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Ethanol treatment of lymphoblastoid cell lines from alcoholics and non-alcoholics causes many subtle changes in gene expression

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A B S T R A C T

To elucidate the effects of a controlled exposure to ethanol on gene expression, we studied lymphoblastoid cell lines (LCLs) from 21 alcoholics and 21 controls. We cultured each cell line for 24 h with and without 75 mM ethanol and measured gene expression using microarrays. Differences in expression between LCLs from alcoholics and controls included 13 genes previously identified as associated with alcoholism or related traits, including *KCNA3*, *DICER1*, *ZNF415*, *CAT*, *SLC9A9*, and *PPARGC1B*. The paired design allowed us to detect very small changes due to ethanol treatment: ethanol altered the expression of 37% of the probe sets (51% of the unique named genes) expressed in these LCLs, most by modest amounts. Ninety-nine percent of the named genes expressed in the LCLs were also expressed in brain. Key pathways affected by ethanol include cytokine, TNF, and NFκB signaling. Among the genes affected by ethanol were *ANK3*, *EPHB1*, *SLC1A1*, *SLC9A9*, *NRD1*, and *SH3BP5*, which were reported to be associated with alcoholism or related phenotypes in 2 genome-wide association studies. Genes that either differed in expression between alcoholics and controls or were affected by ethanol exposure are candidates for further study.

Introduction

Alcoholism is a major health problem around the world (World Health Organization, 2011). It is a complex disease with both genetic and environmental contributions to risk, and the interplay between genes and environment is likely to be important (Edenberg & Foroud, 2006; Enoch, 2012; Meyers & Dick, 2010; Rietschel & Treutlein, 2013). Alcoholism and alcoholic organ damage are consequences of repeated exposures to high levels of ethanol over long periods (Koob & Le Moal, 2005; Laakso et al., 2000; Parry, Patra, & Rehm, 2011). Understanding how cells and organs are affected by ethanol can provide clues about mechanisms of toxicity and protection. Studies of gene expression can also complement linkage and association studies, by pointing to genes that differ in basal expression between alcoholics and controls and also to genes whose expression is altered temporarily or permanently by ethanol exposure. Nicolae et al. (2010) showed that trait-associated single nucleotide polymorphisms (SNPs) are more likely to affect gene expression in LCLs (i.e., to be expression quantitative trait loci [QTLs]), and that application of this information can enhance discovery of trait-associated SNPs for complex phenotypes.

Gene expression has been profiled in post-mortem human brain from alcoholics and controls (Flatscher-Bader et al., 2005; Iwamoto et al., 2004; Liu, Lewohl, Harris, Dodd, & Mayfield, 2007; Liu et al., 2006; Mayfield et al., 2002; McClintick et al., 2013). Those data, while important, do not allow one to disentangle the effects of long-term alcohol exposure and pre-existing expression differences. Animal models have been used to detect both innate differences in gene expression (Edenberg et al., 2005; Kimpel et al., 2007) and differences due to alcohol consumption (Rodd et al., 2008). However, for studies of living humans an accessible tissue such as blood or a cell culture surrogate such as Epstein–Barr virus (EBV) transformed LCLs can be of great value. Thibault, Hassan, and Miles (2005) concluded that *in vitro* assays in human cell lines are valuable for identifying changes in expression profiles upon exposure to ethanol and other drugs of addiction. Gene expression profiles of LCLs are most like the B cells from which they were derived (Min et al., 2010). They can provide insights into immune response

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mechanisms that play an important role in alcoholism and its effects on the brain (Crews, Zou, & Qin, 2011; Mayfield, Ferguson, & Harris, 2013; McClintick et al., 2013). A recent study has shown substantial overlap in expression between blood and many tissues, including many regions of the brain (Sullivan, Fan, & Perou, 2006; Wright et al., 2014), suggesting they also provide a window on many otherwise inaccessible processes. LCLs have been used in the study of other complex diseases, including autism. Nishimura et al. (2007) used expression profiling of LCLs from patients affected with autism and compared the results to controls to find different sets of dysregulated genes for 2 different subtypes of autism.

We have analyzed both basal gene expression and the effects of ethanol on gene expression in LCLs from 21 alcoholics and 21 controls. We have detected differences in gene expression between LCLs from alcoholics and controls and differences caused by the ethanol exposure. Most of the effects of ethanol were modest, but the effects highlighted pathways that have changes in many genes. We have also examined the overlap between the differences we detect in LCL gene expression and the results of expression studies in brain and with data from genome-wide association studies (GWAS) to identify and prioritize promising candidate genes for association and functional studies.

Methods

Cell growth

Immortalized lymphoblastoid cell lines (LCLs) were created from peripheral blood mononuclear cells isolated from subjects recruited as part of the Collaborative Study on the Genetics of Alcoholism (Begleiter et al., 1995; Bierut et al., 2010; Edenberg & Foroud, 2006). Immortalization was by transformation with Epstein–Barr virus and early passage (>12) cultures were used. In a test of the effects of ethanol on cell growth, 2×10^6 LCLs from each of 3 individuals were cultured in the presence of 0, 50, 75, or 100 mM ethanol in 10 mL RPMI1640 medium supplemented with 15% FBS, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C. For each treatment (cell line and ethanol concentration), 5 identical parallel flasks were seeded. At a given time, cells in 1 flask were counted twice, and the average number was used to calculate a growth curve and doubling time for each individual.

Microarray analysis of LCLs

For the microarray experiment, 2×10^6 LCLs from each of 21 alcoholics and 21 non-alcoholics were seeded in 10 mL of RPMI1640 medium supplemented with 15% FBS, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cultures were maintained in tightly capped flasks to minimize evaporation. Alcoholics were defined as meeting DSM-IV criteria for alcohol dependence (American Psychiatric Association, 1994) at age 18 years or younger. Non-alcoholics were defined as having taken at least 1 drink of alcohol and not meeting any of 4 definitions of alcohol dependence: DSM-IV (American Psychiatric Association, 1994), DSM-IIIR (American Psychiatric Association, 1987), ICD-10 (World Health Organization, 1993), or Feighner definite alcoholism (Feighner et al., 1972); none was dependent on any illicit drug. Each phenotypic group (alcoholic or non-alcoholic) contained 12 males and 9 females. Growth of ethanol-treated and untreated cells was parallel by 22 h even up to 100 mM ethanol; we chose 75 mM to be within this range and to offer a good possibility of discerning effects. Cells were cultured in the absence or presence of 75 mM ethanol for 24 h, at which time cells were harvested and lysed with buffer RLT, supplied in the Qiagen RNeasy kit, and RNA extractions were conducted per the manufacturer's protocol.

Reverse transcription and labeling used the Affymetrix 3′ IVT labeling kit and protocols (GeneChip® Expression Analysis Technical Manual, Affymetrix, Santa Clara, CA). Samples were labeled in groups balanced by sex and phenotype to the extent possible; pairs of treated and untreated samples from the same individual were labeled and hybridized at the same time. Samples were hybridized to Affymetrix HG U133 Plus 2 GeneChips® for 17 h, then washed and stained using the standard Affymetrix protocols. GeneChips® were scanned using an Affymetrix Model 3000 scanner controlled by GCOS software (Affymetrix, Santa Clara, CA). MAS5 signals and detection calls were generated by GCOS. Data are available from NCBI GEO, Accession number GSE52553.

To avoid analyzing genes that were not expressed, only probe sets that were called "present" in at least 33% of the arrays in at least 1 experimental group (phenotype, treatment, sex) were selected for analysis (McClintick & Edenberg, 2006). Using these criteria, 31,528 of the 54,675 probe sets on the GeneChips were retained for analysis. The MAS5 data were imported into Partek Genomics Suite (Partek Inc., St. Louis, Mo.). Because we expected cell lines from different individuals to differ, analysis was done using a general linear method with repeated measures for 0 and 75 mM ethanol; the main effects factors were ethanol treatment, phenotype (alcoholic vs. non-alcoholic), sex, and labeling batch. Addition of the 3 interaction terms (sex*treatment, sex*phenotype, and phenotype*treatment) to the model did not improve the results; none of the interaction terms reached significance after correcting for multiple testing. Therefore, we present the data from the simpler model with main effects only. The p values for each factor tested were imported into R to compute false discovery rate (FDR) using the Storey q-value package (Storey & Tibshirani, 2003). Partek Genomics Suite was used for hierarchical clustering of the arrays using Euclidean distance and average linkage.

Genes that were differentially expressed either by alcohol treatment or by phenotype were analyzed using Ingenuity Pathway Analysis (Ingenuity® Systems, spring 2013 release). Duplicate probe sets were eliminated by selecting the entry with the best p value. Parameters were set to use the Ingenuity knowledge base as the reference set. Due to the large number of genes that were differentially expressed after ethanol treatment, we limited the analysis to those genes with FDR $\leq$0.05 and minimum absolute fold change $\geq$1.2; for phenotype, FDR was set at $\leq$0.36 with no minimum fold change. We used the canonical pathway analysis to identify modified pathways and the upstream regulator analysis to identify putative factors responsible for the changes in expression. The upstream regulator analysis looks for transcription factors, cytokines, hormones, vitamins, and other signaling molecules that may be responsible for a portion of the differential expression. IPA uses its knowledge base of causal effects and the list of differentially expressed genes to predict whether a particular regulator could be activated. The activation z-score sign ($\pm$) indicates whether the upstream 'factor' is activated or less active in either the LCLs treated with ethanol or from alcoholics.

Measurement of gene expression by real time PCR

Two micrograms of total RNA (from the same RNA used for microarrays) was reverse-transcribed using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA). An aliquot of the cDNA was amplified for 40 cycles on a GeneAmp 7900HT Sequence Detection System with gene-specific primers designed using the Primer Express software (Applied Biosystems). Sybr Green was used for signal detection. All analyses were carried out in triplicate, and no-template controls and dissociation curves were used to ensure specific amplification. For each primer pair, serial dilutions of a control cDNA were used to determine standard

curves, and curves with $R^2 > 0.98$ were then used to determine the mRNA levels in individual samples. The expression levels were calculated as a ratio of the mRNA level for a given gene relative to the mRNA level for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same cDNA.

Microarray analysis of brain tissues

Samples from 9 different regions of the brains of each of 4 individuals (2 male and 2 female; an alcoholic and a control of each sex) were obtained from the NIAAA-supported brain bank at the Tissue Resource Center located in the Neuropathology Unit of the Department of Pathology, University of Sydney, Australia. We extracted total RNA from each of the 9 regions of each individual brain: prefrontal cortex, cerebral cortex, thalamus, visual cortex, hippocampus, amygdala, caudate nucleus, putamen, and cerebellum. RNA was extracted using Trizol (Invitrogen), with a higher ratio of Trizol to tissue to improve yield and purity (Edenberg et al., 2005), and further purified using RNeasy mini-columns (Qiagen, Valencia, CA). Samples were labeled using the Affymetrix Whole-Transcript labeling protocol starting with 100 ng of total RNA. The labeled samples were hybridized to Human Gene 1.0 ST arrays, then washed, stained, and scanned as described above.

Partek Genomics Suite was used to generate robust multichip average (RMA) (Bolstad, Irizarry, Astrand, & Speed, 2003; Irizarry et al., 2003) data for each of the arrays from brain samples. The average and standard deviation of RMA values were generated for the core probe sets in each brain region. The mean RMA values ranged from 4 to 21,734 (median = 106). Genes with expression levels at or near background (RMA < 16) were excluded from analyses (McClintick & Edenberg, 2006). When multiple probe sets represented 1 gene, the probe set with the largest mean expression was selected. If the mean RMA value was above 16 in at least 1 region, we considered the gene expressed in brain. In supplementary data, we show relative expression as the mean RMA value in the region in which it was highest.

To determine which genes were expressed both in the LCLs and in the brain, we matched gene symbols associated with the probe sets on the 2 different arrays. We were able to match 24,668 of the 26,814 genes that were detectably expressed in at least 1 group of LCL samples (on the Affymetrix HG U133 Plus 2 GeneChips®) with genes on the Human Gene 1.0 ST arrays on which the brain samples were analyzed.

Cross comparison with GWAS and human gene expression results

We compared the LCL results with results from 14 recent genome-wide association studies (GWAS) for alcohol dependence or related phenotypes (Bierut et al., 2010; Edenberg et al., 2010; Foroud et al., 2007; Gelernter et al., 2014; Hack et al., 2011; Johnson, Drgon, Walther, & Uhl, 2011; Kapoor et al., 2013; Kendler et al., 2011; Lind et al., 2010; Treutlein et al., 2009; Wang et al., 2013; Xuei et al., 2006; Zlojutro et al., 2011; Zuo, Gelernter, et al., 2012). These studies used alcohol dependence and/or 1 or more related phenotypes: age of onset of DSM-IV alcohol dependence, DSM-IV symptom count, initial sensitivity to alcohol, alcohol tolerance, withdrawal, craving, and maximum number of drinks within a 24-h period (maxdrinks). Gene symbols were matched to gene names reported by the various groups, which frequently represented genes within a given distance from the SNP.

We also compared the LCL results to a list of genes identified as differentially expressed by 1 or more of 11 post-mortem gene expression studies in humans (Flatscher-Bader, Harrison, Matsumoto, & Wilce, 2010; Flatscher-Bader et al., 2005; Iwamoto et al., 2004; Kryger & Wilce, 2010; Lewohl et al., 2000; Liu et al., 2007, 2006; Mayfield et al., 2002; McClintick et al., 2013; Sokolov, Jiang, Trivedi, & Aston, 2003; Zhou, Yuan, Mash, & Goldman, 2011).

Results

Effects of ethanol treatment on cell growth

To select ethanol concentrations that would not be toxic over the 24-h course of the experiment, the response of 3 LCLs to increasing concentrations of ethanol up to 100 mM were examined. The 3 LCLs differed in their rates of doubling in the absence of ethanol (22, 28, and 35 h). Ethanol prolonged the lag phase before LCLs began logarithmic growth, but in the period from 22 to 70 h after ethanol was added, LCLs treated with 0, 50, 75, or 100 mM ethanol were in log phase. A plot of $\log_{10}$ (cell number) vs. time during this period fit a linear regression with $r^2 \geq 0.98$ for all LCLs with all concentrations of ethanol. The average doubling time in the absence of ethanol was 27.4 h, and it was 27.7 h in 75 mM ethanol (Supplementary Fig. 1). Thus at the time studied, the cells were growing exponentially. Based upon these data, we chose to examine gene expression with and without 24 h exposure to 75 mM ethanol.

Effects of ethanol on gene expression

For a global picture of differential gene expression, we used hierarchical clustering of the arrays. The differences between individuals were greater than the differences due to either ethanol treatment or phenotype: the ethanol-treated and untreated samples from each person invariably clustered together, whether using all 31,522 probe sets expressed or the 5000 most variable probe sets (those with the largest coefficient of variation; data not shown). Although between-person effects were large, the paired design in which ethanol-treated and untreated LCLs from each of 42 individuals were used as repeated measures allowed us to detect the widespread effects of ethanol on gene expression, even when differences were small; each individual cell line acted as its own control, reducing the noise due to inter-individual differences.

Ethanol treatment significantly affected the expression of 11,734 probe sets (37% of the expressed probe sets), representing 7183 unique, named genes, at a stringent Storey FDR ≤ 5% (nominal p value ≤ 0.039). Most of the expression differences, however, were small (Fig. 1). There were 1393 named genes with absolute fold changes ≥1.2, of which 165 had an absolute fold change ≥1.4.

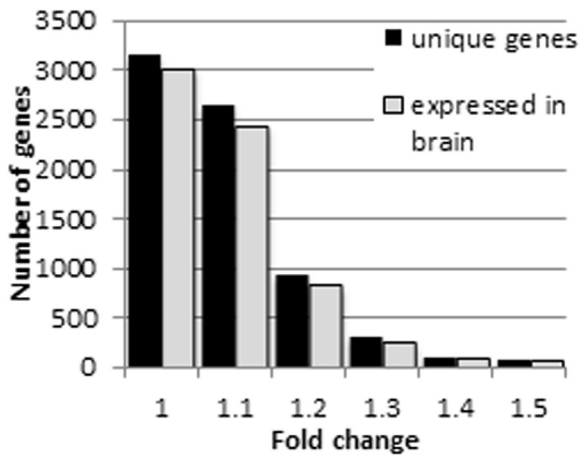

Fig. 1. Genes affected by ethanol exposure. The number of unique, named genes that significantly differed between ethanol-treated and untreated cells is plotted as a function of fold change. 1 = 1.01–1.099, 1.1 = 1.10–1.199, etc. Some genes did not map to the Gene 1.0 ST array used for comparison to brain.

Twenty-three histone genes were all decreased, more than half with absolute fold changes larger than 1.5-fold. A large number of heat shock proteins were affected by ethanol treatment. A list of differentially expressed genes with fold changes ≥1.1 can be found in Supplementary Table 1.

There were 567 probe sets, representing 478 unique named genes, that differed in expression between cell lines derived from alcoholics and cell lines from non-alcoholics (at an FDR ≤ 36%, nominal p value ≤ 0.0076; Fig. 2). Sixty-four percent of the genes that differed by phenotype were also affected by ethanol treatment (305 genes), compared to 51% of named genes being affected by ethanol. Supplementary Table 2 lists the genes differentially expressed between alcoholics and controls.

Not unexpectedly, sex had a significant effect on gene expression: 122 probe sets, associated with 58 unique named genes, were expressed differently in cells from males than in cells from females, FDR ≤0.05 (nominal p value ≤ 2×10^{-4}). This list includes genes such as *XIST* and *EIF1AX,* which are not detectably expressed in males, and *EIF1AY, DDX3Y,* and *NLGN4Y,* which are not detectably expressed in females. Of these 58 loci, 48 mapped to either the X or Y chromosome.

Pathway analysis

The 1393 genes affected by ethanol treatment with an absolute fold change ≥1.2 were used for Ingenuity Pathway Analysis. Forty-one pathways were significantly affected by ethanol treatment (Table 1). Among these were several inflammatory pathways, including IL-6 signaling, dendritic cell maturation, CD40 signaling, IL-10, and IL-9 signaling. TNFR2 (tumor necrosis factor receptor 2) signaling showed mostly increased expression. Four NFκB-related genes (*NFKB2*, *NFKBIA*, *NFKBIE,* and *IKBKE*), all with increased expression, are collectively found in 28 of these pathways, including the NFκB pathway itself. The results from the upstream regulator analysis, shown in Supplementary Table 3, reinforce these findings. NFκB was identified as the most significantly activated upstream regulator. TNF signaling also appears activated; TNFα, which has increased expression, is found in 17 of the pathways. Also affected were 45 cytokines, including IL6 and IL1β. All were activated except 3, 2 of which, IL10 and IL1RN, have known anti-inflammatory effects. Other harbingers of inflammation were seen: activation of interferons and Toll-like receptors.

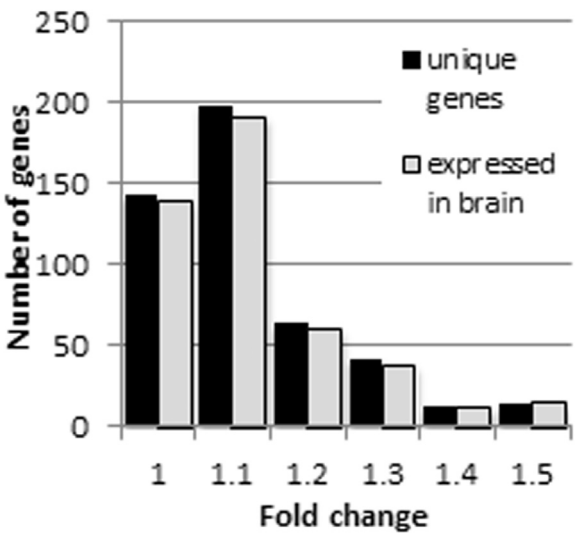

Fig. 2. Genes that differed between alcoholics and controls. The number of unique, named genes that significantly differed between cells from alcoholics and controls is plotted as a function of fold change. 1 = 1.01–1.099, 1.1 = 1.10–1.199, etc. Some genes did not map to the Gene 1.0 ST array used for comparison to brain.

The pathways that differed between cells from alcoholics and controls included phospholipase C signaling, G beta gamma signaling, RAN signaling, signaling by Rho family GTPases, androgen signaling, hypoxia signaling in the cardiovascular system, RhoGDI signaling, netrin signaling, tec kinase signaling, paxillin signaling, telomerase signaling, and ephrin B signaling (Table 2). *RAC1*, *GNG2*, *GNA11,* and *RHOT2*, with decreased expression in alcoholics, were common to several pathways. *GNA13*, *SOS2*, *PRKCE,* and *RHOQ,* with increased expression, were also common to multiple pathways. The upstream regulator analysis of the phenotype differences (Supplementary Table 4) shows increased signaling due to retinoic acid, vitamin D, *TP53,* and *APP.* The growth factors IGF1 (insulin-like growth factor 1) and EGFR (epidermal growth factor receptor), along with transcription factors MYC and MAX, are less active in the alcoholics.

Protein ubiquitination pathway and hypoxia signaling in the cardiovascular system were the only 2 pathways in common for treatment and phenotype. The only affected gene common to these 2 pathways is *UBE2Q,* a ubiquitin-conjugating enzyme, which was decreased in alcoholics and because of treatment by ethanol.

Comparison to brain expression

We detected 20,165 unique genes expressed in at least 1 brain region. Ninety-nine percent of the genes expressed in the LCLs that could be mapped to the Gene 1.0 ST arrays were expressed in at least 1 of the 9 brain regions (Supplementary Tables 1 & 2).

Confirmation by qRT-PCR

qRT-PCR was used to confirm microarray results. Genes that were previously identified by animal or human studies or related to stress or inflammatory response were selected for testing. Of the 22 genes selected for qRT-PCR based on different expression after treatment with ethanol, 20 were confirmed with a p value <0.05, and 1 (*FOXP1*) had a similar fold and direction but with $p = 0.09$ (Supplementary Table 5, Sections A & B). *SRSF11*, which was not confirmed, was measured on the array by 2 non-overlapping probe sets with different results, reflecting different splice variants; the differentially expressed variant contained a longer 3′ UTR that was not captured by the qRT-PCR. The 11 genes selected based on differential expression between alcoholics and controls (8 overlapped with the set affected by ethanol) were confirmed with $p < 0.05$ (Supplementary Table 5, Sections B & C).

Discussion

Analyzing the effects of a 24-h exposure to ethanol on lymphoblastoid cell lines (LCLs) under identical culture conditions allowed us to focus on the direct effects of ethanol on gene expression in a single cell type without complications of organismal environmental variables such as hormonal and nutritional status or different distributions of cell types. The differences in gene expression among individuals were large, but since each individual cell line was its own control, the effects of ethanol could be isolated and measured. Ethanol at 75 mM altered the expression of 37% of the probe sets expressed in LCLs, representing 51% of the unique named genes, which is remarkable, but most changes were small in magnitude (Fig. 1). This concentration, corresponding to a blood level of 0.345 mg%, is within the range seen after heavy drinking by alcoholics (Adachi et al., 1991; Lindblad & Olsson, 1976). Almost all of these genes were also expressed in brain. Given that one cannot sample brain from living subjects, LCLs offer a well-controlled, living cell alternative that can be examined for genes affected by

Table 1
Pathways affected by ethanol exposure.

Canonical pathways	*p* value	Molecules
Type I Diabetes mellitus signaling	4.4E-07	*MAP2K6, HLA-DMA, SOCS1, SOCS3, ICA1, NFKBIE, SOCS2, SOCS6, HLA-DQA1, MAPK9, SOCS4, IKBKE, IL1R1, NFKB2, FAS, NFKBIA, CD80, MAP3K7, IL12B, LTA, GAD1, CD86, TNF*
IL-6 signaling	5.0E-06	*MAP2K6, SOCS3, SOCS1, ABCB1, IL1A, AKT2, TNFAIP6, NFKBIE, MAPK9, IKBKE, IL1R1, NFKB2, STAT3, IL1R2, VEGFA, COL1A1, NFKBIA, MAP3K7, PIK3C3, CSNK2A1, AKT3, TNF*
Dendritic cell maturation	1.2E-05	*IL1A, ICAM1, PDIA3, NFKBIE, HLA-DQA1, CD83, NFKBIA, PIK3C3, AKT3, HLA-DMA, AKT2, RELB, MAPK9, CD58, IKBKE, NFKB2, CREB5, STAT4, COL1A1, CD80, CD40, IL12B, LTA, FSCN1, CD86, TNF, IFNAR1, CCR7*
CD40 signaling	4.2E-05	*MAP2K6, ICAM1, NFKBIE, TNFAIP3, MAPK9, IKBKE, STAT3, NFKB2, NFKBIA, CD40, MAP3K7, PIK3C3, LTA, TRAF1*
TNFR2 signaling	4.6E-05	*NFKBIA, LTA, NFKBIE, TNFAIP3, IKBKE, NFKB2, BIRC3, TNF, TRAF1*
Lymphotoxin β receptor signaling	2.1E-04	*AKT2, VCAM1, NFKBIA, LTA, PIK3C3, RELB, TRAF4, AKT3, IKBKE, NFKB2, TNFSF14, TRAF1*
Crosstalk between dendritic cells and natural killer cells	2.4E-04	*IL3RA, CD69, CD83, NFKB2, FAS, CSF2RB, CD40, CD80, IL12B, LTA, FSCN1, CD226, CD86, TNF, CCR7, IL2RB*
Role of JAK2 in hormone-like cytokine signaling	2.4E-04	*SOCS1, SOCS3, STAT5A, SOCS6, SOCS2, SOCS4, STAT3, PRLR, SIRPA*
Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	2.8E-04	*MAP2K6, SOCS3, SOCS1, IL1A, ICAM1, CAMK4, PDIA3, NFKBIE, CSNK1A1, TCF7, IL18R1, MYC, IL1R2, VEGFA, TLR10, NFKBIA, MAP3K7, PIK3C3, TRAF4, AKT3, TRAF1, VCAM1, AKT2, MAPK9, C5, IKBKE, STAT3, IL1R1, TCF3, CREB5, IL7, CALM1 (includes others), LTA, TLR6, FZD6, TNF, WNT5A*
Acute myeloid leukemia signaling	4.0E-04	*MAP2K6, STAT5A, AKT2, STAT3, NFKB2, TCF3, TCF7, MYC, BRAF, CSF2RB, ARAF, RARA, PIK3C3, AKT3*
Altered T cell and B cell signaling in rheumatoid arthritis	4.1E-04	*HLA-DMA, TLR10, IL1A, SLAMF1, CD40, CD80, IL12B, LTA, RELB, TLR6, HLA-DQA1, CD86, NFKB2, TNF, FAS*
TREM1 signaling	4.2E-04	*STAT5A, TLR10, AKT2, ICAM1, CD40, TLR6, AKT3, CD86, CD83, STAT3, NFKB2, TNF*
Triacylglycerol biosynthesis	4.3E-04	*PPAPDC1B, AGPAT5, ABHD5, PPAP2A, LPCAT4, DGAT2, MBOAT2, AGPAT9, AGPAT3, ELOVL6*
T helper cell differentiation	5.1E-04	*STAT4, HLA-DMA, CD40, CD80, IL12B, IL21R, HLA-DQA1, CD86, IL2RA, IL12RB2, STAT3, TNF, IL18R1*
IL-10 signaling	6.9E-04	*IL1R2, MAP2K6, SOCS3, IL1A, NFKBIA, MAP3K7, BLVRA, NFKBIE, IKBKE, NFKB2, IL1R1, STAT3, TNF*
Protein ubiquitination pathway	7.8E-04	*USP45, HSPA1A/HSPA1B, DNAJC15, HSPA5, TCEB1, ANAPC1, SMURF1, USP3, HSPA4, PAN2, USP7, USP53, USP47, UBE2D4, UCHL5, DNAJB1, DNAJC30, PSMC2, BIRC3, HSPB6, HSPA4L, UBE2Q1, DNAJC27, USP9X, UBE2G2, PSMD11, UBE2L3, USP32, PSMA5, UBE2D3, UBE2I*
B cell development	8.1E-04	*HLA-DMA, CD80, CD40, HLA-DQA1, CD86, IGHM, IL7, IGHD*
Small cell lung cancer signaling	8.9E-04	*FHIT, AKT2, PA2G4, NFKBIE, IKBKE, NFKB2, PTEN, MYC, NFKBIA, PIK3C3, TRAF4, AKT3, TRAF1*
Hypoxia signaling in the cardiovascular system	1.0E-03	*UBE2G2, VEGFA, UBE2L3, NFKBIA, UBE2Q1, SUMO1, NFKBIE, UBE2D4, CREB5, UBE2D3, PTEN, UBE2I*
EIF2 signaling	1.0E-03	*RPL22, AKT2, EIF3H, RPS28, EIF1, RPL37, PPP1CB, EIF4A2, RPL23, RPL35A, RPS23, EIF3M, RPL15, EIF2S2, EIF3F, EIF3B, EIF1AX, PIK3C3, EIF2B5, EIF3A, AKT3, RPS20, RPS15A, RPL13*
NF-κB signaling	1.1E-03	*MAP2K6, AZI2, IL1A, AKT2, RELB, NFKBIE, TNFAIP3, NFKB2, IL1R1, MALT1, IL1R2, TLR10, NFKBIA, CD40, MAP3K7, PIK3C3, LTA, TLR6, CSNK2A1, IGF1R, AKT3, TNF*
il-9 signaling	1.3E-03	*SOCS3, STAT5A, IL9R, PIK3C3, SOCS2, STAT3, NFKB2, TNF*
Role of osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis	1.5E-03	*MAP2K6, IL1A, CAMK4, NFKBIE, CSNK1A1, TCF7, ITGB3, IL18R1, SMURF1, IL1R2, NFKBIA, IGF1, MAP3K7, PIK3C3, AKT3, BIRC3, AKT2, MAPK9, IKBKE, IL1R1, TCF3, IL7, CALM1 (includes others), COL1A1, FZD6, TNF, WNT5A*
Rank signaling in osteoclasts	1.6E-03	*MAP2K6, AKT2, CAMK4, MAP3K13, NFKBIE, MAPK9, IKBKE, NFKB2, CALM1 (includes others), NFKBIA, MAP3K7, PIK3C3, AKT3, BIRC3*
Regulation of eIF4 and p70S6K signaling	2.1E-03	*AKT2, EIF3H, PPP2CA, RPS28, EIF1, EIF4A2, RPS23, EIF3M, EIF2S2, EIF3F, EIF3B, EIF1AX, PIK3C3, EIF2B5, EIF3A, AKT3, PPP2R5C, RPS20, RPS15A, PPP2R5E*
CD28 signaling in T helper cells	2.1E-03	*HLA-DMA, AKT2, CAMK4, NFKBIE, HLA-DQA1, MAPK9, IKBKE, MALT1, NFKB2, CALM1 (includes others), PAK1, ACTR3, NFKBIA, CD80, PIK3C3, CD86, AKT3*
iCOS-iCOSL signaling in T helper cells	2.4E-03	*HLA-DMA, AKT2, CAMK4, NFKBIE, HLA-DQA1, IKBKE, NFKB2, PTEN, CALM1 (includes others), NFKBIA, CD80, CD40, PIK3C3, AKT3, IL2RA, IL2RB*
p53 signaling	2.7E-03	*WT1, PMAIP1, AKT2, GADD45B, JMY, FAS, TP53BP2, PTEN, CHEK1, CCND2, PIK3C3, AKT3, SFN, PIDD*
Toll-like receptor signaling	3.0E-03	*PPARA, MAP2K6, TLR10, NFKBIA, MAP3K7, TRAF4, TLR6, TNFAIP3, NFKB2, TRAF1*
4-1BB signaling in T lymphocytes	3.2E-03	*TNFRSF9, NFKBIA, NFKBIE, MAPK9, IKBKE, NFKB2, TRAF1*
IL-17A signaling in airway cells	3.3E-03	*AKT2, NFKBIA, MAP3K7, PIK3C3, NFKBIE, AKT3, MAPK9, IKBKE, STAT3, NFKB2, PTEN*
IL-22 signaling	3.6E-03	*SOCS3, STAT5A, AKT2, AKT3, MAPK9, STAT3*
JAK/Stat signaling	3.8E-03	*STAT4, SOCS1, SOCS3, STAT5A, AKT2, PIK3C3, SOCS6, SOCS2, AKT3, SOCS4, STAT3*
Death receptor signaling	3.9E-03	*NFKBIA, NFKBIE, IKBKE, HTRA2, TNFSF15, NFKB2, CFLAR, BIRC3, TNF, FAS*
Hepatic fibrosis/hepatic stellate cell activation	4.0E-03	*SMAD2, IGFBP4, VCAM1, IL1A, ICAM1, CXCL9, NFKB2, IL1R1, FAS, VEGFA, IL1R2, COL1A1, IGF1, CD40, IGF1R, TNF, CCR7, IFNAR1*
Induction of apoptosis by HIV1	4.5E-03	*NFKBIA, NFKBIE, MAPK9, IKBKE, HTRA2, NFKB2, BIRC3, TNF, FAS, TRAF1*
IGF-1 signaling	4.8E-03	*IGFBP4, SOCS3, SOCS1, AKT2, IGF1, PIK3C3, SOCS6, SOCS2, CSNK2A1, IGF1R, AKT3, SOCS4, STAT3, SFN*
Production of nitric oxide and reactive oxygen species in macrophages	4.9E-03	*PPARA, AKT2, APOM, PPP2CA, NFKBIE, MAP3K13, PPP1CB, MAPK9, IKBKE, NFKB2, RAP1A, APOL1, NFKBIA, MAP3K7, PIK3C3, NCF2, AKT3, PPP2R5C, PPP2R5E, RHOF, TNF, SIRPA*
ATM signaling	5.0E-03	*GADD45B, NFKBIA, H2AFX, MAPK9, TDP1, CBX5, CREB5, CHEK1, CCNB1, SMC1A*

Genes with FDR ≤ 0.05 and absolute fold change ≥ 1.2 were used for Ingenuity® pathway analysis. In cases where there are multiple probe sets for the same gene, the lowest *p* value was used.

ethanol, and can help in prioritizing findings from genetic studies and biomarker studies of expression in the more complex mixture of blood cells.

Gene expression affected by ethanol

Ethanol activated many pathways related to inflammation (Table 1, Supplementary Tables 1 & 3). The NFκB and TNFα pathways are central to inflammatory responses and alcoholic liver disease (Roh & Seki, 2013; Wang, Gao, Zakhari, & Nagy, 2012). These pathways showed strong increases in expression of many genes, including TNFα, 15 TNF receptors or TNF-associated genes, and 5 NFκB related genes (*NFKB1, NFKB2, NFKBIA or NFKBIE, IKBKE*). It is notable that *NFKB1* was found to be associated with risk for alcoholism (Edenberg et al., 2010). Seventy-seven genes downstream of NFκB and 151 downstream of TNFα were affected, as were

Table 2
Pathways that differ between alcoholics and controls.

Ingenuity canonical pathways	*p* value	Molecules
Molecular mechanisms of cancer	9.8E-04	*RAP2B, JAK1, GNA11, RHOT2, SOS2, RAC1, RALBP1, NBN, RHOQ, MAX, PRKAR1B, PRKCE, ARHGEF2, GNA13, ARHGEF3, CTNNB1, BCL2L11*
Actin nucleation by ARP-WASP complex	1.0E-03	*ARPC1A, RHOQ, RHOT2, SOS2, RAC1, NCK1*
Protein ubiquitination pathway	1.0E-03	*UCHL3, USP14, UBE2Q1, PSMD13, SKP1, HSPA8, PSMB7, UBE2J1, HSP90AB1, PSMB2, UBE2G1, HSPE1, PSMA4, PSMB1*
Phospholipase C signaling	1.6E-03	*CALM1 (includes others), RHOQ, HDAC7, GNG2, SOS2, RHOT2, RAC1, PRKCE, ARHGEF2, MEF2C, GNA13, ARHGEF3, LCP2*
Breast cancer regulation by Stathmin1	2.9E-03	*CALM1 (includes others), TUBB3, SOS2, GNG2, RAC1, PRKAR1B, PRKCE, PPP1R11, ARHGEF2, ARHGEF3, GNA13*
G Beta Gamma signaling	4.4E-03	*KCNJ5, SOS2, GNG2, GNA11, PRKAR1B, PRKCE, GNA13*
RAN signaling	6.6E-03	*KPNA2, TNPO1, RAN*
Signaling by Rho family GTPases	8.9E-03	*ARFIP2, ARPC1A, RHOQ, DIAPH3, RHOT2, GNG2, GNA11, RAC1, ARHGEF2, ARHGEF3, GNA13*
Androgen signaling	9.8E-03	*CALM1 (includes others), GNG2, GNA11, PRKAR1B, PRKCE, POLR2B, GNA13*
Hypoxia signaling in the cardiovascular system	1.1E-02	*UBE2J1, UBE2Q1, HSP90AB1, UBE2G1, CSNK1D*
RhoGDI signaling	1.2E-02	*ARPC1A, RHOQ, RHOT2, GNG2, GNA11, RAC1, ARHGEF2, ARHGEF3, GNA13*
Netrin signaling	1.3E-02	*RAC1, PRKAR1B, NCK1, ABLIM1*
Fcγ receptor-mediated phagocytosis in macrophages and monocytes	1.6E-02	*ARPC1A, RAC1, PRKCE, RAB11A, NCK1, LCP2*
Tec Kinase signaling	1.7E-02	*JAK1, RHOQ, RHOT2, GNG2, GNA11, PRKCE, GNA13, TNFRSF10A*
Paxillin signaling	1.8E-02	*ITGB2, ARFIP2, SOS2, RAC1, NCK1, ITGAL*
Telomerase signaling	1.8E-02	*ELF2, HSP90AB1, SOS2, HDAC7, PTGES3, ELF1*
Ephrin B signaling	2.0E-02	*GNG2, GNA11, RAC1, GNA13, CTNNB1*
Acetyl-CoA biosynthesis I (Pyruvate Dehydrogenase Complex)	2.0E-02	*DLAT, DLD*
Huntington's disease signaling	2.1E-02	*HSPA8, ARFIP2, BDNF, SOS2, HDAC7, GNG2, GNA11, PRKCE, CASP4, POLR2B*
Integrin signaling	2.1E-02	*RAP2B, ITGB2, ARPC1A, RHOQ, RHOT2, SOS2, RAC1, NCK1, ITGAL*
Semaphorin signaling in neurons	2.2E-02	*SEMA4D, RHOQ, RHOT2, RAC1*
Cholecystokinin/Gastrin-mediated signaling	2.2E-02	*RHOQ, RHOT2, SOS2, PRKCE, MEF2C, GNA13*
Cleavage and Polyadenylation of Pre-mRNA	2.5E-02	*PABPN1, CPSF4*
Role of NFAT in regulation of the immune response	3.1E-02	*CALM1 (includes others), SOS2, GNG2, GNA11, CSNK1D, MEF2C, GNA13, LCP2*
CREB signaling in neurons	3.6E-02	*CALM1 (includes others), SOS2, GNG2, GNA11, PRKAR1B, PRKCE, POLR2B, GNA13*
Sertoli Cell-Sertoli cell junction signaling	3.8E-02	*TUBB3, TJAP1, PPAP2B, RAC1, PRKAR1B, MLLT4, YBX3, CTNNB1*
Tight Junction signaling	3.9E-02	*RAC1, PRKAR1B, MLLT4, YBX3, ARHGEF2, CTNNB1, CPSF4*
ERK5 signaling	4.5E-02	*YWHAG, YWHAE, MEF2C, GNA13*
ERK/MAPK signaling	4.6E-02	*ELF2, YWHAG, SOS2, RAC1, PRKAR1B, PRKCE, PPP1R11, ELF1*
Germ Cell-Sertoli cell junction signaling	4.7E-02	*TUBB3, RHOQ, PPAP2B, RHOT2, RAC1, MLLT4, CTNNB1*
PI3K/AKT signaling	4.9E-02	*YWHAG, JAK1, YWHAE, HSP90AB1, SOS2, CTNNB1*
CXCR4 signaling	4.9E-02	*RHOQ, RHOT2, GNG2, GNA11, RAC1, PRKCE, GNA13*

Genes with FDR $\leq$ 0.36 were used for Ingenuity® pathway analysis. In cases where there are multiple probe sets for the same gene, the lowest *p* value was used.

numerous genes downstream of the activated cytokines and more than 120 downstream of the interferons. The Toll-like receptors are also activated by ethanol. *TXNIP* (thioredoxin interacting protein; 1.5-fold higher in LCL from alcoholics) is also increased 10% by ethanol treatment. *TXNIP*, which functionally links ER stress to the inflammasome and activation of NFκB, was found to be 1.7-fold higher in the hippocampus of alcoholics (McClintick et al., 2013). Recently, neuroinflammation has been linked to alcoholism and may play a role in the addiction process (Crews et al., 2011; Mayfield et al., 2013). It has been hypothesized that lipopolysaccharides (LPS) introduced into circulation from the gut may be responsible for neuroinflammation (Mayfield et al., 2013) by activating peripheral TLR4 receptors to produce circulating cytokines that can cross the blood–brain barrier. Others have shown that a robust inflammatory response to ethanol does not require lipopolysaccharides from the gut–liver axis, and that a direct effect of ethanol on Toll-like receptor 4 can initiate neuroinflammation (Fernandez-Lizarbe, Montesinos, & Guerri, 2013). Our data show that a 24-h exposure to ethanol was sufficient to initiate this inflammatory response in LCLs without exposure to LPS.

Among the LCL genes differentially expressed upon exposure to ethanol, 1043 were differentially expressed in brain in 1 or more of 11 post-mortem gene expression studies, 58 of which also differed between alcoholics and controls (Supplementary Table 1). Most GWAS findings are in the non-protein coding portion of the genome, and are thought to influence gene expression. Trait-associated SNPs are more likely to be expression quantitative trait loci (Nicolae et al., 2010). We therefore examined the overlap between genes whose expression in LCLs was altered by ethanol and genes reported in GWAS studies. Two hundred eighty-four were identified by at least 1 GWAS (Supplementary Table 1, GWAS references therein), including 8 that also differed between alcoholics and controls (Supplementary Tables 1 & 2). Among the 284 genes, 12 were reported by 2 GWAS, including 2 genes associated with glutamate uptake. *SLC9A9* (cation proton antiporter 9) is associated with alcohol dependence (Kendler et al., 2011) and alcohol dependence symptom count (Wang et al., 2013); it was also associated with smoking (Vink et al., 2009) and ADHD (Kondapalli et al., 2013). *SLC9A9* expression was also altered in the frontal cortex of alcoholics (Liu et al., 2006; Wang et al., 2013). *SLC1A1* (high affinity glutamate transporter) is associated with alcohol dependence (Edenberg et al., 2010; Kendler et al., 2011); it was also associated with obsessive-compulsive disorder (Wendland et al., 2009) and schizophrenia (Horiuchi et al., 2012). Three SNPs in or near *SLC1A1* are correlated with gene expression levels in LCLs (Wendland et al., 2009), and are associated with increased expression in post-mortem prefrontal cortex (Horiuchi et al., 2012). *ANK3* (ankyrin 3, node of Ranvier) is associated with alcoholism (Kendler et al., 2011) and alcohol plus illegal substance dependence (Johnson et al., 2011), and also with posttraumatic stress disorder and externalizing behavior (Logue et al., 2013), bipolar disorder especially associated with stress (Leussis et al., 2013), and autism susceptibility (Bi et al., 2012). *EPHB1* (ephrin receptor B1) is associated with alcoholism (Edenberg et al., 2010; Kendler et al., 2011) and also shown to differ in expression in the frontal cortex of alcoholics (Liu et al., 2007). *SH3BP5*, which was also differentially expressed in

alcoholics compared to controls, was identified in 2 GWAS related to alcohol dependence (Bierut et al., 2010; Johnson et al., 2011) and has been replicated recently in alcohol and nicotine co-dependence (Zuo, Zhang, et al., 2012).

Gene expression in alcoholics vs. controls

Genes that differ between alcoholics and controls were harder to detect, given the relatively high level of expression heterogeneity observed among all subjects. Such differences could reflect genomic variation between subjects including gene expression differences and gene product variations that contribute to risk, effects of repeated exposure to ethanol in the subject from whom the cells were derived, or gene × environment interactions. Most of the pathways that exhibited expression differences between LCLs from alcoholics and controls are signaling pathways, including ones associated with brain functions (Table 2). *PRKCE* is known to affect ethanol consumption (Olive, Mehmert, Messing, & Hodge, 2000).

Thirteen genes differentially expressed in the alcoholics were associated with alcoholism in at least 1 of 14 GWAS (Supplementary Table 2; references therein). *ZNF415* (Zinc finger 415, a transcriptional regulator) had the largest fold difference between alcoholics and controls (1.9-fold increase) and was previously identified by post-mortem expression (Sokolov et al., 2003) and GWAS (Kendler et al., 2011).

We did not detect significant interaction between alcoholic status and ethanol exposure. After correction of the interaction term for multiple testing, only 1 probe set for an unknown transcript had an FDR <0.95. This may be an issue of power, given the relatively small number of genes detected as differentially expressed between the alcoholics and controls. There was substantial heterogeneity between LCL from different subjects, which reduces power to detect differences between alcoholics and controls but did not greatly interfere with detection of the effects of ethanol because of our paired design.

We have identified genes and pathways that differ in expression between alcoholics and controls, and genes that are affected by ethanol treatment. In a complex disease such as alcoholism, both pre-existing genetic risk factors that might influence gene expression, and expression differences that result from heavy drinking, can contribute to the disease. LCLs are an accessible tissue model, and 99% of the genes differentially expressed in LCLs treated with ethanol that could be mapped to the Gene 1.0 ST array are also expressed in at least 1 part of the brain. Many were also identified in studies of post-mortem brain. These data can be used to prioritize genes reported by GWAS at sub-genome-wide levels.

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Appendix. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.alcohol.2014.07.004.

References

Adachi, J., Mizoi, Y., Fukunaga, T., Ogawa, Y., Ueno, Y., & Imamichi, H. (1991). Degrees of alcohol intoxication in 117 hospitalized cases. *Journal of Studies on Alcohol, 52*, 448–453.

American Psychiatric Association. (1987). *Diagnostic and statistical manual of mental disorders: DSM-iii-r* (3rd ed.). Washington, DC: American Psychiatric Association.

American Psychiatric Association. (1994). *Diagnostic and statistical manual of mental disorders: DSM-iv*. Washington, DC: American Psychiatric Association.

Begleiter, H., Reich, T., Hesselbrock, V., Porjesz, B., Li, T.-K., Schuckit, M. A., et al. (1995). The collaborative study on the genetics of alcoholism. *Alcohol Health & Research World, 19*, 228–236.

Bi, C., Wu, J., Jiang, T., Liu, Q., Cai, W., Yu, P., et al. (2012). Mutations of ANK3 identified by exome sequencing are associated with autism susceptibility. *Human Mutation, 33*, 1635–1638.

Bierut, L. J., Agrawal, A., Bucholz, K. K., Doheny, K. F., Laurie, C., Pugh, E., et al. (2010). A genome-wide association study of alcohol dependence. *Proceedings of the National Academy of Sciences of the United States of America, 107*, 5082–5087.

Bolstad, B. M., Irizarry, R. A., Astrand, M., & Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics (Oxford, England), 19*, 185–193.

Crews, F. T., Zou, J., & Qin, L. (2011). Induction of innate immune genes in brain create the neurobiology of addiction. *Brain, Behavior, and Immunity, 25*(Suppl. 1), S4–S12.

Edenberg, H. J., & Foroud, T. (2006). The genetics of alcoholism: identifying specific genes through family studies. *Addiction Biology, 11*, 386–396.

Edenberg, H. J., Koller, D. L., Xuei, X., Wetherill, L., McClintick, J. N., Almasy, L., et al. (2010). Genome-wide association study of alcohol dependence implicates a region on chromosome 11. *Alcoholism: Clinical and Experimental Research, 34*, 840–852.

Edenberg, H. J., Strother, W. N., McClintick, J. N., Tian, H., Stephens, M., Jerome, R. E., et al. (2005). Gene expression in the hippocampus of inbred alcohol-preferring and -nonpreferring rats. *Genes, Brain, and Behavior, 4*, 20–30.

Enoch, M. A. (2012). The influence of gene-environment interactions on the development of alcoholism and drug dependence. *Current Psychiatry Reports, 14*, 150–158.

Feighner, J. P., Robins, E., Guze, S. B., Woodruff, R. A., Jr., Winokur, G., & Munoz, R. (1972). Diagnostic criteria for use in psychiatric research. *Archives of General Psychiatry, 26*, 57–63.

Fernandez-Lizarbe, S., Montesinos, J., & Guerri, C. (2013). Ethanol induces TLR4/TLR2 association, triggering an inflammatory response in microglial cells. *Journal of Neurochemistry, 126*, 261–273.

Flatscher-Bader, T., Harrison, E., Matsumoto, I., & Wilce, P. A. (2010). Genes associated with alcohol abuse and tobacco smoking in the human nucleus accumbens

and ventral tegmental area. *Alcoholism: Clinical and Experimental Research, 34*, 1291–1302.
Flatscher-Bader, T., van der Brug, M., Hwang, J. W., Gochee, P. A., Matsumoto, I., Niwa, S., et al. (2005). Alcohol-responsive genes in the frontal cortex and nucleus accumbens of human alcoholics. *Journal of Neurochemistry, 93*, 359–370.
Foroud, T., Wetherill, L. F., Liang, T., Dick, D. M., Hesselbrock, V., Kramer, J., et al. (2007). Association of alcohol craving with alpha-synuclein (SNCA). *Alcoholism: Clinical and Experimental Research, 31*, 537–545.
Gelernter, J., Kranzler, H. R., Sherva, R., Almasy, L., Koesterer, R., Smith, A. H., et al. (2014). Genome-wide association study of alcohol dependence: significant findings in African- and European-Americans including novel risk loci. *Molecular Psychiatry, 19*, 41–49.
Hack, L. M., Kalsi, G., Aliev, F., Kuo, P. H., Prescott, C. A., Patterson, D. G., et al. (2011). Limited associations of dopamine system genes with alcohol dependence and related traits in the Irish Affected Sib Pair Study of Alcohol Dependence (IASPSAD). *Alcoholism: Clinical and Experimental Research, 35*, 376–385.
Horiuchi, Y., Iida, S., Koga, M., Ishiguro, H., Iijima, Y., Inada, T., et al. (2012). Association of SNPs linked to increased expression of SLC1A1 with schizophrenia. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics, 159B*, 30–37.
Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., & Speed, T. P. (2003). Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Research, 31*, e15.
Iwamoto, K., Bundo, M., Yamamoto, M., Ozawa, H., Saito, T., & Kato, T. (2004). Decreased expression of NEFH and PCP4/PEP19 in the prefrontal cortex of alcoholics. *Neuroscience Research, 49*, 379–385.
Johnson, C., Drgon, T., Walther, D., & Uhl, G. R. (2011). Genomic regions identified by overlapping clusters of nominally-positive SNPs from genome-wide studies of alcohol and illegal substance dependence. *PLoS One, 6*, e19210.
Kapoor, M., Wang, J. C., Wetherill, L., Le, N., Bertelsen, S., Hinrichs, A. L., et al. (2013). A meta-analysis of two genome-wide association studies to identify novel loci for maximum number of alcoholic drinks. *Human Genetics, 132*, 1141–1151.
Kendler, K. S., Kalsi, G., Holmans, P. A., Sanders, A. R., Aggen, S. H., Dick, D. M., et al. (2011). Genomewide association analysis of symptoms of alcohol dependence in the molecular genetics of schizophrenia (MGS2) control sample. *Alcoholism: Clinical and Experimental Research, 35*, 963–975.
Kimpel, M. W., Strother, W. N., McClintick, J. N., Carr, L. G., Liang, T., Edenberg, H. J., et al. (2007). Functional gene expression differences between inbred alcohol-preferring and -non-preferring rats in five brain regions. *Alcohol, 41*, 95–132.
Kondapalli, K. C., Hack, A., Schushan, M., Landau, M., Ben-Tal, N., & Rao, R. (2013). Functional evaluation of autism-associated mutations in NHE9. *Nature Communications, 4*, 2510.
Koob, G. F., & Le Moal, M. (2005). Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. *Nature Neuroscience, 8*, 1442–1444.
Kryger, R., & Wilce, P. A. (2010). The effects of alcoholism on the human basolateral amygdala. *Neuroscience, 167*, 361–371.
Laakso, M. P., Vaurio, O., Savolainen, L., Repo, E., Soininen, H., Aronen, H. J., et al. (2000). A volumetric MRI study of the hippocampus in type 1 and 2 alcoholism. *Behavioural Brain Research, 109*, 177–186.
Leussis, M. P., Berry-Scott, E. M., Saito, M., Jhuang, H., de Haan, G., Alkan, O., et al. (2013). The ANK3 bipolar disorder gene regulates psychiatric-related behaviors that are modulated by lithium and stress. *Biological Psychiatry, 73*, 683–690.
Lewohl, J. M., Wang, L., Miles, M. F., Zhang, L., Dodd, P. R., & Harris, R. A. (2000). Gene expression in human alcoholism: microarray analysis of frontal cortex. *Alcoholism: Clinical and Experimental Research, 24*, 1873–1882.
Lind, P. A., Macgregor, S., Vink, J. M., Pergadia, M. L., Hansell, N. K., de Moor, M. H., et al. (2010). A genomewide association study of nicotine and alcohol dependence in Australian and Dutch populations. *Twin Research and Human Genetics: The Official Journal of the International Society for Twin Studies, 13*, 10–29.
Lindblad, B., & Olsson, R. (1976). Unusually high levels of blood alcohol? *JAMA: The Journal of the American Medical Association, 236*, 1600–1602.
Liu, J., Lewohl, J. M., Harris, R. A., Dodd, P. R., & Mayfield, R. D. (2007). Altered gene expression profiles in the frontal cortex of cirrhotic alcoholics. *Alcoholism: Clinical and Experimental Research, 31*, 1460–1466.
Liu, J., Lewohl, J. M., Harris, R. A., Iyer, V. R., Dodd, P. R., Randall, P. K., et al. (2006). Patterns of gene expression in the frontal cortex discriminate alcoholic from nonalcoholic individuals. *Neuropsychopharmacology, 31*, 1574–1582.
Logue, M. W., Solovieff, N., Leussis, M. P., Wolf, E. J., Melista, E., Baldwin, C., et al. (2013). The ankyrin-3 gene is associated with posttraumatic stress disorder and externalizing comorbidity. *Psychoneuroendocrinology, 38*, 2249–2257.
Mayfield, J., Ferguson, L., & Harris, R. A. (2013). Neuroimmune signaling: a key component of alcohol abuse. *Current Opinion in Neurobiology, 23*, 513–520.
Mayfield, R. D., Lewohl, J. M., Dodd, P. R., Herlihy, A., Liu, J., & Harris, R. A. (2002). Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. *Journal of Neurochemistry, 81*, 802–813.
McClintick, J. N., & Edenberg, H. J. (2006). Effects of filtering by Present call on analysis of microarray experiments. *BMC Bioinformatics, 7*, 49.
McClintick, J. N., Xuei, X., Tischfield, J. A., Goate, A., Foroud, T., Wetherill, L., et al. (2013). Stress-response pathways are altered in the hippocampus of chronic alcoholics. *Alcohol, 47*, 505–515.
Meyers, J. L., & Dick, D. M. (2010). Genetic and environmental risk factors for adolescent-onset substance use disorders. *Child and Adolescent Psychiatric Clinics of North America, 19*, 465–477.
Min, J. L., Barrett, A., Watts, T., Pettersson, F. H., Lockstone, H. E., Lindgren, C. M., et al. (2010). Variability of gene expression profiles in human blood and lymphoblastoid cell lines. *BMC Genomics, 11*, 96.
Nicolae, D. L., Gamazon, E., Zhang, W., Duan, S., Dolan, M. E., & Cox, N. J. (2010). Trait-associated SNPs are more likely to be eQTLs: annotation to enhance discovery from GWAS. *PLoS Genetics, 6*, e1000888.
Nishimura, Y., Martin, C. L., Vazquez-Lopez, A., Spence, S. J., Alvarez-Retuerto, A. I., Sigman, M., et al. (2007). Genome-wide expression profiling of lymphoblastoid cell lines distinguishes different forms of autism and reveals shared pathways. *Human Molecular Genetics, 16*, 1682–1698.
Olive, M. F., Mehmert, K. K., Messing, R. O., & Hodge, C. W. (2000). Reduced operant ethanol self-administration and in vivo mesolimbic dopamine responses to ethanol in PKCepsilon-deficient mice. *The European Journal of Neuroscience, 12*, 4131–4140.
Parry, C. D., Patra, J., & Rehm, J. (2011). Alcohol consumption and non-communicable diseases: epidemiology and policy implications. *Addiction, 106*, 1718–1724.
Rietschel, M., & Treutlein, J. (2013). The genetics of alcohol dependence. *Annals of the New York Academy of Sciences, 1282*, 39–70.
Rodd, Z. A., Kimpel, M. W., Edenberg, H. J., Bell, R. L., Strother, W. N., McClintick, J. N., et al. (2008). Differential gene expression in the nucleus accumbens with ethanol self-administration in inbred alcohol-preferring rats. *Pharmacology, Biochemistry, and Behavior, 89*, 481–498.
Roh, Y. S., & Seki, E. (2013). Toll-like receptors in alcoholic liver disease, non-alcoholic steatohepatitis and carcinogenesis. *Journal of Gastroenterology and Hepatology, 28*(Suppl. 1), 38–42.
Sokolov, B. P., Jiang, L., Trivedi, N. S., & Aston, C. (2003). Transcription profiling reveals mitochondrial, ubiquitin and signaling systems abnormalities in post-mortem brains from subjects with a history of alcohol abuse or dependence. *Journal of Neuroscience Research, 72*, 756–767.
Storey, J. D., & Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences of the United States of America, 100*, 9440–9445.
Sullivan, P. F., Fan, C., & Perou, C. M. (2006). Evaluating the comparability of gene expression in blood and brain. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics: The Official Publication of the International Society of Psychiatric Genetics, 141B*, 261–268.
Thibault, C., Hassan, S., & Miles, M. (2005). Using in vitro models for expression profiling studies on ethanol and drugs of abuse. *Addiction Biology, 10*, 53–62.
Treutlein, J., Cichon, S., Ridinger, M., Wodarz, N., Soyka, M., Zill, P., et al. (2009). Genome-wide association study of alcohol dependence. *Archives of General Psychiatry, 66*, 773–784.
Vink, J. M., Smit, A. B., de Geus, E. J., Sullivan, P., Willemsen, G., Hottenga, J. J., et al. (2009). Genome-wide association study of smoking initiation and current smoking. *American Journal of Human Genetics, 84*, 367–379.
Wang, H. J., Gao, B., Zakhari, S., & Nagy, L. E. (2012). Inflammation in alcoholic liver disease. *Annual Review of Nutrition, 32*, 343–368.
Wang, J. C., Foroud, T., Hinrichs, A. L., Le, N. X., Bertelsen, S., Budde, J. P., et al. (2013). A genome-wide association study of alcohol-dependence symptom counts in extended pedigrees identifies C15orf53. *Molecular Psychiatry, 18*, 1218–1224.
Wendland, J. R., Moya, P. R., Timpano, K. R., Anavitarte, A. P., Kruse, M. R., Wheaton, M. G., et al. (2009). A haplotype containing quantitative trait loci for SLC1A1 gene expression and its association with obsessive-compulsive disorder. *Archives of General Psychiatry, 66*, 408–416.
World Health Organization. (1993). *International classification of disease*. Geneva: World Health Organization.
World Health Organization. (2011). *Global status report on alcohol and health*. Geneva: WHO Press.
Wright, F. A., Sullivan, P. F., Brooks, A. I., Zou, F., Sun, W., Xia, K., et al. (2014). Heritability and genomics of gene expression in peripheral blood. *Nature Genetics, 46*, 430–437.
Xuei, X., Dick, D., Flury-Wetherill, L., Tian, H. J., Agrawal, A., Bierut, L., et al. (2006). Association of the kappa-opioid system with alcohol dependence. *Molecular Psychiatry, 11*, 1016–1024.
Zhou, Z., Yuan, Q., Mash, D. C., & Goldman, D. (2011). Substance-specific and shared transcription and epigenetic changes in the human hippocampus chronically exposed to cocaine and alcohol. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 6626–6631.
Zlojutro, M., Manz, N., Rangaswamy, M., Xuei, X., Flury-Wetherill, L., Koller, D., et al. (2011). Genome-wide association study of theta band event-related oscillations identifies serotonin receptor gene HTR7 influencing risk of alcohol dependence. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics, 156B*, 44–58.
Zuo, L., Gelernter, J., Zhang, C. K., Zhao, H., Lu, L., Kranzler, H. R., et al. (2012). Genome-wide association study of alcohol dependence implicates KIAA0040 on chromosome 1q. *Neuropsychopharmacology, 37*, 557–566.
Zuo, L., Zhang, F., Zhang, H., Zhang, X. Y., Wang, F., Li, C. S., et al. (2012). Genome-wide search for replicable risk gene regions in alcohol and nicotine co-dependence. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics, 159B*, 437–444.