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Background: Alcohol dependence (AD) shows evidence for genetic liability, but genes influencing risk remain largely unidentified.

Methods: We conducted a genomewide association study in 706 related AD cases and 1,748 unscreened population controls from Ireland. We sought replication in 15,496 samples of European descent. We used model organisms (MOs) to assess the role of orthologous genes in ethanol (EtOH)-response behaviors. We tested 1 primate-specific gene for expression differences in case/control postmortem brain tissue.

Results: We detected significant association in *COL6A3* and suggestive association in 2 previously implicated loci, *KLF12* and *RYR3*. None of these signals are significant in replication. A suggestive signal in the long noncoding RNA *LOC339975* is significant in case:control meta-analysis, but not in a population sample. Knockdown of a *COL6A3* ortholog in *Caenorhabditis elegans* reduced EtOH sensitivity. *Col6a3* expression correlated with handling-induced convulsions in mice. Loss of function of the *KLF12* ortholog in *C. elegans* impaired development of acute functional tolerance (AFT). *Klf12* expression correlated with locomotor activation following EtOH injection in mice. Loss of function of the *RYR3* ortholog reduced EtOH sensitivity in *C. elegans* and rapid tolerance in *Drosophila*. The ryanodine receptor antagonist dantrolene reduced motivation to self-administer EtOH in rats. Expression of *LOC339975* does not differ between cases and controls but is reduced in carriers of the associated rs11726136 allele in nucleus accumbens (NAc).

Conclusions: We detect association between AD and *COL6A3*, *KLF12*, *RYR3*, and *LOC339975*. Despite nonreplication of *COL6A3*, *KLF12*, and *RYR3* signals, orthologs of these genes influence behavioral response to EtOH in MOs, suggesting potential involvement in human EtOH response and AD liability. The associated *LOC339975* allele may influence gene expression in human NAc. Although the functions of long noncoding RNAs are poorly understood, there is mounting evidence implicating these genes in multiple brain functions and disorders.

Key Words: Alcohol Dependence, *COL6A3*, *KLF12*, *LOC339975*, *RYR3*.

ALCOHOL DEPENDENCE (AD) is a major public health burden with substantial costs for individuals and societies (Rice, 1999). Despite robust evidence for genetic influences on risk (Cotton, 1979; Prescott et al., 2005a; Sigvardsson et al., 1996) and heritability estimates of ~50% (Ystrom et al., 2011), the genes influencing AD risk remain largely unidentified.

Prior genomewide association studies (GWAS) of AD and alcohol-related phenotypes in European samples detected novel signals in the *PECR* (Treutlein et al., 2009), *AUTS2* (Schumann et al., 2011), and uncharacterized *c15orf53* (Wang et al., 2013) genes. None of these novel signals were replicated. Two independent signals were detected and replicated around the long-standing candidate gene *ADH1B* (Frank et al., 2012; Gelernter et al., 2014). Three studies of Asian subjects identified signals at *ALDH2* (Baik et al., 2011; Quillen et al., 2014) and the *ADH1B* functional variant H47R (rs1229984) (Park et al., 2013), likely due to the frequencies of functional *ADH* and *ALDH* alleles in Asian populations.

To identify genes influencing alcohol-related phenotypes, we conducted a GWAS of AD. To maximize power, we studied an ethnically homogeneous sample of cases, affected siblings, and unscreened controls from Ireland, correcting for relatedness and lack of control screening analytically. We sought replication in 15,496 subjects of European descent (6,742 case/controls and 8,754 population samples).

Well-developed experimental approaches can test directly whether changes in candidate genes impact behavioral response to ethanol (EtOH) in vertebrate (Crabbe, 2002) and invertebrate (Grotewiel and Bettinger, 2015) model organisms (MOs). Orthologs of genes that affect simple EtOH responses in invertebrates also affect more complex EtOH responses in mammals, including measures of sensitivity and voluntary drinking (Bhandari et al., 2012; Kapfhamer et al., 2008; Liu et al., 2008). This approach has previously been successful in demonstrating functional relevance of genes implicated by GWAS in EtOH-response behaviors (Schumann et al., 2011).

To provide functional support for GWAS candidates, we tested whether perturbation of orthologous genes alters behavioral response to EtOH in *Caenorhabditis elegans* and/or *Drosophila*, depending on the presence of orthologous genes and the availability of genetic reagents and information. In vertebrate MO, we analyzed correlations between candidate gene expression and alcohol phenotypes bioinformatically in curated archival data from recombinant inbred (BXD) mouse lines and we tested the effect of pharmacological antagonism of 1 candidate gene product on motivation to self-administer EtOH in rats after chronic EtOH exposure. For 1 candidate gene with no ortholog outside of primates, we tested for expression differences in alcohol-dependent and control human postmortem brain tissue stratified by clinical status or genotype.

MATERIALS AND METHODS

GWAS Discovery Sample

Participants in the Irish Affected Sib Pair Study of Alcohol Dependence (IASPSAD) were recruited in Ireland and Northern Ireland between 1998 and 2002 (Prescott et al., 2005b). Briefly, probands were ascertained in community alcoholism treatment facilities and public and private hospitals. Probands were eligible for inclusion if they met DSM-IV criteria (American Psychiatric Association, 1994) for lifetime AD and if all 4 grandparents had been born in Ireland, Northern Ireland, Scotland, Wales, or England. Probands, siblings, and parents were interviewed by clinically trained research interviewers, most of whom had extensive clinical experience with alcoholism. We assessed lifetime history of AD using a modified version of the Semi-Structured Assessment of the Genetics of Alcoholism interview, version II (Bucholz et al., 1994), demographic characteristics, other comorbid conditions, alcohol-related traits, personality features, and clinical records. All participants provided informed consent. We included 815 probands and siblings in genotyping.

A total of 2,048 DNA samples from healthy, unpaid volunteers donating blood at the Irish Blood Transfusion Service and obtained from the Trinity Biobank at Trinity College Dublin were used as controls. Biobank controls were eligible if they denied any problems with alcohol or history of mental illness and if all 4 grandparents had been born in Ireland, Northern Ireland, Scotland, Wales, or England. Because of the sample source, controls were not formally screened for AD, but the lack of screening was addressed analytically (see GWAS Statistical Analyses). Information about age and sex was available for these subjects.

GWAS Genotyping, Quality Control, and Imputation

Genomic DNAs passing quality control (QC) standards were genotyped on Affymetrix v6.0 SNP arrays (Affymetrix, Santa Clara, CA). All arrays included in analysis passed standard QC measures. Genotypes were called using BEAGLECALL (Browning and Yu, 2009), followed by rigorous genotype QC. We imputed the 1000 Genomes Project (1000 Genomes Project Consortium, 2010) April 2012 integrated variant reference panel of 36.5 million simple nucleotide polymorphisms (SNPs) using IMPUTE2 (Howie et al., 2009). We converted posterior genotypic probabilities to allelic dosages using GenABEL (Aulchenko et al., 2007). After postimputation QC, 8,344,348 SNPs were available for analysis. Complete details of array, SNP and individual QC, BEAGLECALL genotyping, imputation, and postimputation QC and sample power are included in Appendix S1 and Figs S1–S4.

GWAS Statistical Analyses

We tested individual SNPs for association by Modified Quasi-Likelihood Score (MQLS) (Thornton and McPeek, 2007) because MQLS accepts genotypes in postimputation dosage format and can account for subject relatedness by using a kinship matrix calculated from pedigree data. Unscreened Biobank controls were coded as phenotype unknown. We included an estimated sex-weighted 8.9% population AD prevalence derived from population (Hasin et al., 2007) and unpaid Dutch blood donor (Atsma et al., 2011) data to account for lack of control screening. Varying this estimate from 0% to 12% gave a similar *p*-value distribution for all prevalence estimates. MQLS cannot include covariates. We used a threshold of $p \leq 5 \times 10^{-8}$ for genomewide significant (GWS) results. Odds ratios were not calculated due to the nonindependence of related case alleles. Secondary analytic approaches for gene-based, network, and geneset analyses are described in Supplementary Methods in Appendix S1.

Selection for Further Study

We calculated false discovery rate (FDR) *q*-values (Storey and Tibshirani, 2003) for all SNPs to select loci for further study. For replication and secondary analysis of discovery data, we used a threshold $q < 0.3$ ($p < 1 \times 10^{-5}$ for our *p*-value distribution, 30% of results are false discoveries) to maximize discovery potential. For functional studies, we included any loci achieving GWS signals in discovery or replication (*COL6A3*, *LOC339975*) and any loci with both $q < 0.1$ (10% of results are false discoveries) and prior evidence of involvement in EtOH phenotypes from human and/or MO studies (*KLF12*, *RYR3*).

Assessment of Variants for Potential Functional Impact

For variants with $q < 0.1$, we assessed variant potential to impact function either directly or via linkage disequilibrium (LD) with other variants using GWAS3D (Li et al., 2013). GWAS3D provides an adjusted *p*-value from Fisher's combined probability test incorporating the GWAS evidence of genetic association with evidence that the variant alters (i) coding or (ii) conserved sequence, or (iii) sites of long-range interactions, (iv) binding energy for known transcription factors or lies within (v) promoter, (vi) enhancer, or (vii) insulator elements from ENCODE and other published sources.

GWAS Replication

We conducted replication analyses in $N = 15{,}496$ European subjects from 3 AD case–control samples (Edenberg et al., 2010; Frank et al., 2012; Gelernter et al., 2014; Treutlein et al., 2009) and 1 population sample (Heath et al., 2011). Details of the individual samples, genotyping, and imputation are provided in Appendix S1. We conducted look-up analysis of replication SNPs in each sample and meta-analysis of all replication SNPs first in the 4 ascertained case–control samples, then adding the unascertained population sample, for which AD diagnoses were derived rather than directly assessed (Heath et al., 2011), using METAL (Willer et al., 2010). We weighted meta-analyses by sample size and direction of effect because MQLS does not generate standard errors. Many markers are nonindependent due to LD, so we assessed the 274 replication SNPs for independence using SNAP (Johnson et al., 2008) to determine the number of independent tests.

Invertebrate MO Studies

C. elegans *Studies*. A single, continuous acute exposure of *C. elegans* to 400 mM exogenous EtOH yields an internal concentration of 40 to 50 mM (Alaimo et al., 2012) (~200 mg/dl, within the range observed in humans after heavy drinking (Bond et al., 2010)). A concentration-dependent slowing of locomotion at 10-minute exposure (measuring *initial sensitivity*) is followed at ~30 minutes by an increase in speed of locomotion (measuring *AFT*) (Davies et al., 2003, 2004) despite an increase in the internal tissue concentration of EtOH (Alaimo et al., 2012). Both measures can be independently affected by the loss of individual genes (Bettinger et al., 2012; Bhandari et al., 2012; Davies et al., 2003, 2004; Kapfhamer et al., 2008; Mathies et al., 2015).

C. elegans *Strains*—*C. elegans* strains were maintained as previously described (Brenner, 1974). Strains used in these studies were wild-type N2 (var. Bristol), RB1603 *klf-3(ok1975)*, TR2170 *unc-68 (r1161)*, and TR2171 *unc-68(r1162)*.

RNAi Treatment—RNA interference (RNAi) induction and locomotion assays were performed as previously described (Kamath et al., 2001). Briefly, cultures of bacteria containing RNAi vectors corresponding to genes C16E9.1, C18H7.1, *cutl-23*, or empty vector (L4440) (Geneservice, Cambridge, UK) were plated on nematode growth media (NGM) plates with 1 mM IPTG, and allowed to grow at room temperature for 24 hours. Three to 5 fourth larval stage wild-type N2 worms were placed on the seeded plates and incubated at 20°C and allowed to produce F1 progeny, which were maintained on RNAi cultures to adulthood. First-day adult F1 progeny were collected and subjected to behavioral analysis.

Locomotion Tracking—Locomotion was assayed as previously described (Bettinger et al., 2012). Ten worms for each strain were tested in each assay, and we calculate the average of the speeds of the 10 worms in each iteration of the assay ($n = 1$). Comparisons were only made of animals tested simultaneously on the same plates. Briefly, NGM-containing plates were dried for 2 hours with lids off at 37°C, and then, copper rings were embedded in the surface of the plate to act as corrals. EtOH was added to the plates to a final concentration of 0 or 400 mM, the plates were sealed, and the EtOH was allowed to equilibrate for 2 hours. Worms were placed in the corrals and 2-minute movies were captured at 10 and 30 minutes of exposure using a Retiga 4000R camera (QImaging, Surrey, BC, Canada) on an Olympus SZX-7 microscope (Center Valley, PA). Movies were analyzed using ImagePro Plus (6.2) (MediaCybernetics, Rockville, MD) software. We derived 2 measures of EtOH response: *initial sensitivity* (depression of speed of locomotion at 10-minute exposure compared to the same strain untreated) and AFT (increase in speed at 30-minute exposure compared to the same strain at 10 minutes).

Statistical Analysis—Comparisons were made of animals tested in identical conditions. Relative speeds (treated/untreated × 100) were used in comparisons. We used Prism 5.0 software (GraphPad, La Jolla, CA) to perform 2-way ANOVA comparisons across time of EtOH exposure and genotype with Bonferroni post hoc comparisons at each time point to determine differences between genotypes. Development of AFT was tested using a paired 2-tailed *t*-test.

Drosophila *Studies*. Only 1 candidate locus (*RYR3*) had both a *Drosophila* ortholog and genetic reagents available. Detailed methods for *Drosophila* studies are provided in Supplementary Materials and Methods in Appendix S1.

Mammalian MO Studies

Mouse Studies. We queried selected candidate genes for localization to EtOH behavioral quantitative trait locus (QTL) intervals using the Mouse Genome Informatics (MGI) tool set. We queried published expression microarray and EtOH behavioral response data sets within the curated GeneNetwork web-based resource of genetic, phenotypic, and genomic data for evidence that basal candidate gene expression correlated with measured EtOH behavioral phenotypes in C57BL/6J × DBA/2J BXD mouse lines. All expression data were from Affymetrix M430 microarrays (Affymetrix, Santa Clara, CA). We included saline or air-treated BXD control whole brain (GN113), nucleus accumbens (NAc) (GN156), prefrontal cortex (PFC) (GN135), and ventral tegmental area (VTA) (GN228) (Wolen et al., 2012).

We used the single *Col6a3* probeset (1424131_at_A). We selected the most representative probeset based on GeneNetwork quality scores for *Ryr3* (1427427_at_A) and *Klf12* (1455521_at; we also assessed the better of the 2 probesets showing *Klf12* EtOH response (Wolen et al., 2012), 1439847_s_at). Robust multi-array average normalized expression data were used for Spearman rank correlation analyses with EtOH behavioral phenotypes. Identifiers of probesets and phenotypes used for specific analyses are shown in Results and in Fig. 5. Expression and phenotype data archived in GeneNetwork were produced in different laboratories and at different times; as a result, the N of BXD lines tested in our analyses varies across the tissues and phenotypes analyzed. We use the GeneNetwork default setting to retrieve the top 1,000 correlations for each probeset × tissue pair.

A total of 5,134 traits are present in GeneNetwork, but they are not all independent because of both the multiple related measures made within studies and the partial overlap of BXD lines used between studies. GeneNetwork developers suggest that Bonferroni correction for 200 independent traits approximates an FDR of 0.2 based on data in (Wang et al., 2016)). We apply a more stringent correction for 2,000 independent tests to maintain a conservative significance threshold of 0.05/2,000 = 2.5E-05.

Rat Studies. We studied the effect of pharmacological antagonism of ryanodine receptors on EtOH self-administration in rats. Detailed methods for this single rat study are provided in Supplementary Materials and Methods in Appendix S1.

Human Postmortem Studies

PFC and NAc tissues from 41 AD cases and 41 controls were provided by the New South Wales Tissue Resource Centre. Age, sex, ethnicity, brain weight, brain pH, postmortem interval (PMI), tissue hemisphere, cause of death, blood toxicology, smoking status, neuropathology, and liver pathology were provided for each subject. Confounding effects of all these covariates were controlled by analysis of covariance (ANCOVA; Table S7). Total RNA was isolated from 100 mg frozen tissue using the mirVana-PARIS kit (Life Technologies, Carlsbad, CA) following manufacturer's protocols. RNA concentration was measured using the Quant-iT Broad Range RNA Assay kit (Life Technologies). The RNA integrity number (RIN) was measured on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Quantitative real-time PCR (qRT-PCR) analyses were performed as previously described using SYBR Green (Riley et al., 2010) with primers spanning the *LOC339975* exon 2 to 3 junction. Samples with missing genotypes and outliers (±2 standard deviations [SD] from the mean) were omitted from further analysis.

RESULTS

Irish Case/Control Discovery Data Set

After all QC, the data set consisted of 706 probands and affected siblings, 464 (65.7%) male and 242 (34.3%) female, mean age 41.8 (SD 9.8) years, and 1,748 population controls, 755 (43.2%) male and 993 (56.8%) female, mean age 37.2 (SD 12.6) years, with 8,344,348 SNPs for analysis. The Manhattan plot for case/control analysis of AD is shown in Fig. 1. The QQ plot for the final data set after imputation and all QC is shown in Fig. 2.

Variation in COL6A3 *is Associated with AD*

In single marker analyses, 14 SNPs defining 2 independent genomic intervals achieve genomewide significance, 13 within the collagen VI A3 (*COL6A3*) gene on chromosome 2q37.2 (Fig. 3*A*), including the most significant SNP in our study, rs2256485, $p = 6.17 \times 10^{-9}$ (Table 1). *COL6A3* encodes an extracellular matrix (ECM) protein expressed in brain. Although there is no prior human association evidence for this gene, remodeling of the ECM has been strongly implicated in response to various drugs of abuse (Lubbers et al., 2014). The second interval is defined by a single SNP (rs150268941, $p = 1.65 \times 10^{-8}$, Table 1) on chromosome 3q26.31 (Fig. S5*A*).

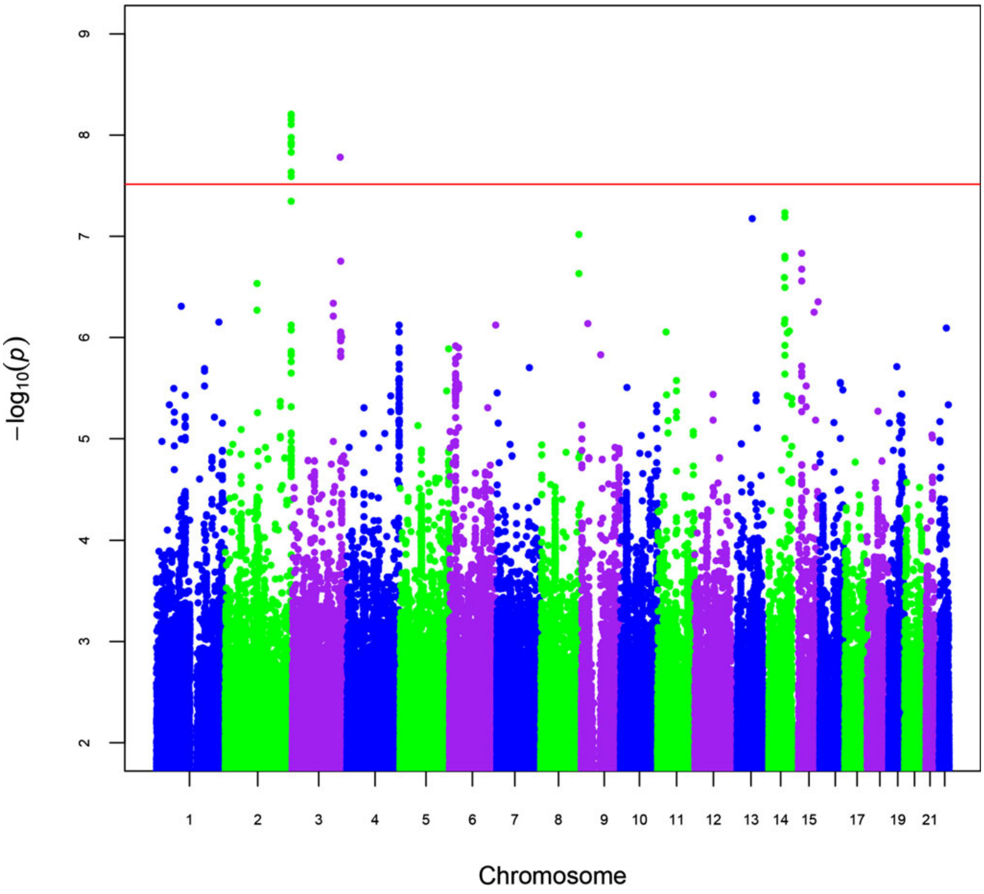

Fig. 1. Manhattan plot for case/control analysis of alcohol dependence. Horizontal red line indicates genomewide significance (5×10^{-8}).

Additional Loci are Suggestively Associated with AD

A total of 28 SNPs in 7 LD-independent loci had $q < 0.1$ (Table 1). These include SNPs in 2 loci with prior support from both human and MO alcohol studies (the ryanodine receptor 3 [*RYR3*] gene, chr. 15q14, rs4780153, $p = 1.47 \times 10^{-7}$, Fig. 3*B*, and the Krueppel-like factor 12 [*KLF12*] gene, chr. 13q22.1, rs117695261, $p = 6.63 \times 10^{-8}$, Fig. 3*C*). Four additional regions with suggestive signals are shown in Fig. S5*B*–*E*.

If the Fisher's combined probability test from GWAS3D provided increased evidence based on potential function of the test SNP or a SNP in LD with the test SNP, the combined *p*-value is also shown in Table 1. Of the 28 SNPs with $q < 0.1$ input to GWAS3D, 14 showed more significant combined *p*-values after incorporating evidence of function. Of these 14 results, 12 were based on evidence of functional impact for the GWAS target SNP itself. For the 2 sites where the increased evidence was based on putative functional impact of a variant in LD with the GWAS signal, the LD SNP rsID, r^2 with the GWAS target and functional evidence are shown. GWAS3D analysis yields strongly enhanced signals in *COL6A3* (rs2646265: $p = 1.47 \times 10^{-8}$, combined $p = 1.40 \times 10^{-12}$; rs2256485: r^2=1 with rs2646265, $p = 6.17 \times 10^{-9}$, combined $p = 6.18 \times 10^{-13}$) and *RYR3* (rs4780153: r^2=0.867 with rs2076954, $p = 1.47 \times 10^{-7}$, combined $p = 7.92 \times 10^{-10}$).

The signals at 3q26.31 (Fig. S5*A*) and *KLF12* (Fig. 3*C*) are with single imputed SNPs of low minor allele frequency (MAF). In 1000 Genomes Phase 3 data from U.K. subjects (GBR, British residents of England and Scotland), rs117695261 (MAF 0.03) in *KLF12* has no r^2>0.2 with any other SNP, consistent with the lack of correlated signals in Irish subjects. By contrast, rs150268941 on 3q26.31 is tagged by rs148750402, which was imputed and analyzed in our sample (0.034, $p = 0.00147$, $q = 0.754464$). Direct genotyping of rs150268941 also did not support the observed association (see Appendix S1), and we did not consider the chromosome 3q26.1 region further.

Human Replication

A total of 274 SNPs had discovery $q < 0.3$, and were included in replication. SNAP analysis indicated that 121 represent LD-independent tests, setting replication significance at 0.05/121 = 0.0004. Lookup replication and meta-analysis results arranged by chromosome and base pair are also shown for all 28 SNPs with $q < 0.1$ in Table 1. Table S1 shows these results for all 274 SNPs with $q < 0.3$. No

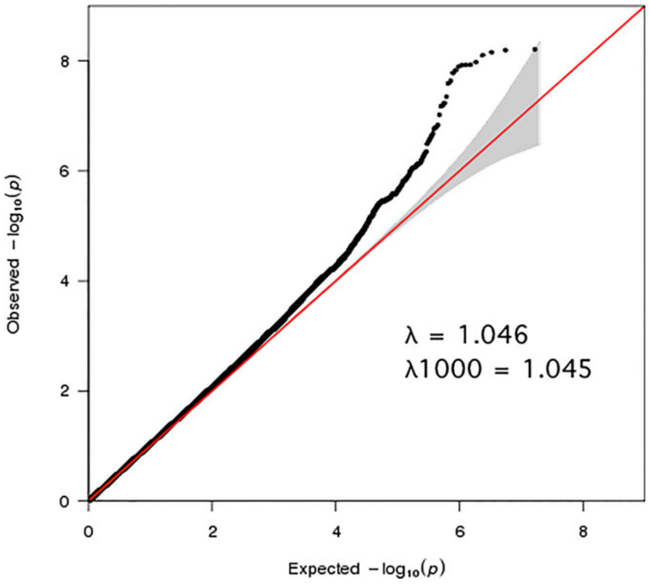

Fig. 2. QQ plot for final postimputation data set after all quality control and exclusions. Gray shading indicates 95% confidence interval for expected values. The postimputation lambda (1.046) and sample size-standardized lambda1000 (1.045) indicate there is little inflation of test statistics.

individual SNP achieved $p < 0.0004$ in any sample. In meta-analysis of the 4 case–control samples, 1 SNP in a second novel locus, the long noncoding RNA (lncRNA) gene *LOC339975* showed a GWS signal (chr. 4q35.2, rs11726136, $p = 7.52 \times 10^{-7}$, $q = 0.149$ in the discovery sample, Fig. 3*D*, and GWS $p = 4.20 \times 10^{-8}$ in meta-analysis of the 4 case/control samples, Table 1 and Table S1). Because of differences in ascertainment and severity of affection, we performed replication analyses including the Australian population sample separately; this signal was not significant when the Australian sample was included (Table S1).

We also assessed evidence in our discovery data for association with loci identified in prior AD GWAS in subjects of European ancestry. We detect modest signals in *PECR* (minimum $p = 0.0017$), *AUTS2* (minimum $p = 0.0009$), and ADH1B (minimum $p = 0.00166$), but none of these signals are with the SNPs originally reported (Table S2). We detect no evidence of signal in c15orf53 (minimum $p = 0.2058$).

Secondary Analyses

Results of secondary analyses of discovery GWAS data are shown in Table S3 (gene-based analyses), Fig. S6 and

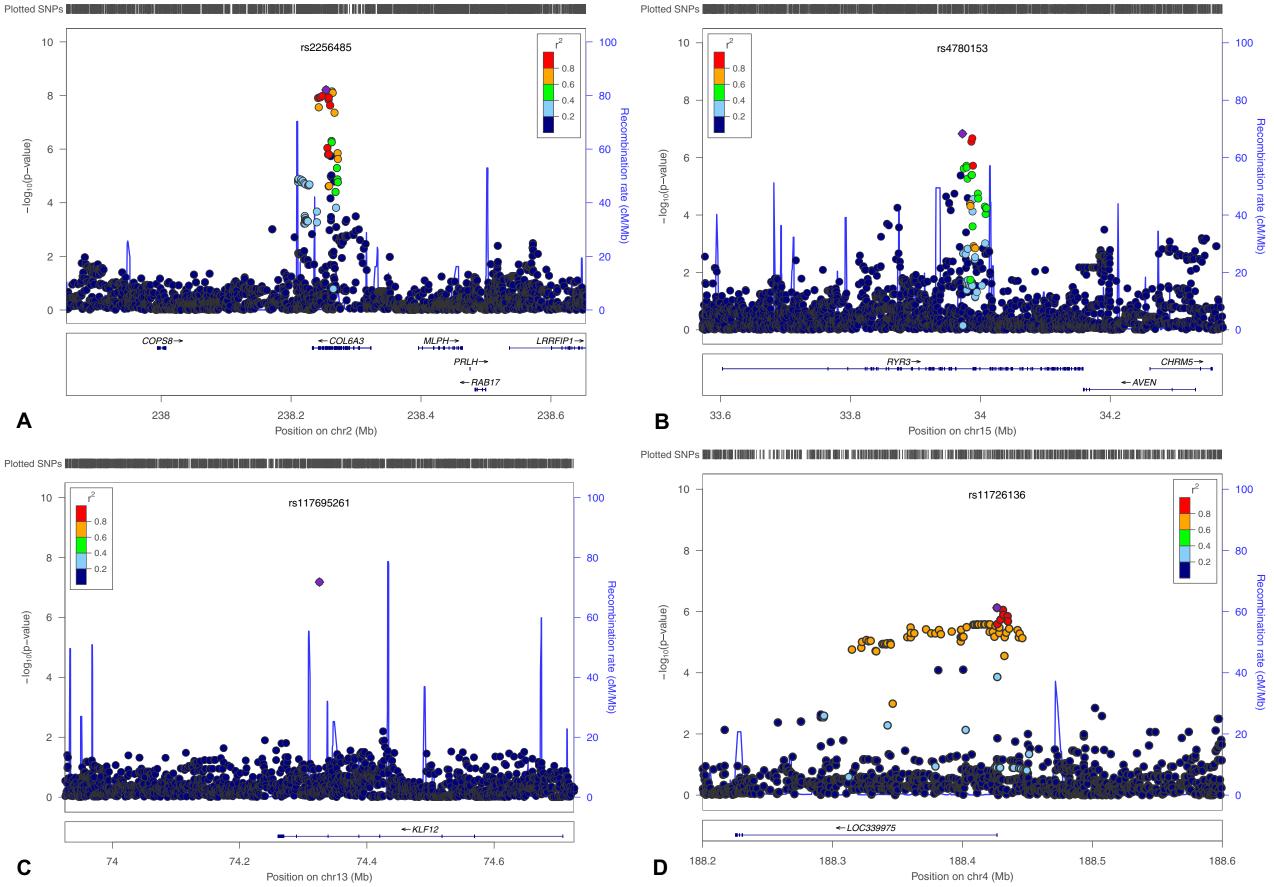

Fig. 3. LocusZoom plots of *COL6A3*, *RYR3*, *KLF12* and *LOC339975*. Genomewide significant results in *COL6A3* (**A**) and 3 other regions of suggestive association supported by additional data, *RYR3* (**B**), *KLF12* (**C**), and *LOC339975* (**D**).

Table 1. Results of GWAS Analyses

A. Irish discovery MQLS analysis						B. GWAS3D	C. Independent Case:Control Replication and Meta-analysis						D. Irish Plus Independent Case/ Control Meta-analysis				E. All Case/Control + Population Sample Meta-analysis				
Chr	bp	SNP	Gene	*p*	*q*	Combined p	COGA p	GESGA p	Yale/ Penn p	*N*	*Z*	Meta 1 p	*N*	*Z*	Meta 2 p	Sign Test	OZALC p	*N*	*Z*	Meta 3 p	Sign Test
2	114,506,030	rs72833664	SLC35F5	2.91E-07	0.088	5.01E-08^{T,E}	NA	NA	0.500	1,842	−0.67	5.00E-01	4,303	−4.32	1.56E-05	−??−	NA	4,303	−4.32	1.56E-05	−??−?
2	**238,240,863**	**rs56310758**	**COL6A3**	**1.26E-08**	**0.011**		0.043	0.431	0.521	6,742	−1.82	6.81E-02	9,203	1.38	1.67E-01	+−−−	0.556	17,957	1.40	1.61E-01	+−−−+
2	**238,241,881**	**rs75561681**	**COL6A3**	**2.57E-08**	**0.016**		0.010	0.433	0.518	6,742	2.07	3.85E-02	9,203	−1.11	2.68E-01	−+++	0.706	17,957	−1.06	2.91E-01	−+++−
2	**238,243,285**	**rs2270671**	**COL6A3**	**1.18E-08**	**0.011**	6.40E-10^{S,T,E,C}	0.063	0.424	0.250	6,742	−2.02	4.30E-02	9,203	1.22	2.24E-01	+−−−	0.545	17,957	1.29	1.96E-01	+−−−+
2	**238,244,559**	**rs10929226**	**COL6A3**	**1.17E-08**	**0.011**	1.37E−09^{T,E}	0.061	0.426	0.453	6,742	−1.82	6.87E-02	9,203	1.39	1.64E-01	+−−−	0.576	17,957	1.39	1.66E-01	+−−−+
2	**238,247,257**	**rs112523013**	**COL6A3**	**1.05E-08**	**0.011**	1.28E-09^{T,E}	0.067	0.352	0.213	6,742	2.16	3.11E-02	9,203	−1.11	2.66E-01	−+++	0.586	17,957	−1.18	2.39E-01	−+++−
2	**238,253,149**	**rs2646258**	**COL6A3**	**6.38E-09**	**0.011**	5.03E-10^{S,T,E,C}	0.049	0.352	0.200	6,742	−2.24	2.52E-02	9,203	1.09	2.77E-01	+−−−	0.622	17,957	1.12	2.62E-01	+−−−+
2	**238,253,930**	**rs2256485**	**COL6A3**	**6.17E-09**	**0.011**	6.18E-13^{*,S,T,E,C} (rs2646265, 1)	0.033	0.345	0.204	6,742	2.31	2.07E-02	9,203	−1.03	3.05E-01	−+++	0.602	17,957	−1.10	2.72E-01	−+++−
2	**238,257,013**	**rs2646265**	**COL6A3**	**1.47E-08**	**0.012**	1.40E-12^{S,T,E,C}	0.075	0.332	0.180	6,742	−2.21	2.70E-02	9,203	1.04	3.00E-01	+−−−	0.629	17,957	1.08	2.80E-01	+−−−+
2	**238,257,213**	**rs2646264**	**COL6A3**	**1.20E-08**	**0.011**		0.095	0.359	0.237	6,742	2.04	4.15E-02	9,203	−1.20	2.29E-01	−+++	0.632	17,957	−1.20	2.32E-01	−+++−
2	**238,259,387**	**rs2646261**	**COL6A3**	**2.31E-08**	**0.015**		0.081	0.445	0.184	6,742	2.04	4.13E-02	9,203	−1.14	2.53E-01	−+++	0.640	17,957	−1.15	2.52E-01	−+++−
2	**238,262,254**	**rs2645764**	**COL6A3**	**6.99E-09**	**0.011**		0.177	0.298	0.355	6,742	−1.85	6.45E-02	9,203	1.41	1.58E-01	+−−−	0.388	17,957	1.61	1.07E-01	+−−−+
2	**238,263,299**	**rs2645763**	**COL6A3**	**7.86E-09**	**0.011**		0.171	0.298	0.339	6,742	−1.87	6.10E-02	9,203	1.38	1.67E-01	+−−−	0.389	17,957	1.59	1.12E-01	+−−−+
2	**238,266,146**	**rs2645777**	**COL6A3**	**4.47E-08**	**0.026**		0.133	0.295	0.323	6,742	−1.96	5.06E-02	9,203	1.16	2.48E-01	+−−−	0.349	17,957	1.48	1.38E-01	+−−−+
3	**172,575,312**	**rs150268941**	**intergenic**	**1.65E-08**	**0.012**		0.202	NA	0.327	3,241	0.10	9.21E-01	5,702	3.78	1.55E-04	++?−	NA	5,702	3.78	1.55E-04	++?−?
8	175,451,990	rs142645748	NAALADL2	1.75E-07	0.065	3.91E-09^{T}	NA	NA	0.447	1,842	0.76	4.47E-01	4,303	4.45	8.66E-06	+??+	NA	4,303	4.45	8.66E-06	+??+?
8	137,895,174	rs79048468	intergenic	2.32E-07	0.079		0.525	NA	0.591	3,241	−0.01	9.90E-01	5,702	−3.41	6.57E-04	−−?+	0.095	14,456	−3.44	5.86E-04	−−?+−
8	137,907,672	rs117687198	intergenic	9.57E-08	0.043	6.03E-08^{T}	0.405	NA	0.515	3,241	0.06	9.55E-01	5,702	3.55	3.89E-04	++?−	0.098	14,456	3.52	4.38E-04	++?−+
13	74,325,506	rs117695261	KLF12	6.63E-08	0.031		0.825	0.804	0.054	6,742	1.09	2.77E-01	9,203	3.72	1.97E-04	+−++	NA	9,203	3.72	1.97E-04	+−++?
14	77,100,157	rs117727648	intergenic	2.53E-07	0.083	1.21E-08^{T}	0.892	NA	0.323	3,241	0.84	4.04E-01	5,702	4.02	5.91E-05	++?+	NA	5,702	4.02	5.91E-05	++?+?
14	77,641,605	rs113653607	intergenic	1.64E-07	0.064	1.39E−09^{T}	0.386	NA	0.591	3,241	−0.98	3.30E-01	5,702	2.71	6.83E-03	+−?−	0.084	14,456	3.04	2.36E-03	+−?−+
14	77,750,946	rs142687658	POMT2	6.46E-08	0.032	3.40E-08^{T,E}	NA	NA	0.651	1,842	0.45	6.51E-01	4,303	4.38	1.17E-05	+??+	0.190	4,303	4.38	1.17E-05	+??+?
14	77,755,799	rs150915059	POMT2	3.20E-07	0.093		NA	NA	0.643	1,842	0.46	6.43E-01	4,303	4.17	3.06E-05	+??+	NA	4,303	4.17	3.06E-05	+??+?
14	77,806,492	rs150017190	intergenic	1.57E-07	0.064	3.31E-09^{T,E}	NA	NA	0.619	1,842	0.50	6.19E-01	4,303	4.29	1.78E-05	+??+	0.149	4,303	4.29	1.78E-05	+??+?
14	77,828,430	rs56198483	TMED8	5.85E-08	0.032	3.09E-08^{T}	0.083	NA	0.539	3,241	0.68	4.98E-01	5,702	4.07	4.63E-05	++?−	0.878	5,702	4.07	4.63E-05	++?−?
15	33,972,420	rs4780153	RYR3	1.47E-07	0.063	7.92E-10^{*,T,E} (rs2076954, 0.87)	0.951	0.930	0.276	6,742	−0.61	5.45E-01	9,203	2.20	2.78E-02	++−−	0.730	17,957	1.33	1.82E-01	++−−−
15	33,984,848	rs1353348	RYR3	2.77E-07	0.087		0.761	0.985	0.164	6,742	0.60	5.47E-01	9,203	−2.14	3.22E-02	−−++	0.805	17,957	−1.36	1.74E-01	−−+++
15	33,986,294	rs939432	RYR3	2.11E-07	0.075		0.814	0.813	0.156	6,742	0.81	4.21E-01	9,203	−1.99	4.61E-02	−−++	0.779	17,957	−1.23	2.18E-01	−−+++
4	188,426,585	rs11726136	LOC339975	7.52E-07	0.149		**0.090**	**NA**	**0.014**	**3,241**	**−2.96**	**3.07E-03**	**5,702**	**−5.48**	**4.2E-08**	−−**?**−	0.779	14,456	−3.66	2.50E-04	−−?−−

A. SNPs from Irish discovery GWAS with *q*-values < 0.1 ($p < 1 \times 10^{-5}$) or significant replication results. Genomewide significant *p*-values $< 5 \times 10^{-8}$ are shown in bold. Chr: chromosome; bp: base pair position; SNP: dbSNP identifier; Gene: SNP is within or in LD with the given locus, or is intergenic. *p*: *p*-value in Irish discovery GWAS; *q*: FDR *q*-value in Irish discovery genomewide association studies; B. SNPs with functional evidence showing increased signal from GWAS3D combined *p*-value. All 28 SNPs with $q < 0.1$ were assessed with GWAS3D for evidence of functional impact of the target SNP or SNPs in high LD with the target. Combined p: GWAS3D Fisher's combined probability test *p*-value is shown where signal is increased by evidence for SNP function; Superscripts: *: functional evidence comes from a SNP in LD with the tested SNP (functional SNP rsID and r^2 are shown in parentheses below), otherwise functional evidence comes from the tested SNP itself; ^{S}: synonymous coding variant; ^{T}: significant change in transcription factor binding energy; ^{E}: SNP lies within an ENCODE-defined enhancer region; ^{C}: evidence of conservation from GERP score. C. Lookup replication and meta-analysis of independent case/control samples. COGA, GESGA, and Yale/Penn p: lookup replication *p*-values in COGA, GESGA, and Yale/U Penn samples, respectively; NA indicates SNP was not available in the specific data set; *N*, *Z*, and Meta 1 p: meta-analysis sample *N*, *Z*-score and *p*-value, respectively, in meta-analysis of independent case/control samples. D. Meta-analysis of all case/control samples including the Irish discovery sample. N, Z, and Meta 2 p: meta-analysis sample *N*, Z-score and *p*-value, respectively, in meta-analysis of Irish plus independent case/control samples; Sign test: direction of effect in Irish, COGA, GESGA, and Yale/U Penn samples, respectively; signs indicate that direction of effect is same (+), opposite (−) or data point unavailable (?) in each sample. E. Meta-analysis of all samples including the Australian population sample. OZALC *p*: *p*-value in look-up replication in the Australian population sample; *N*, *Z*, and Meta 3 p: meta-analysis sample *N*, *Z*-score and *p*-value, respectively, in meta-analysis of 4 case/control samples plus the Australian population sample; Sign test: direction of effect in Irish, COGA, GESGA, Yale/U Penn and OZALC samples, respectively; signs indicate that direction of effect is same (+), opposite (−), or data point unavailable (?) in each sample.

Table S4 (network analyses) and Table S5 (geneset analyses). Gene-based analyses assess over representation of case–control allele frequency differences in all SNPs within defined genic regions (with LD pruning or weighting as described in Supplementary Materials and Methods in Appendix S1). The hybrid set-based test incorporated in Knowledge-Based Mining System for Genome-Wide Genetic Studies (KGG) shows strong gene-based association of AD with *COL6A3* ($p = 7.30 \times 10^{-9}$, $q = 0.00016$), *RYR3* ($p = 2.62 \times 10^{-7}$, $q = 0.0029$), and *LOC339975* ($p = 5.31 \times 10^{-6}$, $q = 0.0248$) (Table S3).

Selection for Functional Studies

We undertook functional studies of 4 candidate loci, *COL6A3,* and *LOC339975* based on the GWS signals in discovery or replication analyses and *KLF12* and *RYR3* based on discovery $q < 0.1$ and prior evidence of implicating these loci in alcohol-related phenotypes (reviewed below). We used established behavioral paradigms to test orthologs of *COL6A3*, *KLF12,* and *RYR3* for effects on behavioral responses to EtOH in *C. elegans* and *Drosophila* where orthologous genes were present in the models and genetic reagents and information were available. We analyzed correlation between brain gene expression and alcohol-related phenotypes in BXD mouse data for all 3 genes. All these studies are summarized in Table 2, and we report here the results of all studies performed. We additionally tested the effect of the ryanodine receptor antagonist dantrolene on EtOH self-administration in rats. The fourth gene, *LOC339975*, is primate-specific and was taken forward for study in human postmortem brain.

COL6A3*: Regulation of EtOH Sensitivity in* C. elegans *and Correlation with Handling-Induced Convulsions in Mice*

We tested 3 *C. elegans* genes with equally high orthology to human *COL6A3* for effects on initial sensitivity and AFT. RNAi knockdown of *C16E9.1* decreased initial sensitivity compared to control RNAi animals ($p < 0.05$, Fig. 4*A*) but did not affect the development of AFT. RNAi knockdown of the other *COL6A3* orthologs (*C18H7.1* and *cutl-23*) produced no significant differences in either measure (Fig. 4*B,C*). Statistics and uncorrected basal speed data for all *C. elegans* experiments are shown in Table S6.

In mice, *Col6a3* is located within the Alcw5 QTL interval (MGI:3037048) for handling-induced convulsions (HIC) following 72-hour EtOH vapor exposure (Bergeson et al., 2003). The Alcw5 QTL maps to 39.16 centiMorgans (cM) on mouse chromosome 1, with a support interval of 28 to 47 cM. After converting cM to megabase pairs (Mb) for the latest version of the mouse genome (GRCm38/mm10), this yields a physical location of the Alcw5 QTL peak of 75.57 Mb and a QTL support interval of 62.12 to 107.66 Mb. *Col6a3* is located at mouse chr1:90766860–90843971, within the defined Alcw5 QTL support interval.

In GeneNetwork, the strongest correlation observed for mouse *Col6a3* basal whole brain expression (GN113, probeset 1424131_at_A) is with total HIC score (sum of baseline subtracted HIC at 4, 6, and 7 hours) after 4 g/kg IP EtOH in males (Philip et al., 2010) (trait 11,382, correlation rank = 1, rho = 0.959, $p = 1.05 \times 10^{-9}$, $N = 13$ strains, Fig. 5*A*), which surpasses our Bonferroni-corrected significance level of $p = 2.5 \times 10^{-5}$. HIC at 7 hours in males (trait 11,380, correlation rank = 2, rho = 0.835, $p = 3.11 \times 10^{-6}$, $N = 18$ strains) and in males and females (trait 11,894, correlation rank = 3, rho = 0.780, $p = 5.11 \times 10^{-5}$, $N = 18$ strains) (Philip et al., 2010) are also strongly correlated with *Col6a3* expression, and all 3 HIC measures are highly correlated (phenotypic rho = 0.91 to 0.95). *Col6a3* expression correlated negatively with 2-bottle choice EtOH preference (Phillips et al., 1994) (trait 10,479, correlation rank = 67, rho = −0.569, $p = 0.0124$, $N = 18$ strains, Fig. 5*B*). While this is not significant after multiple test correction, it is consistent with the expectation that factors increasing HIC will decrease voluntary consumption (Metten et al., 1998).

KLF12: Regulation of AFT to EtOH in C. elegans *and Gene Expression Correlation with Locomotor Activity in Mice*

There is significant prior evidence for a role of *KLF12* in EtOH-response behaviors across species. In BXD mice, *Klf12* is regulated by acute EtOH in PFC, NAc, and VTA, and is a hub in a network of EtOH-responsive genes (Wolen et al., 2012). In humans, KLF12 acts in combination with the co-repressor CTBP1 (Schuierer et al., 2001), and in *C. elegans*, the *ctbp-1* gene is required for the development of AFT (Bettinger et al., 2012). The closest *C. elegans* ortholog to human *KLF12* is *klf-3*, and this evidence collectively suggests KLF-3 is likely to act together with CTBP-1 to regulate AFT in worms.

We tested a strong loss-of-function allele in *klf-3*. There was no difference in initial sensitivity between wild-type N2 and *klf-3(ok1975)* mutants (Fig. 4*D*). While wild-type N2 animals demonstrated normal AFT at 30 minutes, *klf-3* mutants failed to develop AFT (Fig. 4*D*, *t*-test of degree of speed recovery between 10 and 30 minutes, 400 mM EtOH: N2 versus *klf-3(ok1975)*, $t_3 = 8.99$, $p < 0.001$). These data strongly suggest that the transcriptional regulation provided by KLF-3 is required for the development of AFT in worms.

Based on patterns of regulation of mouse *Klf12* by EtOH (Wolen et al., 2012), we analyzed correlation between basal *Klf12* expression in mouse PFC (GN135), NAc (GN156), VTA (GN228), and whole brain (GN113) data sets and BXD panel phenotypes in GeneNetwork. The strongest correlations observed for basal *Klf12* expression in PFC (GN135, probeset 1455521_at) were with locomotor activity 0 to 5 minutes (trait 11,708, correlation rank = 1, rho = 0.756, $p = 1.69 \times 10^{-5}$, $N = 22$ strains, Fig. 5*C*) and 0 to 20 minutes (trait 11,705, correlation rank = 3,

Table 2. Summary of Evidence for 10 GWAS Loci with $q < 0.1$

A. GWAS and literature			B. *Caenorhabditis elegans* studies					C. *Drosophila* studies					D. Mouse bioinformatics analyses							E. Rat studies				
Locus	*q*	Support	Ortholog	Reagent	Tested	PT	*p*	Ortholog	Reagent	Tested	PT	*p*	Ortholog	Tissue	PT	*N*	Rho	Rank	*p*	Ortholog	Reagent	Tested	PT	*p*
SLC35F5	0.088	No																						
COL6A3	0.011	GWS	***C16E9.1***	**RNAi**	**Yes**	**IS**	**<0.05**	No					***Col6a3***	**Brain**	**HIC**	**13**	**0.959**	**1**	**1.05E-09**	Col6a3	n/a	No		
			C18H7.1	RNAi	Yes	None								Brain	2BC	18	−0.569	67	0.0124					
			cutl-23	RNAi	Yes	None																		
ECT2	0.012	No																						
SPATA16	0.012	No																						
NAALADL2	0.065	No																						
KLF12	0.031	Prior	***klf-3***	***ok1975***	**Yes**	**AFT**	**<0.001**	No					***Klf12***	**PFC**	**LA_5**	**22**	**0.756**	**1**	**1.69E-05**	Klf12	n/a	No		
														PFC	LA_{20}	22	0.747	3	2.54E-05					
														NAc	AFT	25	0.560	29	0.003					
														Brain	None									
														VTA	None									
POMT2	0.032	No																						
TMED8	0.032	No																						
RYR3	0.063	Prior	***unc-68***	***r1161***	**Yes**	**IS**	**<0.01**	***RyR***	***d03686***	**Yes**	**RT**	**<0.0001**	*Ryr3*	Brain	None					**Ryr3**	**Dantrolene**	**Yes**	**ESA**	**<0.05**
				r1162	**Yes**	**IS**	**<0.01**		***k04913***	**Yes**	**RT**	**<0.0001**												
LOC339975	0.149	GWS	No					No					No							No				

Shaded rows indicate the 4 human loci taken forward for further study. Bold text indicates significant results for the test displayed, with significance levels for each analysis as described in the text. A. Summary of genomewide association studies (GWAS) or literature evidence for loci with genomewide significant results or $q < 0.1$ (Locus: Gene symbol for each locus; *q*: FDR *q*-value from the GWAS analysis for loci with $q < 0.1$ shown; Support: No: no genomewide significant results in this study OR suggestive results in this study but no prior support, and locus not considered further; GWS: locus displays genomewide significant results in primary analysis (*COL6A3*) or replication (*LOC339975*); Prior: locus displays $q < 0.1$ in this study AND has prior support from the literature. Only loci marked GWS or PRIOR were taken forward for study in model organisms. B, C, E. Summary of *C. elegans* (B), *Drosophila* (C) and rat (E) studies (Ortholog: gene symbol for ortholog(s) of the 4 loci taken forward from A; No indicates no ortholog present and no further consideration of that gene in that species); Reagent: mutant strain identifier for existing loss-of-function mutations, RNAi indicating availability of RNAi reagents for the gene, or name of pharmacological antagonist; Tested: Yes: tested in this study; PT: phenotype affected by genetic manipulation (IS: initial sensitivity; AFT: acute functional tolerance; RT: rapid tolerance; ESA: ethanol self-administration; None: no phenotype affected); *p*: *p*-value for tests showing a significant effect on phenotype. D. Summary of mouse bioinformatics studies (Ortholog: mouse gene symbol for ortholog(s) of the 4 loci taken forward from A; No indicates no ortholog in mice); Tissue: tissue tested for correlation between basal gene expression and behavioral phenotypes (Brain: whole brain; PFC: prefrontal cortex; NAc: nucleus accumbens; VTA: ventral tegmental area); PT: phenotype correlated with basal gene expression (HIC: handling-induced convulsions; 2BC: voluntary ethanol consumption in 2 -bottle choice design; LA_5, LA_{20}: locomotor activation 0 to 5 minutes or 0 to 20 minutes (respectively) after ethanol administration; None: no phenotype correlated with basal gene expression); N: N of BXD strains with available data included in analysis; Rho: Spearman rank correlation coefficient; Rank: correlation rank; *p*: *p*-value for tests showing a significant effect on phenotype.

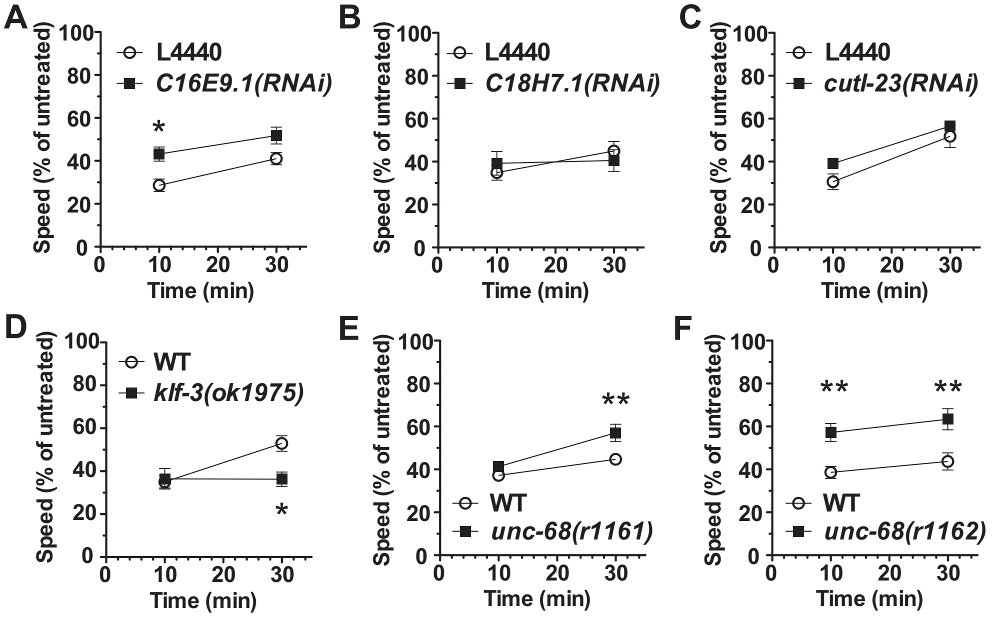

Fig. 4. Mutations in *Caenorhabditis elegans* orthologs of human candidate genes cause ethanol (EtOH)-response phenotypes. Speed of locomotion was measured at 10 and 30 minutes, and expressed as a percent of the untreated control speed. Control worms were tested simultaneously on the same plates. Worms were treated with 400 mM exogenous EtOH. The waxy cuticle of worms excludes most of the exogenous EtOH; tissue concentrations are approximately 12% of the exogenous dose (~48 mM). (**A, B, C**) RNAi-induced gene knockdown reduced sensitivity to EtOH for *C16E9.1* (a *COL6A3* ortholog) but not for 2 other orthologous genes relative to untreated worms. (**D**) Loss of function of the *KLF12* ortholog, *klf-3*, prevented the development of acute functional tolerance between the 10- and 30-minute time points relative to wild-type N2. (**E, F**) *unc-68* mutant animals demonstrate reduced sensitivity to EtOH relative to wild-type N2. Statistical significance is shown for 2-way ANOVA followed by post hoc comparisons across genotypes ($*p < 0.05$, $**p < 0.01$).

rho = 0.747, $p = 2.54 \times 10^{-5}$, $N = 22$ strains) after 2.25 g/kg IP EtOH in females (Philip et al., 2010). The first result remains significant, and the second falls just below significance after Bonferroni correction. Basal *Klf12* expression in mouse NAc (GN156, probeset 1439847_s_at) was positively correlated with AFT (Kirstein et al., 2002) (trait 10,348, correlation rank = 29, rho = 0.560, $p = 0.003$, $N = 25$ strains, Fig. 5*D*). While not significant after Bonferroni correction, this is consistent with the failure to develop AFT in *C. elegans klf-3* mutants.

RYR3: Regulation of Initial Sensitivity to EtOH in C. elegan*s, Rapid Tolerance to EtOH in* Drosophila*, and Motivation to Self-Administer Alcohol in Rats*

Previous studies have implicated ryanodine receptors (RyR) in EtOH phenotypes: In humans, *RYR3* was implicated in a GWAS of alcohol response (Joslyn et al., 2010). Ryr1 and Ryr2 up-regulation in mouse brain is observed following acute exposure to multiple drugs including alcohol (Kurokawa et al., 2010, 2013) and behavioral changes like conditioned place preference and withdrawal expected following acute exposure are blocked by the RyR antagonist dantrolene (Kurokawa et al., 2010, 2013).

C. elegans has 1 RyR gene, *unc-68*. We tested the effect of EtOH on 2 strains carrying different *unc-68* mutations (*r1161* and *r1162*). Loss of *unc-68* confers reduced sensitivity to EtOH (minimum $p < 0.001$ for *r1162*, Fig. 4*E*, *F*). We also observed consistent effects of mutations in 2 additional genes with products involved in calcium regulation and known to interact with UNC-68 (Supplementary Materials and Methods, Supplementary Results, and Fig. S7 in Appendix S1).

There is a single RYR3 ortholog in *Drosophila*, *RyR*. We found that 2 insertional mutations that cause partial loss of function in *RyR* reduce the development of rapid tolerance to EtOH with no obvious effects on initial sensitivity (Supplementary Materials and Methods, Supplementary Results and Fig. S8 in Appendix S1).

The mouse *Ryr3* gene is localized to the support intervals for a complex group of EtOH behavioral QTL mapped to Chr 2 but *Ryr3* basal whole brain expression (GN113; probeset 1427427_at) is not strongly correlated with EtOH-related phenotypes.

The availability of dantrolene, a pharmacological antagonist of ryanodine receptors, allowed us to assess the effect of antagonism of RyRs on the complex behavior of EtOH self-administration in rats. We found that in rats, dantrolene dose dependently reduced motivation to self-administer EtOH after 50 contiguous days of chronic EtOH self-administration (Supplementary Materials and Methods, Supplementary Results, and Fig. S9 in Appendix S1).

rs11726136 Genotype Alters LOC339975 *Expression in Human NAc*

LOC339975 shows homology only with sequences from other primates. To assess the potential functional impact of

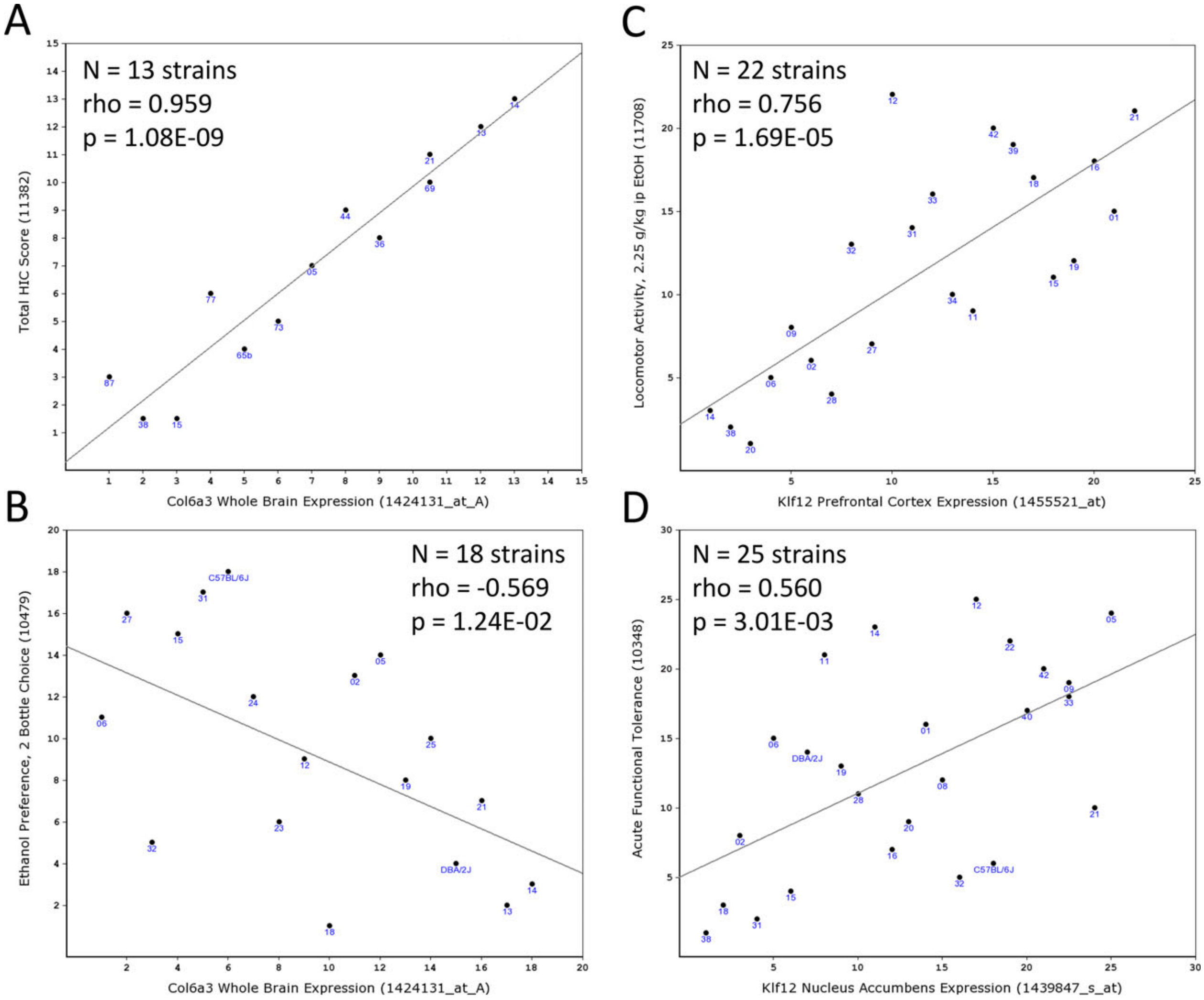

Fig. 5. Bioinformatic analysis of correlation of *Col6a3*, *Klf12,* or *Ryr3* expression with ethanol (EtOH) behaviors in C57BL/6J x DBA/2J recombinant inbred (BXD) mouse lines**.** The GeneNetwork (GN) web-based analysis platform was used to identify correlations between basal expression levels of *Col6a3*, *Klf12,* or *Ryr3* and EtOH behaviors. **Panels A–D** display Spearman ranked order correlations between microarray gene expression (*x*-axis) and behavioral phenotypes (*y*-axis). *Col6a3* (probeset 1424131_at) in GN113 whole brain expression data set showed significant positive correlation with total handling-induced convulsion (HIC) score (sum of baseline subtracted HIC at 4, 6 and 7 hours) after 4 g/kg intraperitoneal (IP) EtOH in males (GN record 11382; **Panel A**) and negative correlation with EtOH 2-bottle choice voluntary consumption (GN record 10479; **Panel B**). *Klf12* basal expression in prefrontal cortex (GN135, probeset 1455521_at) was significantly positively correlated with locomotor activity 0 to 5 minutes after 2.25 g/kg IP EtOH (GN record 11708; **Panel C**) and in nucleus accumbens (GN156, probeset 1439827_s_at) was positively correlated with EtOH acute functional tolerance (GN record 10348; **Panel D**).

alleles at rs11726136, we therefore tested AD case and control postmortem tissue from PFC and NAc for differences in *LOC339975* expression by clinical status or genotype. The final numbers available for analysis were for PFC, 28 cases and 30 controls and 50 reference allele T/T and 5 T/G genotypes, and for NAc, 34 cases and 35 controls and 58 T/T and 7 T/G genotypes (Table S7).

We included age, sex, ethnicity, brain weight, brain pH, PMI, tissue hemisphere, cause of death, blood toxicology, smoking status, neuropathology, and liver pathology as covariates in analysis. Neuropathology and brain weight were both significantly associated with expression level in the NAc; no covariates were associated with expression level in the PFC (Table S7). We detected no difference in expression level between AD cases and controls in either NAc (p = 0.75) or PFC (p = 0.23) (Fig. 6*A,B* and Table S7*A,B*). After controlling for covariates, expression of *LOC339975* is significantly reduced in carriers of the associated nonreference allele in NAc (p = 0.003, Fig. 6*C*, Table S7*C*) but did not differ by genotype in PFC (p = 0.54, Fig. 6*D*, Table S7*D*). Alternative regulation in NAc and PFC is consistent with the presence of several distinct transcription factor binding sites upstream of lncRNA genes (Alam et al., 2014). Although we do not detect case/control differences in expression, our data suggest the associated allele of rs11726136 has functional consequence based on the reduced

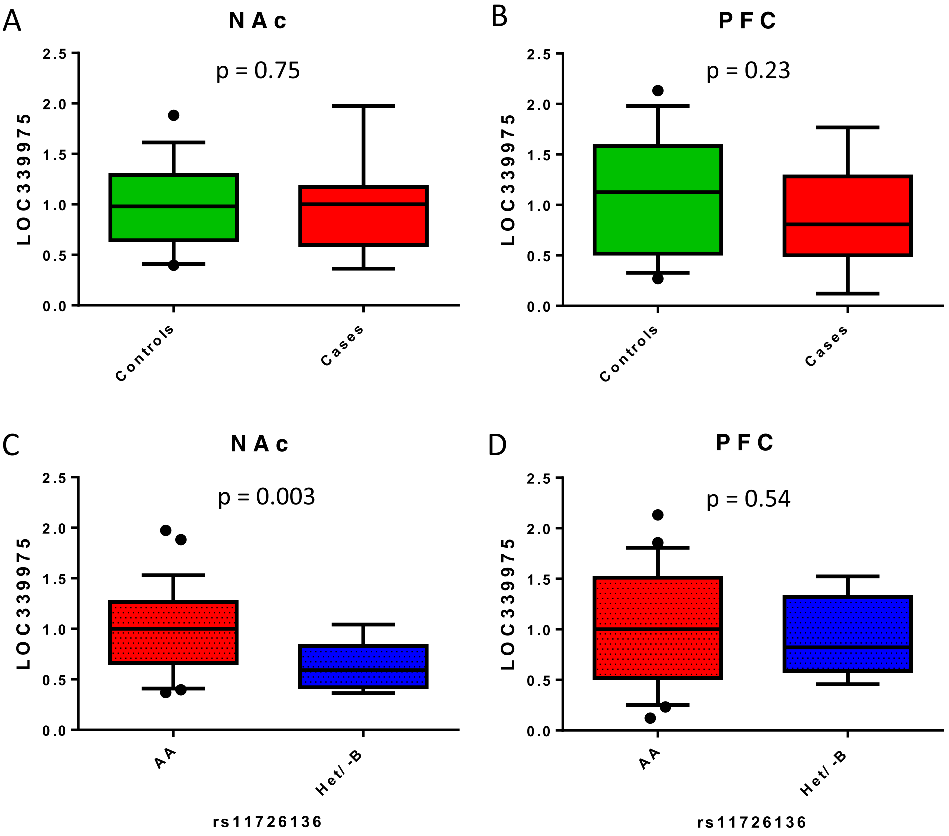

Fig. 6. Expression of long noncoding RNA *LOC339975* in human postmortem nucleus accumbens (NAc) and dorsolateral prefrontal cortex (PFC) stratified by diagnostic status and by genotype. Control mean RIN was 5.6 (SD = 1.8), case mean RIN was 5.8 (SD = 1.5), and did not differ between cases and controls (Mann–Whitney $p = 0.78$). Samples that did not amplify (NAc, $N = 13$; PFC, $N = 24$) and 4 samples missing rs11726136 genotypes were excluded. Case:control comparisons of *LOC339975* expression in (**A**) 34 case and 35 control NAc samples, and (**B**) 28 case and 30 control PFC samples. There were no significant differences in expression between diagnostic groups. Genotypic comparisons of *LOC339975* expression in (**C**) 58 reference (T/T) homozygote and 7 T/G heterozygote NAc samples, and (**D**) 50 T/T homozygote and 5 T/G heterozygote PFC samples. While no significant differences in expression by genotype were observed in the PFC, NAc expression was significantly reduced in carriers of the associated nonreference G allele compared to reference allele homozygotes ($F = 9.72$, $p = 0.003$).

LOC339975 expression observed in the NAc in heterozygotes.

DISCUSSION

We identified 2 novel GWS association signals in *COL6A3* and *LOC339975*, and suggestive signals in a number of loci, including 2 genes with prior support, *KLF12* and *RYR3*. *COL6A3*, *RYR3*, and *LOC339975* are further supported by gene-based analyses (Table S3). We detect evidence of human replication only for *LOC339975* but we observe consistent evidence across multiple MOs that *COL6A3*, *KLF12,* and *RYR3* orthologs modulate behavioral response to EtOH. Human postmortem studies show that the AD-associated allele reduces *LOC339975* expression in NAc.

Collagen VI A3 (COL6A3)

Collagen IV (Joslyn et al., 2010) and VIII (Edenberg et al., 2010) genes were implicated in EtOH response and AD. *COL6A3* is located in a QTL interval for alcohol withdrawal identified in the IASPSAD sample (Kuo et al., 2006). Although this signal did not replicate, our GWS association with potentially functional SNPs (GWAS3D results, Table 1) is supported by the reduced sensitivity after RNAi knockdown in *C. elegans* (Fig. 4*A*), mapping to the Alcw5 QTL for HIC in mice and the strong correlation between expression and HIC in BXD lines (Fig. 5*A*). *COL6A3* encodes a component of the ECM, and there is mounting evidence (Lubbers et al., 2014) that multiple substances of abuse increase ECM remodeling and that remodeling is required for the expected behavioral changes following exposure. EtOH dose dependently induces tissue plasminogen activator (tPA), required for ECM remodeling, which enhances EtOH reward (Bahi and Dreyer, 2012). Withdrawal seizures are reduced in tPA-deficient mice following chronic EtOH administration (Pawlak et al., 2005). Inhibition of proteolytic enzymes that degrade the ECM block escalated responding during acute withdrawal in dependent animals (Smith et al., 2011). Collectively, these results argue that ECM structural components (like COL6A3) and remodeling enzymes are important determinants of EtOH-induced neuroadaptation. We hypothesize that *Col6a3* may underlie the Alcw5 HIC QTL.

Krueppel-Like Factor 12 (KLF12)

Klf12 is regulated by acute EtOH in mouse brain and is a hub in a network of EtOH-responsive genes (Wolen et al., 2012) including many implicated in EtOH response (e.g., *Grm3* (Gass and Olive, 2008), *Kcnma1* (Davies et al., 2003), and *Gsk3b* (French and Heberlein, 2009)). Orthologs of *KLF12* (Fig. 4*D*) and its binding partner *CTBP1* (Bettinger et al., 2012) are required for the development of AFT in *C. elegans*. The targets of KLF12 regulation are not yet known, but the convergent evidence argues strongly they are central to acute EtOH response and potentially relevant to AD risk.

Ryanodine Receptor 3 (RYR3)

RYR3 was implicated in a GWAS of EtOH response (Joslyn et al., 2010) and our observed association may be driven by functional SNPs (GWAS3D analysis, Table 1). In *C. elegans*, loss of the single RyR gene *unc-68* reduces initial sensitivity to EtOH (Fig. 4*E,F*). This mutation would be predicted to decrease intracellular Ca^{2+}. Consistent with this observation, we found that mutations in *csq-1,* which would be predicted to increase intracellular Ca^{2+} concentration, increase initial sensitivity (Fig. S7*A*). Mutations in the ether-a-go-go-related potassium channel gene *unc-103,* which has a genetic interaction with unc-68, also decrease sensitivity to EtOH (Fig. S7*D*). In *Drosophila*, reduction in function of the *RyR* gene blunts rapid tolerance (Fig. S8*B*).

The RyR antagonist dantrolene reduces cocaine (Kurokawa et al., 2011) and methamphetamine (Kurokawa et al., 2010) induced conditioned place preference, EtOH withdrawal symptoms (Kurokawa et al., 2013), and motivation to self-administer EtOH after chronic exposure in a dose-dependent manner in rats (Fig. S9), but these studies are limited by the nonspecificity of dantrolene, an antagonist of both ryanodine and inositol triphosphate receptors, the latter functioning upstream of RyR activation. In myocytes, RyRs provide the Ca^{2+} ions that activate BK channels (Lifshitz et al., 2011), which have strong effects on EtOH response in vivo and in vitro (Davies et al., 2003; Martin et al., 2008). EtOH modulates BK channel function in a calcium-dependent manner (Liu et al., 2008), and we hypothesize that RyRs may be involved in this calcium-dependent modulation of BK channel function.

lncRNA LOC339975

lncRNA are of emerging importance in the function and dysfunction of the brain (Roberts et al., 2014). Expression of the estimated 25 to 50K lncRNA genes in the human genome (Hangauer et al., 2013) is widespread in the brain and highly regulated (Guttman et al., 2011; Mercer et al., 2008). lncRNA are implicated in multiple neurodevelopmental, neurodegenerative, and neuropsychiatric diseases, including schizophrenia (Barry et al., 2014), Alzheimer's (Faghihi et al., 2008), autism (Kerin et al., 2012), and neuronal excitability and epilepsy (Barry et al., 2017). A recent GWAS of AD detected GWS association in the lncRNA *LOC100507053* (Gelernter et al., 2014). While this signal is part of the larger *ADH* gene cluster signal reported and associated SNPs could only be analyzed in African Americans due to MAF or imputation information, *LOC100507053* is anti-sense to multiple *ADH* genes, and has potential to regulate their expression. Although the role of these transcripts remains unclear, the growing number of reported associations argues that lncRNA genes are also important in human health and disease. Our data suggest the associated allele of rs11726136 may have functional consequence based on the reduced *LOC339975* expression observed in the NAc.

Limitations

Unscreened controls: While the use of unscreened controls is common in studies of traits with low population prevalence, unscreened controls are not ideal for traits with the high population prevalence of AD (Wellcome Trust Case Control Consortium, 2007). However, the most likely impact of this lack of screening is for a proportion of controls to be unrecognized and phenotypically misclassified cases. This will reduce the contrast between cases and controls, increase type II error and reduce study power to detect effects. Importantly, the use of unscreened controls is not expected to increase type I error and produce spurious positive results.

Lack of strong human replication: The lack of strong human replication is a serious limitation of our study. However, nonreplication of novel signals is common in GWAS of AD and may have several causes. Specific to our study, differences in sampling, data production, QC, imputation, or analysis can confound meta-analysis. The GESGA sample was imputed to an older, smaller HapMap reference panel, has many missing data points, and shows the least consistent sign tests (Table 1 and Table S1). The OZALC population sample was genotyped at multiple sites, a well-known source of systematic genotype bias. We note that our strongest replication signals come from analyses of the 3 samples (Irish, COGA, and Yale/Penn) with the most consistent ascertainment and genotyping. More generally, this pattern of nonreplication may reflect the multiple independent domains of risk for AD (Kendler et al., 2012), including variation in (i) alcohol-specific physiological measures like initial sensitivity and tolerance (Schuckit et al., 1997), (ii) brain reward circuitry implicated in substance use and other pathological behaviors (Volkow et al., 2012), and (iii) personality traits like internalizing and externalizing behaviors (Harford et al., 2013), which are unlikely to be influenced by the same genes. As in other complex traits, these issues will be overcome primarily by increasing sample size and power. Finally, as both *KLF12* and *RYR3* were previously implicated in substance phenotypes, our findings may be considered as replication evidence for these signals.

Limited phenotypic consilience: Across our MO studies, many different phenotypes are affected by manipulation of candidate orthologs, with little consilience between species. Although mammalian and invertebrate nervous systems show extensive molecular and functional conservation (Bargmann, 1998; Brownlee and Fairweather, 1999) and many drugs mediate their behavioral effects through orthologous target proteins (Kaletta and Hengartner, 2006; Matthews and Kopczynski, 2001), phenotypic consilience and consistent direction of effect following manipulation of a specific gene are not always observed across species (e.g., manipulations of chloride intracellular channel 4 (*Clic4*) orthologs altered sensitivity in flies and mice but in different directions (Bhandari et al., 2012). There are also differences in EtOH-response measures available for different MOs (e.g., AFT has not been demonstrated in flies despite direct efforts to elicit this response (Chan et al., 2014)). Within species, we observe consilience across studies for effects of (1) *Klf-3* and binding partner *Ctbp1* on AFT and (2) genes influencing intracellular calcium levels on initial sensitivity in worms, and (3) *Col6a3* on HIC in mice.

CONCLUSIONS

Our combined data implicate *COL6A3*, *KLF12*, *RYR3,* and *LOC339975* in response to EtOH across multiple species and/or AD risk in humans. Our data are also consistent with prior work implicating remodeling of the ECM (*COL6A3*), regulation of EtOH-responsive genes (*KLF12*), and regulation of intracellular calcium release (*RYR3*) in response to EtOH.

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AUTHOR CONTRIBUTIONS

BPR conceived and designed the study, acquired funding, organized replication, and wrote the manuscript with AEA and LMH. AEA and LMH organized the study and performed primary analyses of the data; TBB performed imputation with assistance from FA for imputation of COGA data; access to COGA data was provided by DMD; AM undertook QC analyses; BTW oversaw data cleaning, QC, imputation, and analysis of genotypic data with assistance from BSM and SAB. VSW, GOM, MM, and VIV undertook bioinformatic and postmortem brain studies of *LOC339975*. ACE performed geneset enrichment analysis. RCR, JTA, GGB, LDM, AGD, and JCB undertook *C. elegans* studies. MFM and BPR performed GeneNetwork analyses of archival BXD mouse data. RFC, PB, and MG undertook *Drosophila* studies. RSP, BR, and MSB undertook dantrolene studies in rats. DGP, DW, CAP, and KSK organized the collection of samples in Ireland and Northern Ireland. The COGA Consortium, JBW, GZ, GWM, AKH, NGM, ACH, and PAFM (for the OZALC Consortium), JF, MR, NW, MS, PZ, MI, MMN, FK, MR, and the GESGA

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1000 Genomes Project: http://www.1000genomes.org; accessed April, 2016.

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GeneNetwork: http://www.genenetwork.org/webqtl/main.py; accessed February, 2016.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Supplementary information.

Fig. S1. GWAS data QC processes.

Fig. S2. Impact of BeagleCall on pre-imputation genotypic data quality.

Fig. S3. Post-QC Ancestry Analysis using MDS.

Fig. S4. Power of the post-QC sample of 706 AD cases and 1,748 controls.

Fig. S5. LocusZoom plots for additional loci with individual SNPs displaying $q < 0.1$.

Fig. S6. GeneMania network derived from the set of 45 genes annotated for the 274 SNPs with $q < 0.3$.

Fig. S7. Mutations in *C. elegans* orthologs of genes involved in calcium regulation cause EtOH response phenotypes.

Fig. S8. The *RyR* locus and behavioral responses to EtOH in flies.

Fig. S9. Dantrolene dose dependently reduced the motivation to self-administer EtOH.

Table S1. All 274 SNPs with $q < 0.3$ ($p < 1 \times 10^{-5}$) in Irish AD GWAS discovery data with results of replication in 3 independent samples, and meta-analyses of independent case/control samples, all case control samples and all samples.

Table S2. Lookup replication of AD genomewide association signals previously reported in samples of European descent.

Table S3. Results of gene-based analyses with KGG.

Table S4. Genetic interactions between associated loci identified in GENEMANIA network analysis.

Table S5. Results of iGSEA4GWAS analysis of signal over representation by functionally related sets of genes for 3 significant gene sets.

Table S6. Uncorrected basal speeds and 2-way ANOVA comparisons across time of EtOH exposure and genotype with Bonferroni post hoc comparisons at each time point. Development of AFT was tested using a paired 2-tailed *t*-test.

Table S7. Analysis of *LOC339975* expression controlling for covariates in post mortem brain tissue.