson Laboratory animals. All experimental procedures were approved by Virginia Commonwealth University Institutional Animal Care and Use Committees in accordance with National Institutes of Health guidelines.

Light–dark box behavioral protocol and apparatus

Genetic variation in anxiety- or anxiolytic-like behavior following saline or ethanol was measured across the BXD RI strains using the light–dark transition model of anxiety. Behavioral testing occurred between 1000 and 1300 h in multiple cohorts over a 12-month period. All behavioral testing was carried out by a male investigator. Each cohort contained a different combination of BXD strains with each strain balanced for saline or ethanol treatment. Following a 1-h acclimation period to the behavioral testing room, animals were restrained in a 50 ml conical tube for 15 min followed by intraperitoneal (IP) injections with either physiological saline (0.9%) or 1.8 g/kg ethanol (12.8% wt/v) in 0.9% saline. A mild restraint stress was employed to create an artificial baseline level of anxiety-like behavior. Fifteen minutes of immobilization has been shown to activate the sympathoadrenal system, as indicated by marked increases in plasma adrenocorticotropic hormone, epinephrine, norepinephrine and corticosterone (Tjurmina *et al.* 2002). Interestingly, ethanol antagonizes these neurochemical responses, including immobilization-induced plasma levels of epinephrine and norepinephrine (DeTurck & Vogel 1982; Patel & Pohorecky 1988; Popper *et al.* 1977). Given this biochemical evidence for ethanol-induced anxiolysis, a mild restraint stress was incorporated into our behavioral paradigm to increase anxiety-like behavior and the robustness of anxiolytic-like responses. This method provided a common environmental stressor across all mice just prior to studying anxiolytic-like effects from a control (saline) or ethanol injection. This restraint stress produced slight increases in anxiety-like behavior in the progenitor B6 and D2 strains, compared to no restraint, and trended toward increasing within-strain reproducibility of anxiety-like measures (data not shown). An activating dose of ethanol was chosen for this study based on its ability to elicit a significant anxiolytic-like response in the progenitor strains and allow for considerable phenotypic variation across the BXD RI strains. Following a 5-min delay from the time of injection, each animal was placed in the light chamber facing the entrance to the dark chamber of the light–dark box. Once the animal entered the dark compartment, anxiety-like scores were collected in 5-min intervals for a total of 10 min. Behavioral measures were recorded in both chambers of the light–dark box and included distance traveled (cm) and time spent in each compartment and total locomotor activity (TLA). Anxiety-like measures were reported as percent time spent in the light (TSL) and percent distance traveled in light (DTL) to control for locomotor activity. An increase in either measure was interpreted as decreased anxiety-like behavior.

Light–dark box studies were carried out with a commercial product adapted from the originally described apparatus (Crawley & Goodwin 1980). An open field arena ($27.3 \times 27.3 \times 20.3\ cm^3$) was divided into two equally sized compartments using an opaque black plastic box insert with an opening in the middle to allow for light–dark transitions (catalog nos. ENV-510 and ENV-511, Med Associates Inc, St. Albans, VT, USA). The arena was enclosed in a sound-attenuating box (catalog no. MED-OFA-022) equipped with overhead lighting (two 100 mA bulbs; 22 lux in light compartment) and fan ventilation. The system was interfaced with Med Associates software (catalog no. SOF-811) enabling automatic measurement of activity using a set of 16 infrared beam sensors along the *X–Y* plane. The arena was cleaned with 5% ammonia solution between animals.

Microarray data generation

Mice used for behavioral studies above were sacrificed by cervical dislocation 4 h following IP injection. Immediately thereafter, brains were extracted and chilled for 1 min in iced phosphate buffer before being microdissected into eight constituent regions as previously described (Kerns *et al.* 2005). These included PFC, NAc and VMB, which contains ventral tegmental area and substantia nigra. Excised regions were placed in individual tubes, flash-frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from four to five pooled brain region samples, processed and hybridized to Mouse Genome 430 2.0 microarrays (Affymetrix, Santa Clara, CA, USA, part #900497) according to the manufacturer's protocol as previously

described (Kerns *et al.* 2005; Wolen & Miles 2012). In total, this study incorporated PFC tissue from 27 BXD strains, NAc and VMB tissue from 35 BXD strains and B6 and D2 tissue from all three regions in triplicate. A detailed description and network analysis of these microarray data were previously reported (Wolen *et al.* 2012).

Statistical analysis

BXD strain genotypes used for all analyses consisted of approximately 4000 genetic markers obtained from GeneNetwork (www.genenetwork.org/genotypes/BXD.geno). All behavioral measures collected across the BXD strains and their inbred progenitors were evaluated for strain, treatment and strain-by-treatment differences using analysis of variance (ANOVA) with Tukey HSD (honest significant difference) *post hoc* analysis. Narrow-sense heritability (h^2) estimates for all behavioral measures were calculated as previously described by Belknap *et al.* (1996). Behavioral QTL were identified by strain-mean single locus interval mapping performed with the R/qtl package for R (versions 2.11.1–3.1.1) (Broman *et al.* 2003; Chesler *et al.* 2004). The QTL genome-wide adjusted *P*-values were calculated empirically by permutation analysis with 1000 iterations (Churchill & Doerge 1994). Significant and suggestive thresholds corresponded to LOD scores with genome-wide *P*-values <0.05 and 0.63, respectively (Lander & Kruglyak 1995). The QTL support intervals were estimated using the Bayes credible interval approach implemented in R/qtl with a 97% probability of coverage (Manichaikul *et al.* 2006).

To examine genotype-by-treatment interactions, a single marker mixed-model QTL analysis was conducted using the lme4 package for R (Bates *et al.* 2015). With genotype treated as a fixed effect, three models were fit using maximum likelihood to estimate the parameters. The *reduced model* approximated the previous strain-mean mapping, testing genotype effect only, on either the combined data (saline and ethanol) or separate data sets for each group. The *additive model* tested for genotype effects in addition to treatment effects from the reduced model. The *full model* included all parameters from the additive model and an interaction term that incorporated genotype-by-treatment effects. To show the treatment-specific effects, the interaction model was analyzed by comparison of additive model parameters to those from the full model. The QTL were identified as those markers with a Benjamini–Hochberg false discovery rate (FDR) <10% in at least one measure of anxiety-related behavior across any model. To show the most parsimonious model, nested models were compared using the likelihood ratio test ($\chi^2 = 10.8$, $P = 0.001$) with degrees of freedom equal to differences in the number of parameters tested between models.

Statistical significance for molecular studies was examined for each gene or protein using a Student's *t*-test between the two strains or genotypes tested, with Bonferroni correction for multiple testing as necessary.

Bioinformatic approaches to identify candidate genes

To identify candidate genes underlying QTL, multiple standard bioinformatic approaches were utilized. First, PFC, NAc and VMB gene expression datasets from BXD RI strains were correlated to light–dark box phenotypes following saline or ethanol treatment. Genes or predicted open-reading frames located within the QTL support interval and found to be significantly correlated (Spearman's rank) with an anxiety-related behavioral measure were considered genes of interest. The significance of a correlation was determined by converting correlation coefficients to *Z*-scores with the Fisher transformation and using the FDR to adjust raw ρ-values in order to account for the multiple tests conducted within each brain region (Benjamini & Hochberg 1995).

Next, microarray expression data were utilized to identify genetic loci regulating each gene's level of expression in the three profiled brain regions by mapping expression QTL (eQTL) using GeneNetwork (Chesler *et al.* 2004). Any eQTL located within 5 Mb of its associated gene was considered a *cis* regulatory locus, or *cis* eQTL. Genes with *cis* eQTL overlapping a behavioral QTL were considered as high priority candidates, especially if their expression levels strongly correlated with the behavior of interest. Genes with potentially spurious *cis* eQTL caused by D2 single nucleotide polymorphisms (SNPs) overlapping probe binding sites (Alberts *et al.* 2007) were evaluated and removed from further consideration.

Each gene of interest was examined for polymorphisms using the B6 and D2 genome sequences. Genes carrying non-synonymous or putative functional polymorphisms, were considered as high priority candidates. In order to assess the likelihood that a coding SNP would affect protein function, we employed the web-based bioinformatics tool, Variant Effect Predictor (VEP), which predicts whether an amino acid change might carry functional consequence for the protein (McLaren *et al.* 2010). Finally, genes satisfying all criteria were examined for biological significance using evidence from literature associations with anxiety- and/or ethanol-related phenotypes.

Molecular characterization of candidate genes containing cis eQTL

To further investigate candidate genes containing *cis* eQTL on microarray analysis, B6 and D2 mRNA expression levels of each gene were compared by using quantitative real-time polymerase chain reaction (qRT-PCR) in the brain region of interest, NAc ($n = 8$ NAc per strain). If significant expression differences between B6 and D2 mice were verified, allele-specific sequencing of B6D2F1/J NAc tissue ($n = 3$ biological replicates of two pooled NAc) was performed in order to confirm *cis* regulation. Functional differences between B6 and D2 mice for the top priority candidate gene (*Nin*), evidenced as differences in protein expression levels, were examined by immunoblotting from NAc samples ($n = 5$ NAc per strain). Additional details are provided in Appendix S1.

Results

Anxiolytic-like response to ethanol in B6 and D2 progenitors

Acute ethanol elicited a significant anxiolytic-like response in B6 and D2 progenitor mouse strains, as measured using percent distance traveled in the light (DTL) in the light–dark transition model of anxiety (Fig. 1a; $P < 0.05$ vs. within-strain saline; see Table S1 for full statistical results). There also was a significant main effect of treatment across both DTL and TSL (see Table S1). Although there was no significant strain × treatment effect on ANOVA for DTL or TSL, following *post hoc* testing of the B6 strain showed significant ethanol anxiolytic-like responses with both DTL and TSL, whereas D2 were only significant with the DTL measure. Consistent with the earlier reports (Phillips *et al.* 1995; Tritto & Dudek 1994), D2 and B6 strains showed contrasting TLA in response to ethanol (Fig. 1b). While ethanol induced locomotor activation in D2 mice, B6 mice lacked this response. Considering that the B6 strain showed a significant anxiolytic-like response to ethanol with the absence of locomotor activation, ethanol-induced anxiolysis, as measured by DTL or TSL, did not appear to be strictly dependent on ethanol induction of locomotor activity. Factor analysis of BXD strain phenotypes supports this observation, as detailed in the following section.

Anxiety-related behaviors across BXD RI strains

To identify provisional QTL contributing to genetic variance in ethanol-induced anxiolysis, anxiety-related behaviors in response to saline or ethanol were measured across 27 BXD RI strains and the progenitor B6 and D2 strains, as described in *Materials and methods*. Behavioral phenotypes

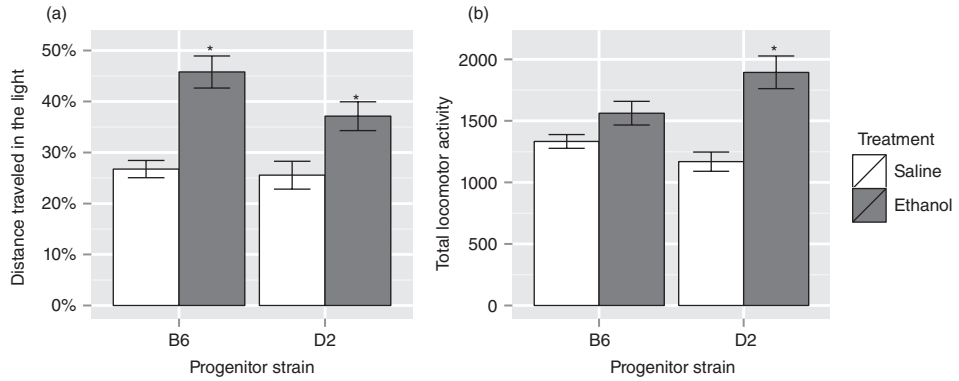

Figure 1: Ethanol-induced phenotypes in the light–dark transition model of anxiety. The DTL (a) following saline or 1.8 g/kg ethanol in B6 and D2 progenitors. An increase in the distance traveled in the light represents an anxiolytic-like response to ethanol. TLA (b) following saline or 1.8 g/kg ethanol was simultaneously recorded. Significance was determined by ANOVA with Tukey *post hoc* analysis (* $P < 0.05$ vs. within-strain saline).

used for QTL mapping from the 0–5 to 0–10-min intervals are summarized in Table S2. As expected, results for DTL, TSL or TLA were tightly correlated between the 0- to 5- and 0- to 10-min data points (Pearson's $r > 0.96$ for DTL, TSL, TLA, $P < 0.0001$). We therefore focus discussion of QTL analysis on the 0- to 5-min interval because of the greater anxiolytic-like responses to ethanol across all strains at this time interval. However, qualitatively identical results were obtained for QTL analysis of the 0- to 5- and 0- to 10-min intervals. Distributions of the 0- to 5-min saline and ethanol phenotypic (DTL, TLA) strain-means across the BXD RI strains are displayed graphically in Fig. 2. Strikingly, nearly all BXD RI strains exhibited ethanol-induced anxiolytic-like responses. As mentioned above, the anxiolytic-like response to ethanol was not strictly dependent upon ethanol-induced locomotor activation, as demonstrated in B6 mice as well as in other BXD RI strains (Fig. 2a vs. Fig. 2b; e.g. BXD12 and BXD2). We examined this more formally by factor analysis of post-ethanol behavioral phenotypes, which showed that DTL and TLA load on different factors (Fig. S1).

The ANOVA across DTL and TLA showed significant ($P < 0.0001$) strain ($F_{(26, 364)} = 6.91$; $F_{(26, 364)} = 16.11$, respectively) and treatment effects ($F_{(1, 364)} = 309.3$; $F_{(1, 364)} = 357.2$, respectively) across the BXD strains. Significant strain-by-treatment effects were also identified in DTL and TLA ($F_{(26, 364)} = 2.31$, $P < 0.0001$; $F_{(26, 364)} = 1.98$, $P < 0.01$, respectively). Within-strain statistics for DTL, TSL and TLA are provided in Table S3. As shown in Table 1, narrow-sense heritability estimates for DTL, TSL and TLA following saline or ethanol ranged from 0.30 to 0.58. Table 1 also displays a phenotypic correlation matrix for DTL, TSL and TLA recorded following saline or ethanol treatment. According to these data, both anxiety-related measures (DTL and TSL) were tightly correlated (Pearson's $r > 0.93$, $P < 0.001$) within each treatment group, suggesting that these behaviors measure similar anxiety-related phenotypes. We therefore focus our further discussion on DTL in additional studies on anxiety-like traits but results from TSL are displayed in Tables 2, S1 and S2 and Figs. S1,S5.

Strain-mean QTL mapping

Strain-mean single factor interval mapping with permutation-corrected significance levels showed multiple QTL influencing phenotypic variation in anxiety-related behaviors and TLA in response to saline or ethanol injections following mild restraint stress (Table 2). Three suggestive ($P < 0.63$) QTL altering DTL following saline were detected on chromosome (Chr) 1, 6 and 11 (Fig. 3, Table 2). The Chr 1 and Chr 11 loci also modified TSL following saline; the Chr 11 QTL (**Sal**ine **an**xiety-like QTL 1, *Salanq1*), exceeded the threshold for statistical significance ($P < 0.05$; Table 2). In addition, the post-saline TLA phenotype produced a significant locus ($P < 0.05$) on Chr 1 and a suggestive QTL ($P < 0.63$) on Chr 9 (Fig. S2). Although significant QTL were observed following saline injection for anxiety-like phenotypes, most notably the QTL on Chr 11 (*Salanq1*) that modified both DTL and TSL, the remaining analyses were focused on ethanol-induced phenotypes.

In contrast to the results seen following saline injection, QTL modifying DTL following ethanol mapped to a suggestive locus ($P < 0.63$) on Chr 1 and a significant ($P < 0.05$) QTL on Chr 12 (Fig. 3, Table 2). Interestingly, the Chr 11 locus that altered saline DTL and TSL did not appear to significantly influence ethanol-induced anxiolysis. For ethanol-induced TLA, a significant QTL ($P < 0.05$) was identified on Chr 1 and two suggestive loci were found on Chr 2 and Chr 18 (Table 2). The Chr 1 QTL that modified ethanol-induced TLA mapped to the same locus that alters TLA following saline. Because support intervals for all identified Chr 1 QTL influencing TLA following saline (159.8 Mb) and ethanol (157.9 Mb), saline anxiety-like behavior (128.4 Mb) and ethanol-induced anxiolysis (182.5 Mb) overlap, further analyses are required to determine whether these QTL represent distinct or overlapping loci. To focus on QTL more specific to ethanol-induced anxiolysis, anxiety-related QTL that overlapped TLA modifying QTL were omitted from additional analyses. Thus, the best provisional QTL altering the anxiolytic-like response to ethanol was the statistically significant ($P < 0.05$) QTL on Chr 12 [**et**hanol-induced

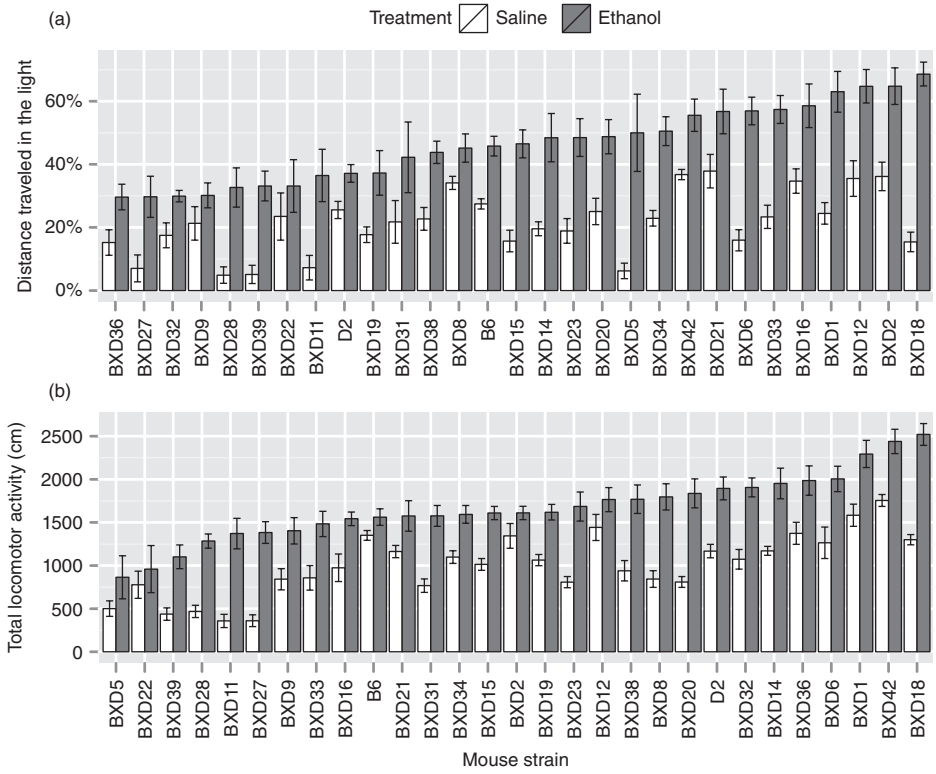

Figure 2: Distribution of behavioral strain means. The DTL (a) and TLA (b) strain means + SE obtained in the light–dark box across 27 BXD RI strains and their progenitor strains following saline or 1.8 g/kg ethanol. Strains are sorted by ethanol strain means.

Table 1: Phenotype correlations

	Saline TLA	Saline DTL	Saline TSL	Ethanol TLA	Ethanol DTL	Ethanol TSL
Saline TLA	1	7.84e−04	4.06e−04	1.99e−06	4.20e−03	5.39e−03
Saline DTL	0.589	1	2.70e−13	5.29e−02	5.91e−03	2.82e−03
Saline TSL	0.613	0.931	1	8.00e−02	2.71e−02	1.69e−02
Ethanol TLA	0.757	0.363	0.33	1	4.83e−03	1.16e−02
Ethanol DTL	0.516	0.499	0.41	0.509	1	2.22e−22
Ethanol TSL	0.503	0.534	0.44	0.462	0.986	1
Heritability (h^2)	0.58	0.35	0.3	0.44	0.28	0.23

Values in the lower diagonal represent Pearson's correlation coefficients between indicated phenotypes; *P*-values (upper diagonal) were obtained using Fisher's *z*-transformation.

anxiolysis **Q**TL **1** (*Etanq1*)] that modified both DTL and TSL (Table 2, Fig. S5).

Mixed-model QTL mapping

Although QTL studies measuring the effect of drug treatment have used difference values by calculating strain mean post-drug minus pre-drug scores, this method entails a variety of complications including increased error and spurious correlations (Nagoshi *et al.* 1986). An alternative approach to identify QTL influencing drug treatment-specific responses is through a nested model. Therefore, as described in *Materials and methods*, we employed a single marker mixed-model QTL mapping procedure to examine genotype-by-treatment interactions across individual animals, thereby identifying saline and ethanol-specific effects on anxiety-related behaviors and TLA (Tables 3 and S5).

Strain-mean QTL indicated that, with the exception of the Chr 1 QTL influencing TLA, most loci were treatment-specific, altering anxiety-like behavior in response to either ethanol or saline injections. In contrast, the mixed model analysis showed that these loci influenced anxiety-like behavior following both saline and ethanol, although to different degrees. For example, as shown in Table 3, isolating saline and ethanol responses by examining reduced model *P*-values for the Chr 6 (140.2 Mb) QTL showed a significant QTL for anxiety-related behavior following saline injection and

Table 2: Strain-mean QTL mapping results of light–dark box phenotypes

Phenotype	Peak marker	Chr	Location	LOD	*P*-value	Support interval
Saline TLA	rs3696645	1	159.9	3.99	**3.0e–02**	147.39–184.42
	rs3689326	9	53.77	2.39	5.2e–01	29.75–106.32
Ethanol TLA	UT_1_113.684537	1	157.9	3.71	**1.5e–02**	147.39–184.42
	rs3675564	2	164.2	2.48	3.7e–01	9.75–172.85
	rs13481014	11	47.93	2.16	6.2e–01	4.41–120.82
	rs4231742	18	11.08	2.68	2.5e–01	3.52–86.75
Saline DTL	D1Mit139	1	128.4	2.41	5.6e–01	29.23–184.42
	gnf06.140.234	6	140.1	2.74	3.7e–01	16.01–149.21
	rs13481251	11	116.9	2.7	4.0e–01	8.74–121.41
Ethanol DTL	rs13474399	1	182.5	2.59	4.5e–01	13.98–197.13
	rs3716547	12	68.67	4.32	**2.7e–02**	53.91–71.66
Saline TSL	rs13476098	1	128.5	3.35	9.8e–02	96.87–169.91
	rs13481251	11	116.9	3.75	**4.1e–02**	114.53–120.82
Ethanol TSL	rs13474399	1	182.5	2.9	2.8e–01	14.79–190.74
	rs3716547	12	68.67	4.37	**2.3e–02**	53.91–71.66

Suggestive (*P*-value < 0.63) and significant (*P*-value < 0.05; bolded) QTL identified by single locus genome scans of strain-mean phenotypes. Support intervals define regions with a 97% probability of harboring the true QTL.

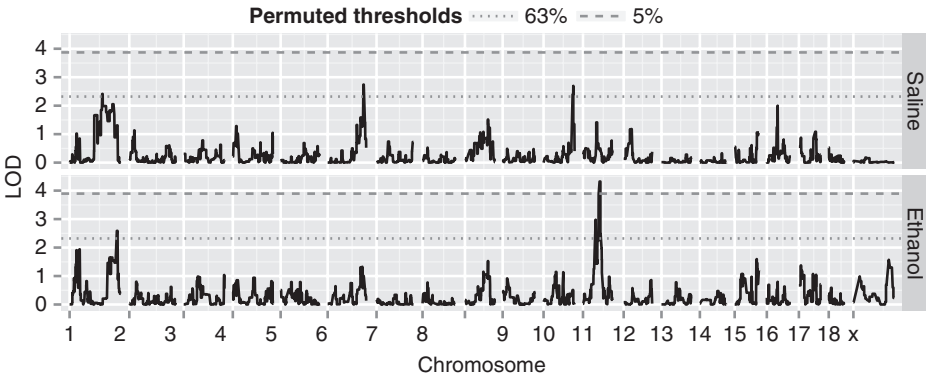

Figure 3: Anxiety-like behavior interval maps. BXD strain-mean interval maps for DTL following saline (upper panel) or 1.8 g/kg ethanol (lower panel). Permuted genome-wide significance thresholds for falsely rejecting the null hypothesis (no linkage in the genome) at probabilities of 5% and 63% (corresponding to $P < 0.05$ and $P < 0.63$) are denoted by the dashed and dotted lines, respectively.

a suggestive QTL following ethanol. Therefore, similarly to the TLA-related Chr 1 (156–158 Mb) QTL, it appears ethanol may partially elicit locomotor activation and anxiolytic-like effects through mechanisms involved in the basal (restraint stress + saline injection) regulation of these phenotypes. Regarding ethanol-specific anxiolytic-like effects, the interaction model validated the previously identified and significant Chr 12 QTL (68.67 Mb, $P < 0.001$). Although the Chr 12 QTL appeared unique to ethanol-induced anxiolysis following strain-mean genetic mapping, mixed-model QTL analysis showed a trend following saline injection also at this locus ($P = 0.13$).

Replication of ethanol anxiolytic-like QTL

We utilized novel BXD RI strains (BXD ARI) derived from two independent advanced intercrosses between the B6 and D2 progenitor strains (Peirce *et al.* 2004) for initial confirmation of the significant *Etanq1* on Chr 12 (68.67 Mb). Using the peak genetic marker within *Etanq1*, three BXD ARI strains (BXD 43, 90 and 98) containing the B6 allele were chosen, together with three strains (BXD 63, 66 and 67) with the D2 allele. All the six BXD ARI strains were balanced for genotype at the two significant post-saline anxiety-like QTL on Chr 1 and Chr 11, to minimize the influence of these loci on ethanol-induced anxiolysis. Anxiety-related behaviors in response to saline or ethanol were measured across each BXD ARI strain, as described in *Materials and methods*. ANOVA showed that two of the three BXD ARI strains with the B6 allele showed a significant anxiolytic-like response to ethanol, whereas the BXD ARI strains with the D2 allele did not show significant anxiolytic-like responses to ethanol (Fig. 4a). Measuring TSL in response to saline or ethanol resulted in similar findings (data not shown). Collapsing each of the three BXD ARI strains into genotypic groups identified a statistically significant difference in DTL following ethanol between strains with the B6 allele vs. the D2 allele at the peak genetic marker underlying *Etanq1* (Fig. 4b). As expected, this response was ethanol-specific with no significant differences in anxiety-like behaviors between the two genotypic groups following saline injections.

Fine-mapping of ethanol anxiolytic-like QTL

We examined the haplotype structure of *Etanq1*'s support interval using genotype data for all the 93 BXD strains to calculate the r^2 measure of linkage disequilibrium (LD) for all pairwise combinations of Chr 12 genetic markers. Results

Table 3: Mixed-model QTL mapping results of light–dark box DTL and TLA phenotypes

Phenotype	Chr	Peak marker	Location	Saline	Ethanol	Additive	Interaction
DTL	1	rs13476098	126.5	1.3e−03	7.6e−01	8.4e−02	**7.4e−03**
	1	rs13474399	180.6	1.9e−02	**3.1e−04**	**2.3e−04**	**1.0e−02**
	6	gnf06.140.234	140.2	**6.2e−04**	2.5e−02	**9.4e−04**	7.6e−01
	11	rs13481251	117	**9.9e−04**	2.0e−01	1.5e−02	1.5e−01
	12	rs3716547	68.67	1.3e−01	**4.3e−06**	**2.3e−04**	**1.0e−05**
TLA	1	UT_1_113.684537	156.1	**6.0e−05**	**2.3e−04**	**2.4e−05**	9.1e−01
	1	rs3696645	157.9	**6.0e−05**	**2.3e−04**	**2.4e−05**	9.1e−01
	2	rs8275857	164.4	1.3e−02	**4.2e−04**	**1.0e−03**	**6.4e−03**
	9	rs13480205	54.53	3.6e−03	4.5e−03	**2.1e−03**	2.3e−01
	11	rs13481014	48.12	6.4e−03	9.0e−04	**1.1e−03**	3.3e−02
	18	rs4231742	11.08	4.0e−03	1.3e−03	**9.3e−04**	4.3e−01

Bold values indicate a statistically significant QTL (FDR ≤ 10%).

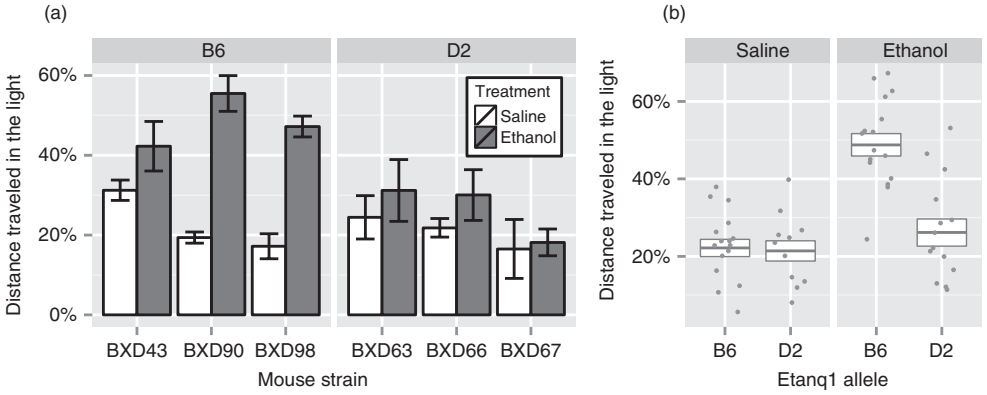

Figure 4: Replication of chromosome 12 ethanol-related anxiety QTL. (a) Differences in DTL in response to ethanol across six BXD ARI strains containing either the D2 (BXD 63, 66 and 67) or B6 (BXD 43, 90 and 98) allele at the peak genetic marker (rs3716547, 68.67 Mb) within the chromosome 12 QTL, *Etanq1*. The ANOVA with Tukey *post hoc* test showed a significant anxiolytic-like response to ethanol in most BXD strains containing the B6 allele (BXD43: $P = 0.84$, BXD90: $P = 7.1e{-}05$, BXD98: $P = 3.4e{-}04$), whereas the lowest *P*-value among strains containing the D2 allele was 0.97. (b) Collapsing post-ethanol DTL scores across BXD ARI strains containing the D2 or B6 allele showed a significant effect of the B6 allele on ethanol-induced anxiolysis ($P = 2.9E{-}06$ vs. D2 allele ethanol, ANOVA with Tukey *post hoc* analysis).

from this analysis showed *Etanq1*'s provisional support interval was largely comprised of three haplotype blocks (Fig. 5c). The independent effects of these haplotype blocks on ethanol-induced anxiolysis could not be discerned using only the BXD strains assayed for the provisional mapping of the QTL.

We therefore refined the *Etanq1* support interval by conducting anxiety-related behavioral measurements for additional ARI BXD strains. These strains were chosen to contain informative recombination events within *Etanq1*'s support interval determined from the provisional and replication mapping studies (Fig. S3). The addition of these strains (BXD 50, 70 and 75) increased the peak LOD score of *Etanq1* (DTL following ethanol) on interval mapping and proximally shifted the location of the linkage peak by ≈2 Mb (Fig. 5a). Inclusion of the additional strains also narrowed *Etanq1*'s support interval by nearly 80%, to a region that spans from 69.13 to 72.56 Mb across Chr 12 (Fig. 5b). To a lesser extent, the additional strains improved the Chr 1 QTL, increasing its peak LOD score from 2.59 to 3.03, but showed a significant interaction with *Etanq1* (Fig. S4).

The fine-mapped *Etanq1* support interval centered on a very polymorphic region (70.9–71.5 Mb) flanked by areas comprising relatively few SNPs. The proximal region of the refined support interval was particularly SNP poor and may be identical by descent between B6 and D2. As shown in Fig. 5c, the refined support interval comprises a large portion of a single haplotype in which the r^2 for all genetic markers is ≥0.87. As such, continuing to assay novel strains from the currently available BXD panel is unlikely to further resolve *Etanq*1 without additional genetic manipulation.

Bioinformatic analysis of Etanq1 candidate genes

According to the Mouse Genome Informatics database (www.informatics.jax.org), *Etanq1*'s fine-mapped support interval harbors 41 protein-coding genes or non-coding RNAs. Given the limited potential for further refinement of the *Etanq1* support interval by genetic mapping, we

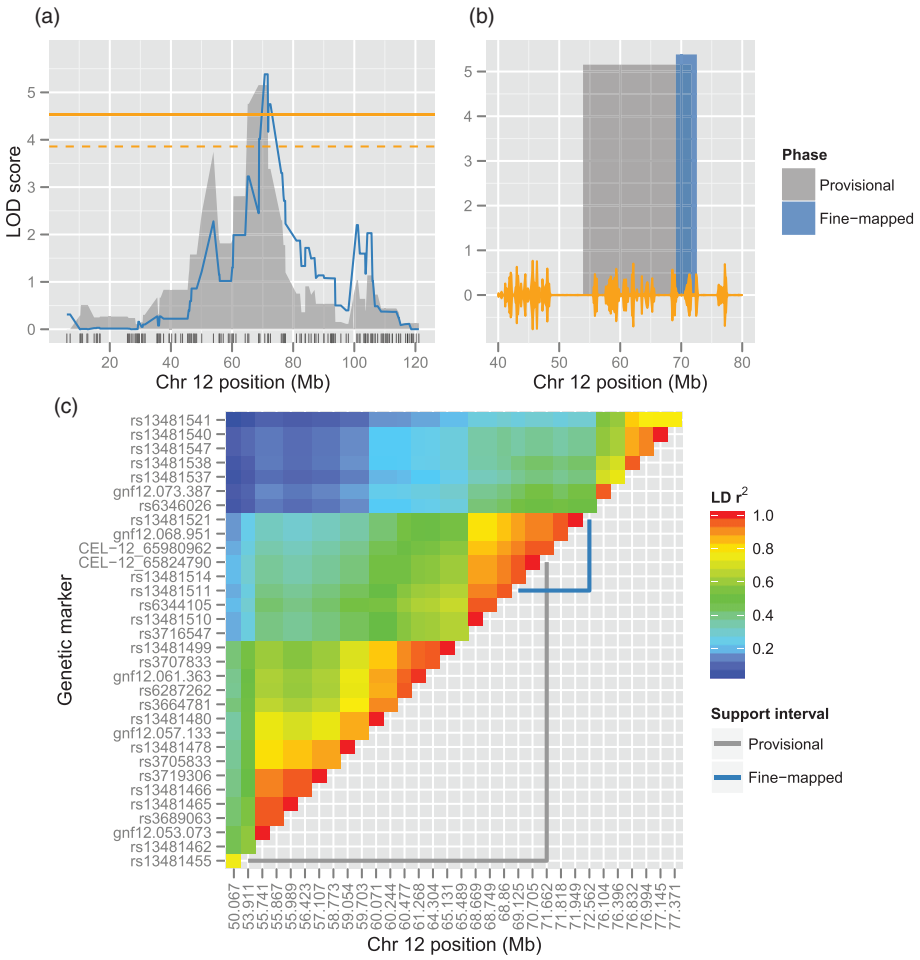

Figure 5: *Etanq1's* fine-mapped support interval. The original (blue) and fine-mapped (red) 97% support intervals for *Etanq1* (DTL) across a subsection of Chr 12. Gray seismograph across the *x*-axis indicates the number of polymorphisms per kilobase between B6 and D2.

prioritized these positional candidates through a series of integrative analyses that combined the anxiety-like phenotype data with genomic sequence data from the B6 and D2 progenitor strains and genome-wide mRNA expression profiles of the mesocorticolimbic reward circuit: PFC, NAc and VMB.

SNP analysis of Etanq1 candidate genes

Sequence variation analysis was performed between the B6 and D2 genomes for all *Etanq1* positional candidates (Table S4). There were a total of 3484 SNPs within the *Etanq1* support interval. While 62.3% of these SNPs fell within a transcript, only 130 occurred within an exon. *Trim9* was the most polymorphic gene in the region, harboring over 400 B6/D2 SNPs, but only a few of these SNPs occur within a coding region. When considering only coding and untranslated region SNPs (Table S4), *Nin* and *Abhd12b* were the most polymorphic genes within the *Etanq1* support interval.

We used the Ensembl VEP (Version 73) script to determine whether any of these identified coding SNPs are likely to affect protein function (McLaren *et al.* 2010). The SNPs positions were re-mapped to the UCSC mm10 build of the genome using liftOver (https://genome.ucsc.edu/cgi-bin/hgLiftOver), in order to match the GRCm38.p1-based positions used by VEP. A total of 10 unique missense mutations were identified across 6 genes; *Nin* contained 4, *Dact1* contained 2 and *Atp5s*, *Abhd12b*, *Pygl* and *2700049A03Rik* each contained 1 (Table S4b). Of these 10, the SIFT algorithm (Ng & Henikoff 2003) identified only one SNP (rs29159683) within *Nin* as being potentially deleterious, located within the 16th exon (ENSMUST00000085314) of the Nin-001 transcript (ENSMUST00000085314).

Expression QTLs

In addition to the functional changes in protein structure, genetic variation can affect gene expression levels and thus, modify behavioral phenotypes. Thus, eQTL analysis with brain expression data was utilized to identify genes with expression levels regulated by local (*cis* eQTL) B6/D2 polymorphisms. Only five positional candidate genes within the *Etanq1* support interval were associated with significant *cis* eQTL in the PFC, NAc or VMB: *Sos2*, *Nin*, *Map4k5*, *Atp5s* and *Trim9*. All are located within the previously discussed polymorphic region near the center of the *Etanq1* support interval (Fig. 5c). However, polymorphisms overlapping microarray probe binding sites can produce false-positive *cis*

Table 4: Genes with significant *cis* eQTL within Etanq1

Gene	Mb	Region	Marker	LOD	*P*-value
Sos2	70.72	NAc	rs6344105	9.11	1.0e−04
Sos2	70.72	VMB	rs6344105	10.24	6.0e−05
Atp5s	70.83	NAc	rs3716547	5.15	2.9e−03
Nin	71.11	NAc	rs6344105	4.09	1.3e−02
Trim9	71.35	NAc	rs6344105	4.71	1.4e−02

Genes, location, brain region, peak marker, LOD scores and *P*-values are shown (saline-treated only). Genes (Map4k5) with likely false-positive *cis* eQTL were excluded.

eQTL-like effects by altering transcript hybridization in an allele-specific manner. We therefore used the previously published list of B6/D2 SNPs (Wolen *et al.* 2012) coincident with Affymetrix Mouse Genome 430 2.0 probe binding sites to identify such spurious *cis* eQTLs. Three of the probes within a *Map4k5* probe-set (1440059_at) and one probe within the *Sos2* probe-set (1443057_at) overlapped D2 SNPs. Removing the affected probes and repeating the QTL analysis nullified the *cis* eQTL for *Map4k5*, but not those for *Sos2*, narrowing the high priority candidate genes to four: *Sos2*, *Nin*, *Atp5s* and *Trim9* (Table 4).

Correlation analysis with Etanq1 candidate genes

Further prioritization of positional candidate genes was completed by examining the association between DTL in response to ethanol and transcript abundance following saline or ethanol in the PFC, NAc or VMB. The strongest associations were observed in NAc (Fig. 6). After correcting for multiple testing, significant phenotype/mRNA correlations (FDR ≤ 0.1) were detected for only three genes: *Sos2*, *Trim9* and *Nin* (Table 5). The strongest was a negative correlation between DTL post-ethanol and basal (saline) *Nin* expression in the NAc ($P = -0.67$).

Biological relevance of Etanq1 candidate genes

To summarize the candidate gene list derived from bioinformatic analyses, six genes contained missense SNPs: *Nin*, *Dact1*, *Atp5s*, *Abhd12b*, *Pygl* and *2700049A03Rik*; four genes contained significant *cis* eQTL: *Sos2*, *Nin*, *Atp5s* and *Trim9*; and only three genes had mRNA expression significantly correlated to the post-ethanol DTL phenotype: *Sos2*, *Trim9* and *Nin*. The *Sos2* (*Drosophila Son of sevenless homolog 2*) encodes a RAS guanine nucleotide exchange factor affecting RAS/MAPK signal transduction (Bowtell *et al.* 1992). The *Trim9* (*tripartite motif containing 9*) is a brain-specific E3 ligase that self-ubiquitinates to target proteasomal degradation (Tanji *et al.* 2010). The *Nin* anchors microtubules in the centrosome (Bouckson-Castaing *et al.* 1996; Mogensen *et al.* 2000) and influences neocortical axonal branching and growth (Srivatsa *et al.* 2015). Although the biological functions of these genes are still being discovered, their expression in the central nervous system, along with roles in signal transduction and neuronal plasticity make them all plausible candidate genes. Interestingly, of the three, only *Nin* satisfies all the bioinformatic criteria

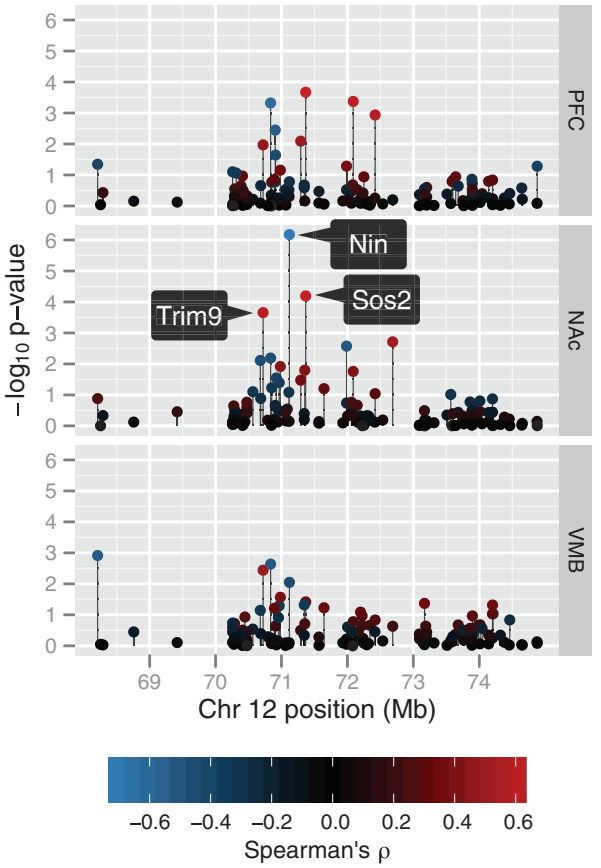

Figure 6: Correlation of ethanol-induced anxiolysis and candidate gene expression in the mesocorticolimbic reward circuit. The *y*-axis indicates significance (Spearman ρ) of correlation between DTL following ethanol and microarray expression values in the PFC, NAc or VMB for candidate genes within the Etanq1 interval. Gene positions on Chr 12 are plotted on the *x*-axis. Correlation direction/significance is represented by point color. Gene symbols are provided for phenotype correlations significant on FDR analysis (q-value < 0.05).

and has been shown to interact with *Gsk3b* (Hong *et al.* 2000; Howng *et al.* 2004) a known ethanol-responsive gene (French & Heberlein 2009; Luo 2009; Wolen *et al.* 2012)

Expression of Ninein in the NAc

To further prioritize our list of candidate genes, differential expression of *Nin*, *Sos2* and *Trim9* was examined between B6 and D2 mice using qRT-PCR analysis. The RNA from NAc samples were used because transcript abundance from the NAc had the strongest correlation with ethanol-induced anxiolysis, as described above. Only expression of *Nin* (Fig. 7a) was significantly different between D2 and B6 progenitor strains ($P < 0.01$, $n = 8$/strain, Student's *t*-test between strain). Allele-specific RT-PCR pyrosequencing was performed to confirm *cis* regulation for *Nin*. Within the NAc of untreated B6D2F1/J mice, pyrosequencing of

Table 5: Gene expression and phenotype correlations

Region	Treatment	Symbol	Probe-set	Position	*n*	ρ	*P*-value
NAc	Saline	*Sos2*	1443057_at	70.72	35	0.56	3.4e−04
	Ethanol				35	0.56	4.0e−04
	Saline	*Nin*	1419078_at	71.12	35	−0.67	3.6e−06
	Saline	*Trim9*	1434595_at	71.37	35	0.63	3.4e−05
	Ethanol				35	0.64	2.0e−05
PFC	Ethanol	*Trim9*	1434595_at	71.37	29	0.68	1.9e−05
VMB	Saline	*Sos2*	1443057_at	70.72	35	0.63	3.1e−05
	Ethanol				35	0.63	2.8e−05
	Ethanol	*Trim9*	1454886_x_at	71.35	35	−0.57	2.5e−04

Significant (FDR < 10%) Spearman's rank correlations between expression of Etanq1-region genes and distance traveled in the light following saline or ethanol. Within experimental group, only the most highly correlated probe-set is listed for each gene.

two missense SNPs within the *Nin* gene (rs29192398 and rs29159683 at 71.144373 and 71.144376 Mb of mouse build mm9, respectively) showed a significantly greater frequency of the D2 alleles compared with B6 alleles ($P < 0.01$, $n = 2$ NAc pooled per biological replicate, $n = 3$ biological replicates, $n > 4000$ reads per group), confirming *cis* regulation of *Nin* (Fig. 7b). Finally, we performed Western blot analysis of basal Ninein expression and confirmed significantly higher basal levels of two NIN protein isoforms in the NAc of D2 mice compared with B6 mice ($P < 0.05$, Student's *t*-test, $n = 5$/group, Fig. 7c,d), suggesting functional differences of NIN in the NAc between the B6 and D2 genotypes.

Discussion

Because ethanol's effects on anxiety are thought to play an important role in the genesis of AUD and recidivism in alcoholics, our work sought to identify candidate genes underlying the acute anxiolytic-like response to ethanol in mice. Based on the overall design of this QTL mapping study, we not only gained insight into the genetic mechanisms underlying ethanol anxiolysis but also into the multiple behaviors including saline- and ethanol-induced locomotor activity, and basal (saline) anxiety. The result is a fine-mapped ethanol anxiolysis QTL, *Etanq1* and a narrowed list of underlying candidate genes, one of which, *Nin*, is a high priority target based on comprehensive SNP, phenotype-expression correlation, eQTL and molecular analyses.

Most of the rodent models of anxiety-like behavior are at least partially activity-based, complicating the identification of QTL specifically related to anxiety. This issue was demonstrated in a recent behavioral genetics correlation analysis of multiple anxiety-like behavior assays across a panel of inbred mouse strains (Milner & Crabbe 2008). However, a detailed F2 mouse genetic analysis of multiple behavioral models of anxiety-like behavior suggested both separate and shared genetic loci contributing to each measure of anxiety and locomotor activity (Turri *et al.* 2001). A similar concept regarding anxiety-related responses to ethanol has also been previously proposed. Studies in selectively-bred mouse lines suggest that ethanol-induced locomotor activity and anxiolytic-like responses to ethanol are controlled by only partially overlapping sets of genes (Boehm *et al.* 2002). Furthermore, in a detailed bioinformatic of loci on Chr 1 contributing to the anxiolytic-like response to ethanol in the elevated zero-maze model of anxiety, showed at least one locus unique to ethanol anxiolytic-like activity (Cook *et al.* 2015).

In our studies described here, although anxiolytic-like responses to ethanol in the light–dark box were not dependent on ethanol-induced locomotor activation, as demonstrated in B6 progenitors (Fig. 1) and various BXD RI mouse strains (Fig. 2), we found significant phenotypic correlations between some anxiety-like measures and TLA in response to saline and ethanol (Table 1), and some overlap between anxiety-related and TLA QTL (see Table 2 and below). While the dissection of anxiety-related QTL in such regions may require extensive analysis (Cook *et al.* 2015; Willis-Owen & Flint 2006), our results also identified anxiety-like QTL that appear distinct from locomotor influences. The most significant of these potentially unique anxiety-related loci consisted of a Chr 11 post-saline anxiety-like QTL (*Salanq1*) and a Chr 12 locus (*Etanq1*) that influenced anxiety-like behavior following ethanol (Fig. 3, Tables 2 and 3). Although the statistical power of mixed-model QTL analysis enabled the detection of additional loci involved in anxiety-related behavior, QTL identified by strain-mean mapping and confirmed using the mixed-model were given emphasis to minimize false positives. Of note, a recent report using the diversity outbred genetic mouse panel mapped an anxiety-like QTL in the light–dark transition model at a Chr 11 location (95.01–96.05 Mb) very close to the support interval identified here for *Salanq1* (Logan *et al.* 2013). It should be recalled, however, that our protocol employed here used a mild restraint stress prior to either saline or ethanol injection and LD box testing. Thus, the anxiety-like QTL seen following saline actually represent the sum of basal anxiety, restraint-stress and saline injection stress effects, rather than merely 'basal' anxiety. Regardless, further genetic dissection of *Salanq1* may produce novel insight into the molecular neurobiology of anxiety.

Following ethanol treatment, we observed anxiolytic-like responses across nearly all BXD RI strains and their progenitors with a continuous pattern of phenotypic variation,

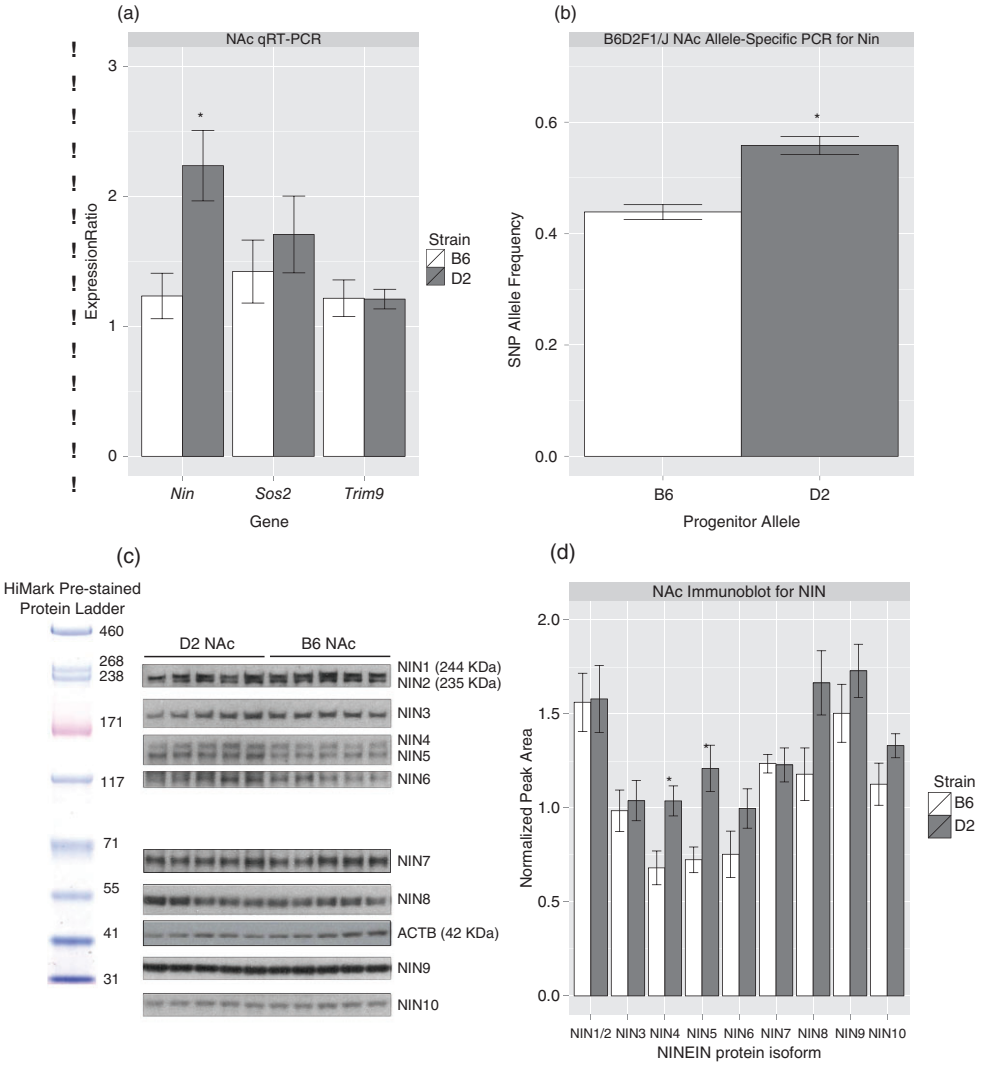

Figure 7: Confirmation of differential regulation of *Nin* in the NAc. (a) Basal mRNA levels of *Nin* were significantly greater in D2 mice than B6 mice (*$P < 0.01$, $n = 8$ per group, Student's *t*-test between strain) as confirmed by qRT-PCR. The *Nin* expression was normalized to *Ppp2r2a*. (b) Pyrosequencing of NAc samples of B6D2F1/J hybrid mice for an amplicon of *Nin* containing two progenitor SNPs confirmed *cis* regulation of *Nin* (*$P < 0.01$, $n = 2$ pooled per sample, $n = 3$ per group, $n > 4000$ reads per group, Student's *t*-test). (c and d) Immunoblotting of NAc samples from untreated B6 and D2 mice showed significantly higher levels of two isoforms (NIN4 and NIN5, ~120 kDa) in D2 mice compared with B6 (*$P < 0.05$, Student's *t*-test, $n = 5$/strain). The D2 mice also show a trend for higher levels of NIN6, NIN8 and NIN10 isoforms ($P = 0.1703$, $P = 0.0589$, $P = 0.1516$, respectively, Student's *t*-test). The NIN levels were normalized to ACTB (beta-actin) protein levels.

suggesting a multi-gene quantitative trait (Fig. 2). Loci influencing the anxiolytic-like response to ethanol consisted of a suggestive Chr 1 locus and a significant Chr 12 QTL, referred to as *Etanq1* (Fig. 3, Table 2). Particularly apparent in the strain-mean mapping, *Salanq1* was absent from our genetic mapping results in the ethanol cohort. This degree of genetic segregation of major loci influencing anxiety-like behavior following saline or ethanol suggests either that ethanol treatment abrogates the role of the influence of *Salanq1*, or that genetic and environmental variance following ethanol does not allow detection of *Salanq1*. Although a variety of ethanol-related behaviors, including acute and chronic ethanol withdrawal (Buck *et al.* 1997, 2000), and acute ethanol sensitivity phenotypes (Cook *et al.* 2015; Radcliffe *et al.* 2000), were previously mapped to similar loci on Chr 1, this complex region appeared to alter a variety of non anxiety-related phenotypes, as mentioned above. Therefore, these reports, along with the existence of large overlapping support intervals, suggest that speculation of candidate genes underlying this locus would be of limited value prior to further analysis.

In contrast, the *Etanq1* Chr 12 locus was identified as a potentially exclusive ethanol anxiolysis-related QTL, distinct from TLA loci. An initial confirmation study completed using BXD RI strains derived from two independent advanced intercrosses between the B6 and D2 progenitor strains (Peirce *et al.* 2004) verified the existence of *Etanq1* and the importance of the B6 allele in increasing ethanol-induced anxiolysis at this locus (Fig. 4). Interestingly, mixed-model QTL analysis showed that although *Etanq1* appeared unique to ethanol-induced anxiolysis, a suggestive effect of saline exists at this locus (Table 3). This result is supported by a previous report that identified a similar Chr 12 locus modifying basal anxiety-like behavior in the light–dark box (Turri *et al.* 2001) and suggests that identifying genes underlying *Etanq1* may provide insight into basic mechanisms of anxiety, as well as ethanol-induced anxiolysis.

Because our provisional mapping and subsequent confirmation studies were performed in BXD RI strains, loci identified here may only represent a portion of natural genetic variation affecting the anxiolytic properties of ethanol. Similarly, our use of only the light–dark box for measurement of anxiety-like phenotypes clearly only captures some of the genetic variance linked to ethanol-induced anxiolysis, as seen with basal anxiety measures (Belzung & Le Pape 1994; File 1995; Flint 2003; Ramos *et al.* 1997; Turri *et al.* 2001). Thus, ongoing studies in our laboratory are assessing the role of ethanol-induced anxiolysis QTL mapped here with other models of anxiety-like behavior. An additional factor possibly limiting the QTL identified here is the absence of female mice, thereby eliminating our ability to map sex-specific QTL as observed with other behavioral responses to ethanol (Downing *et al.* 2006; Radcliffe *et al.* 2000). Fluctuations in sex hormones during the ovarian cycle have been shown to influence anxiety-related behavior (Galeeva *et al.* 2003; Maguire *et al.* 2005). Using our behavioral paradigm in the light–dark box, we confirmed significant within-strain variation of anxiety-like behavior between male and female BXD mice (data not shown). Thus, to reduce total phenotypic variation and possibly increase our ability to successfully identify QTL, only male mice were used for this initial genetic analysis of ethanol-induced anxiolysis. Given the importance of sex-specific QTL to anxiety and behavioral responses to ethanol, sex-specific QTL will be elucidated in future studies but conducting those studies simultaneous, and on the same scale, with the current analysis would have been infeasible in terms of manpower and resources. Regardless of these limitations, this study is the first to report a confirmed QTL for ethanol-induced anxiolysis.

We performed extensive bioinformatic analyses to narrow down candidate genes for *Etanq1*. For molecular studies, we focused on the three genes with putative *cis* eQTL whose NAc expression significantly correlated to the DTL phenotype: *Sos2*, *Trim9* and *Nin* (Tables 4 and 5). Of these candidate genes, missense mutations within *Sos2* have been associated with Noonan syndrome (Cordeddu *et al.* 2015; Yamamoto *et al.* 2015), an autosomal dominant disorder caused by functional dysregulation of RAS/MAPK signal transduction pathways; however, because *Sos2* lacked missense mutations between the B6 and D2 progenitor sequences it was considered a less likely candidate gene. The *Trim9* is expressed in both developing and adult neurons (Winkle *et al.* 2014), with its highest expression found in the cortex and hippocampus. Reported associations between *Trim9* expression or variants and disease are limited to severe repression of *Trim9* in the brain of patients with Parkinson's and Lewy body dementia (Tanji *et al.* 2010).

Of the top three candidates, *Nin* was the only gene to show PCR confirmed differential expression between the B6 and D2 progenitor strains in the NAc (Fig. 7a). Additional analyses confirmed *cis* regulation of *Nin* using allele-specific PCR/sequencing in B6D2F1/J mice (Fig. 7b) and showed differential expression of protein isoforms between progenitors in the NAc (Fig. 7c,d). *Nin* was also the only gene to possess missense mutation SNPs in a protein coding region predicted to alter protein function. In addition to these molecular data, biomedical literature further supports *Nin* as the strongest candidate gene underlying *Etanq1*. In the brain, Nin is known to function as a microtubule binding protein and an important regulator of neocortical axonal outgrowth and branching (Bouckson-Castaing *et al.* 1996; Mogensen *et al.* 2000; Srivatsa *et al.* 2015). Recent studies have reported an association between genetic variation within the *Nin* gene or altered expression patterns of *Nin* with a number of diseases and genetic disorders, including breast cancer (Olson *et al.* 2011), a type of skeletal dysplasia (Grosch *et al.* 2013), microcephalic primordial dwarfism (Dauber *et al.* 2012), polycystic ovary syndrome and non-alcoholic fatty liver disease (Baranova *et al.* 2013). This suggests that certain genetic variants of *Nin* might be a risk factor for multiple disease phenotypes.

As previously mentioned, Nin has been shown to interact with Gsk3β (Hong *et al.* 2000; Howng *et al.* 2004), a known ethanol-responsive gene (French & Heberlein 2009; Luo 2009; Wolen & Miles 2012). The *Gsk3b* is also a hub (highly inter-correlated) gene in a major ethanol-responsive gene network identified from the same microarray studies used in this report (Wolen *et al.* 2012). The two mouse Nin major protein isoforms share an overall ~78% sequence similarity to the human NIN protein, but strikingly, 94% similarity to the human phosphorylation binding site for Gsk3β suggesting conserved regulation of Nin by Gsk3β in mice (Howng *et al.* 2004). Interestingly, *Gsk3b* has also been shown to regulate anxiety-related behaviors in animals (Latapy *et al.* 2012; O'Brien *et al.* 2004) and *Gsk3b* is associated with mood and psychiatric disorders including schizophrenia and bipolar disorder (Jope & Roh 2006) and a known target of neuromodulators and psychotropic drugs (Li & Jope 2010). In total, our findings suggest *Nin* may contribute strongly to genetic variance in ethanol-anxiolytic like activity in the BXD panel through genotype-specific differential transcriptional regulation, protein function or both.

In conclusion, we used QTL mapping across the BXD RI panel to identify loci that influence anxiety-like responses to ethanol in the light–dark transition model of anxiety. The *Etanq1* QTL represents the first confirmed QTL influencing ethanol anxiolysis. We suggest *Nin* as a top priority candidate gene underlying *Etanq1* and future studies confirming *Nin's* role could provide valuable insight into the mechanisms of ethanol anxiolysis. Given the contribution of anxiety in drinking initiation and recidivism in alcoholics, *Nin* or other

candidate genes elucidated here may ultimately aid the identification of new pharmacotherapies for AUDs.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supplementary methods.

Figure S1: Factor analysis of post-ethanol behavioral phenotypes from the 0- to 5-min interval across the 27 provisional BXD strains and their progenitors shows that distance traveled in the light and TLA load on different factors.

Figure S2: The BXD strain-mean interval maps for TLA following saline (upper panel) or 1.8 g/kg ethanol (lower panel). Permuted genome-wide significance thresholds for falsely rejecting the null hypothesis (no linkage in the genome) at probabilities of 5% and 63% (corresponding to $P < 0.05$ and $P < 0.63$) are denoted by the dashed and dotted lines, respectively.

Figure S3: The BXD genotypes within the *Etanq1* support interval. Only BXD strains harboring recombination events within the region are included. Shapes along the left axis indicate whether a particular strain was assayed as part of the provisional mapping phase, replication study or fine-mapping effort. Black vertical lines along the *x*-axis indicate position of B6/D2 SNPs.

Figure S4: Epistatic interaction between QTL on Chr 1 and Chr 12. The DTL at 5-min in response to ethanol. Points indicate the average DTL based on progenitor alleles at rs13474399 (Chr 1) and rs3716547 (Chr 12). Two-way ANOVA for DTL based on progenitor allele indicates significant interaction between the Chr 1 and Chr 12 loci.

Figure S5: Comparison of DTL and TSL measures for ethanol anxiolytic-like activity. Figure displays DTL and TSL interval maps (a) or Spearman ranked correlations for ethanol-treated samples. Interval maps document very similar QTL profiles for the two behavioral measures but with slightly higher significance obtained at the *Etanq1* locus on Chr 12 for DTL. (b) The extremely tight rank order correlations between the two measures, consistent with the data in Fig. S1.

Table S1: The ANOVA results for light–dark box behavioral phenotypes in progenitor mouse strains following saline or ethanol treatment.

Table S2: Anxiety-related behavior strain means.

Table S3: The ANOVA for light–dark box behavioral phenotypes across BXD RI mouse strains in provisional QTL mapping study.

Table S4: *Etanq1* SNP analysis.

Table S5: Mixed model QTL analysis for DTL and TLA.