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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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Abstract

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. 99% of the named genes expressed in the LCLs were also expressed in brain. Key pathways affected by ethanol include cytokine, TNF and NF- κ B 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 two 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 and Foroud,

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2006; Enoch, 2012; Meyers and Dick, 2010; Rietschel and Treutlein, 2012). Alcoholism and alcoholic organ damage are consequences of repeated exposures to high levels of ethanol over long periods (Koob and Le Moal, 2005; Laakso et al., 2000; Parry et al., 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. (Nicolae et al., 2010) showed that trait-associated SNPs are more likely to affect gene expression in LCLs (i.e., to be expression QTLs), and that application of this information can enhance discovery of trait-associated SNPs for complex phenotypes.

Gene expression has been profiled in postmortem human brain from alcoholics and controls (Flatscher-Bader et al., 2005; Iwamoto et al., 2004; Liu et al., 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 lymphoblastoid cell lines (LCLs) can be of great value. Thibault et al. (Thibault et al., 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 mechanisms that play an important role in alcoholism and its effects on the brain (Crews et al., 2011; Mayfield et al., 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 et al., 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. (Nishimura et al., 2007) used expression profiling of LCLs from patients affected with autism and compared to controls to find different sets of dysregulated genes for two 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 LCL from alcoholics and controls and differences caused by the ethanol exposure. Most of the effects of ethanol were modest, but highlighted pathways which 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 and 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 three 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 μ g/ml streptomycin at 37C. For each treatment (cell line and ethanol concentration) 5 identical parallel flasks were seeded. At a given time, cells in one flask were counted twice, and the average number 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 μ g/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 one drink of alcohol and not meeting any of four definitions of alcohol dependence: DSM-IV (American Psychiatric Association 1994), DSM-III-R (American Psychiatric Association, 1987), ICD-10 (World Health Organization, 1993), or Feighner definite alcoholism (Feighner, 1972); none were 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). 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 one experimental group (phenotype, treatment, sex)

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were selected for analysis (McClintick and 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, with the main effects factors ethanol treatment, phenotype (alcoholic vs. non-alcoholic), sex and labeling batch. Addition of the three 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. P-values for each factor tested were imported into R to compute false discovery rate (FDR) using the Storey q-value package (Storey and Tibshirani, 2003). Partek Genomics Suite was used for hierarchical clustering of the arrays using Euclidean distance and average linkage.

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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 < 0.05 and minimum absolute fold change > 1.2; for phenotype, FDR was set at < 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 actors 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 (+/-) indicates whether the upstream 'actor' is activated or less active in either the LCLs treated with ethanol or from alcoholics.

Measurement of gene expression by real time PCR

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Two micrograms of total RNA (from same RNA used for microarrays) was reverse transcribed using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, Calif., USA). 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 nine 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 RMA (robust multichip average (Bolstad et al., 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 and Edenberg, 2006). When multiple probe sets represented one gene, the probe set with the largest mean expression was selected. If the mean RMA value was above 16 in at least one 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 two different arrays. We were able to match 24,668 of the 26,814 genes that were detectably expressed in at least one 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., 2013; Hack et al., 2011; Johnson et al., 2011; Kapoor et al., 2013; Kendler et al., 2011; Lind et al., 2010; Treutlein et al., 2009; Wang et al., 2012; Xuei et al., 2006; Zlojutro et al., 2011; Zuo et al., 2012a). These studies used alcohol dependence and/or one or more related phenotypes: age of onset of DSM4 alcohol dependence, DSM4 symptom count, initial sensitivity to alcohol, alcohol tolerance, withdrawal, craving and maximum number of drinks within a 24 hour 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 one or more of 11 post-mortem gene expression studies in humans (Flatscher-Bader et al., 2010; Flatscher-Bader et al., 2005; Iwamoto et al., 2004; Kryger and Wilce, 2010; Lewohl et

al., 2000; Liu et al., 2007; Liu et al., 2006; Mayfield et al., 2002; McClintick et al., 2013; Sokolov et al., 2003; Zhou et al., 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 three LCLs to increasing concentrations of ethanol up to 100 mM were examined. The three LCLs differed in their rates of doubling in the absence of ethanol (22, 28 and 35 hours). Ethanol prolonged the lag phase before LCLs began logarithmic growth, but in the period from 22-70 hours 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 $r^2 = 0.98$ 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 Figure 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 7,183 unique, named genes, at a stringent Storey FDR 5% (nominal p-value = 0.039). Most of the expression differences, however, were small (**Figure 1**). There were 1,393 named genes with absolute fold changes ≥ 1.2 , of which 165 had an absolute fold change ≥ 1.4 . 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; **Figure 2**). 64% 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*, *EIF1AX*, which are not detectably expressed in males, and *EIF1AY*, *DDX3Y* and *NLGN4Y*, which were 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 NF- κ B related genes (*NFKB2*, *NFKBIA*, *NFKBIE* and *IKBKE*), all with increased expression, are collectively found in 28 of these pathways, including the NF- κ B pathway itself. The results from the upstream regulator analysis, shown in **Supplementary Table 3**, reinforces these findings. NF- κ B was identified as the most significantly activated upstream regulator. TNF signaling also appears activated; TNF α , which has increased expression, is found in 17 of the pathways. Also affected were 45 cytokines, including IL6 and IL1 β . All were activated except three, two of which, IL10 and IL1RN, have known anti-inflammatory effects. Other harbingers of inflammation were seen: activation of interferons and Toll-like receptors.

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*, *GNAI1* and *RHOT2*, with decreased expression in alcoholics, were common to several pathways. *GNAI3*, *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 two pathways in common for treatment and phenotype. The only affected gene common to these two pathways is *UBE2Q*, a ubiquitin-conjugating enzyme, which was decreased in alcoholics and as a result of treatment by ethanol.

Comparison to Brain expression

We detected 20,165 unique genes expressed in at least one brain region. 99% of the genes expressed in the LCLs that could be mapped to the Gene 1.0 ST arrays were expressed in at least one of the 9 brain regions (**Supplementary Tables 1 and 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 on the basis of different expression after treatment with ethanol, 20 were confirmed with a p -value < 0.05 , and one (*FOXPI*) 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 which was not captured by the qRT-PCR. The eleven genes selected on the basis of 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 (**Figure 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 and 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 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 NF- κ B and TNF α pathways are central to inflammatory responses and alcoholic liver disease (Wang, Gao et al. 2012, Roh and Seki 2013). These pathways showed strong increases in expression of many genes, including TNF α , 15 TNF receptors or TNF associated genes, and 5 NF- κ B 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). 77 genes downstream of NF- κ B and 151 downstream of TNF α were affected, as were 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 NF- κ B, 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 et al., 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 one or more of eleven post mortem gene expression studies, fifty-eight 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. 284 were identified by at least one GWAS (**Supplementary Table 1**, GWAS references therein), including 8 that also differed between alcoholics and controls (**Supplementary Table 1 & 2**). Among the 284 genes, twelve were reported by two GWAS, including two genes associated with glutamate uptake. *SLC9A9* (cation proton antiporter 9) is associated with alcohol dependence (Kendler et al., 2011), alcohol dependence symptom count (Wang et al., 2012); it was also associated with smoking (Vink et al., 2009) and ADHD (Kondapalli et al., 2013). *SLC9A9* expression was also altered in frontal cortex of alcoholics (Liu et al., 2006) (Wang et al., 2012). *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* correlated with gene expression levels in LCLs (Wendland et al., 2009), and are associated with increased expression in postmortem 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 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 et al., 2012b).

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 x 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 et al., 2000).

Thirteen genes differentially expressed in the alcoholics were associated with alcoholism in at least one 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 one 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 preexisting 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 one 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 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.

Figure 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.

Table 1

Pathways affected by ethanol exposure.

Canonical Pathways	p-value	Molecules
Type 1 Diabetes Mellitus Signaling	4.4E-07	<i>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</i>
IL-6 Signaling	5.0E-06	<i>MAP2K6, SOCS3, SOCS1, ABCB1, IL1A, AKT2, TNFAIP6, NFKBIE, MAPK9, IKBKE, IL1R1, NFKB2, STAT3, IL1R2, VEGFA, COL1A1, NFKBIA, MAP3K7, PIK3C3, CSNK2A1, AKT3, TNF</i>
Dendritic Cell Maturation	1.2E-05	<i>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</i>
CD40 Signaling	4.2E-05	<i>MAP2K6, ICAM1, NFKBIE, TNFAIP3, MAPK9, IKBKE, STAT3, NFKB2, NFKBIA, CD40, MAP3K7, PIK3C3, LTA, TRAF1</i>
TNFR2 Signaling	4.6E-05	<i>NFKBIA, LTA, NFKBIE, TNFAIP3, IKBKE, NFKB2, BIRC3, TNF, TRAF1</i>
Lymphotoxin β Receptor Signaling	2.1E-04	<i>AKT2, VCAM1, NFKBIA, LTA, PIK3C3, RELB, TRAF4, AKT3, IKBKE, NFKB2, TNFSF14, TRAF1</i>
Crosstalk between Dendritic Cells and Natural Killer Cells	2.4E-04	<i>IL3RA, CD69, CD83, NFKB2, FAS, CSF2RB, CD40, CD80, IL12B, LTA, FSCN1, CD226, CD86, TNF, CCR7, IL2RB</i>
Role of JAK2 in Hormone-like Cytokine Signaling	2.4E-04	<i>SOCS1, SOCS3, STAT5A, SOCS6, SOCS2, SOCS4, STAT3, PRLR, SIRPA</i>
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.8E-04	<i>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</i>
Acute Myeloid Leukemia Signaling	4.0E-04	<i>MAP2K6, STAT5A, AKT2, STAT3, NFKB2, TCF3, TCF7, MYC, BRAF, CSF2RB, ARAF, RARA, PIK3C3, AKT3</i>
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	4.1E-04	<i>HLA-DMA, TLR10, IL1A, SLAMF1, CD40, CD80, IL12B, LTA, RELB, TLR6, HLA-DQA1, CD86, NFKB2, TNF, FAS</i>
TREM1 Signaling	4.2E-04	<i>STAT5A, TLR10, AKT2, ICAM1, CD40, TLR6, AKT3, CD86, CD83, STAT3, NFKB2, TNF</i>
Triacylglycerol Biosynthesis	4.3E-04	<i>PPAPDC1B, AGPAT5, ABHD5, PPAP2A, LPCAT4, DGAT2, MBOAT2, AGPAT9, AGPAT3, ELOVL6</i>
T Helper Cell Differentiation	5.1E-04	<i>STAT4, HLA-DMA, CD40, CD80, IL12B, IL21R, HLA-DQA1, CD86, IL2RA, IL12RB2, STAT3, TNF, IL18R1</i>
IL-10 Signaling	6.9E-04	<i>IL1R2, MAP2K6, SOCS3, IL1A, NFKBIA, MAP3K7, BLVRA, NFKBIE, IKBKE, NFKB2, IL1R1, STAT3, TNF</i>
Protein Ubiquitination Pathway	7.8E-04	<i>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</i>
B Cell Development	8.1E-04	<i>HLA-DMA, CD80, CD40, HLA-DQA1, CD86, IGHM, IL7, IGHG</i>
Small Cell Lung Cancer Signaling	8.9E-04	<i>FHIT, AKT2, PA2G4, NFKBIE, IKBKE, NFKB2, PTEN, MYC, NFKBIA, PIK3C3, TRAF4, AKT3, TRAF1</i>
Hypoxia Signaling in the Cardiovascular System	1.0E-03	<i>UBE2G2, VEGFA, UBE2L3, NFKBIA, UBE2Q1, SUMO1, NFKBIE, UBE2D4, CREB5, UBE2D3, PTEN, UBE2I</i>
EIF2 Signaling	1.0E-03	<i>RPL22, AKT2, EIF3H, RPS28, EIF1, RPL37, PPP1CB, EIF4A2, RPL23, RPL35A, RPS23, EIF3M, RPL15, EIF2S2, EIF3F, EIF3B, EIF1AX, PIK3C3, EIF2B5, EIF3A, AKT3, RPS20, RPS15A, RPL13</i>
NF- κ B Signaling	1.1E-03	<i>MAP2K6, AZI2, IL1A, AKT2, RELB, NFKBIE, TNFAIP3, NFKB2, IL1R1, MALTI, IL1R2, TLR10, NFKBIA, CD40, MAP3K7, PIK3C3, LTA, TLR6, CSNK2A1, IGF1R, AKT3, TNF</i>
IL-9 Signaling	1.3E-03	<i>SOCS3, STAT5A, IL9R, PIK3C3, SOCS2, STAT3, NFKB2, TNF</i>
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	1.5E-03	<i>MAP2K6, IL1A, CAMK4, NFKBIE, CSNK1A1, TCF7, ITGB3, IL18R1, SMURF1, IL1R2, NFKBIA, IGF1, MAP3K7, PIK3C3, AKT3, BIRC3, AKT2, MAPK9,</i>

Canonical Pathways	p-value	Molecules
		<i>IKBKE, IL1R1, TCF3, IL7, CALM1 (includes others), COL1A1, FZD6, TNF, WNT5A</i>
RANK Signaling in Osteoclasts	1.6E-03	<i>MAP2K6, AKT2, CAMK4, MAP3K13, NFKBIE, MAPK9, IKBKE, NFKB2, CALM1 (includes others), NFKBIA, MAP3K7, PIK3C3, AKT3, BIRC3</i>
Regulation of eIF4 and p70S6K Signaling	2.1E-03	<i>AKT2, EIF3H, PPP2CA, RPS28, EIF1, EIF4A2, RPS23, EIF3M, EIF2S2, EIF3F, EIF3B, EIF1AX, PIK3C3, EIF2B5, EIF3A, AKT3, PPP2R5C, RPS20, RPS15A, PPP2R5E</i>
CD28 Signaling in T Helper Cells	2.1E-03	<i>HLA-DMA, AKT2, CAMK4, NFKBIE, HLA-DQA1, MAPK9, IKBKE, MALTI, NFKB2, CALM1 (includes others), PAK1, ACTR3, NFKBIA, CD80, PIK3C3, CD86, AKT3</i>
iCOS-iCOSL Signaling in T Helper Cells	2.4E-03	<i>HLA-DMA, AKT2, CAMK4, NFKBIE, HLA-DQA1, IKBKE, NFKB2, PTEN, CALM1 (includes others), NFKBIA, CD80, CD40, PIK3C3, AKT3, IL2RA, IL2RB</i>
p53 Signaling	2.7E-03	<i>WT1, PMAIP1, AKT2, GADD45B, JMY, FAS, TP53BP2, PTEN, CHEK1, CCND2, PIK3C3, AKT3, SFN, PIDD</i>
Toll-like Receptor Signaling	3.0E-03	<i>PPARA, MAP2K6, TLR10, NFKBIA, MAP3K7, TRAF4, TLR6, TNFAIP3, NFKB2, TRAF1</i>
4-1BB Signaling in T Lymphocytes	3.2E-03	<i>TNFRSF9, NFKBIA, NFKBIE, MAPK9, IKBKE, NFKB2, TRAF1</i>
IL-17A Signaling in Airway Cells	3.3E-03	<i>AKT2, NFKBIA, MAP3K7, PIK3C3, NFKBIE, AKT3, MAPK9, IKBKE, STAT3, NFKB2, PTEN</i>
IL-22 Signaling	3.6E-03	<i>SOCS3, STAT5A, AKT2, AKT3, MAPK9, STAT3</i>
JAK/Stat Signaling	3.8E-03	<i>STAT4, SOCS1, SOCS3, STAT5A, AKT2, PIK3C3, SOCS6, SOCS2, AKT3, SOCS4, STAT3</i>
Death Receptor Signaling	3.9E-03	<i>NFKBIA, NFKBIE, IKBKE, HTRA2, TNFSF15, NFKB2, CFLAR, BIRC3, TNF, FAS</i>
Hepatic Fibrosis / Hepatic Stellate Cell Activation	4.0E-03	<i>SMAD2, IGFBP4, VCAM1, IL1A, ICAM1, CXCL9, NFKB2, IL1R1, FAS, VEGFA, IL1R2, COL1A1, IGF1, CD40, IGF1R, TNF, CCR7, IFNAR1</i>
Induction of Apoptosis by HIV1	4.5E-03	<i>NFKBIA, NFKBIE, MAPK9, IKBKE, HTRA2, NFKB2, BIRC3, TNF, FAS, TRAF1</i>
IGF-1 Signaling	4.8E-03	<i>IGFBP4, SOCS3, SOCS1, AKT2, IGF1, PIK3C3, SOCS6, SOCS2, CSNK2A1, IGF1R, AKT3, SOCS4, STAT3, SFN</i>
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	4.9E-03	<i>PPARA, AKT2, APOM, PPP2CA, NFKBIE, MAP3K13, PPP1CB, MAPK9, IKBKE, NFKB2, RAPIA, APOL1, NFKBIA, MAP3K7, PIK3C3, NCF2, AKT3, PPP2R5C, PPP2R5E, RHOF, TNF, SIRPA</i>
ATM Signaling	5.0E-03	<i>GADD45B, NFKBIA, H2AFX, MAPK9, TDP1, CBX5, CREB5, CHEK1, CCNB1, SMC1A</i>

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.

Table 2

Pathways that differed between alcoholics and controls.

Ingenuity Canonical Pathways	pvalue	Molecules
Molecular Mechanisms of Cancer	9.8E-04	<i>RAP2B, JAK1, GNA11, RHOT2, SOS2, RAC1, RALBP1, NBN, RHOQ, MAX, PRKAR1B, PRKCE, ARHGEF2, GNA13, ARHGEF3, CTNNB1, BCL2L1</i>
Actin Nucleation by ARP-WASP Complex	1.0E-03	<i>ARPC1A, RHOQ, RHOT2, SOS2, RAC1, NCK1</i>
Protein Ubiquitination Pathway	1.0E-03	<i>UCHL3, USP14, UBE2Q1, PSMD13, SKP1, HSPA8, PSMB7, UBE2J1, HSP90AB1, PSMB2, UBE2G1, HSPE1, PSMA4, PSMB1</i>
Phospholipase C Signaling	1.6E-03	<i>CALM1 (includes others), RHOQ, HDAC7, GNG2, SOS2, RHOT2, RAC1, PRKCE, ARHGEF2, MEF2C, GNA13, ARHGEF3, LCP2</i>
Breast Cancer Regulation by Stathmin1	2.9E-03	<i>CALM1 (includes others), TUBB3, SOS2, GNG2, RAC1, PRKAR1B, PRKCE, PPP1R11, ARHGEF2, ARHGEF3, GNA13</i>
G Beta Gamma Signaling	4.4E-03	<i>KCNJ5, SOS2, GNG2, GNA11, PRKAR1B, PRKCE, GNA13</i>
RAN Signaling	6.6E-03	<i>KPNA2, TNPO1, RAN</i>
Signaling by Rho Family GTPases	8.9E-03	<i>ARFIP2, ARPC1A, RHOQ, DIAPH3, RHOT2, GNG2, GNA11, RAC1, ARHGEF2, ARHGEF3, GNA13</i>
Androgen Signaling	9.8E-03	<i>CALM1 (includes others), GNG2, GNA11, PRKAR1B, PRKCE, POLR2B, GNA13</i>
Hypoxia Signaling in the Cardiovascular System	1.1E-02	<i>UBE2J1, UBE2Q1, HSP90AB1, UBE2G1, CSNK1D</i>
RhoGDI Signaling	1.2E-02	<i>ARPC1A, RHOQ, RHOT2, GNG2, GNA11, RAC1, ARHGEF2, ARHGEF3, GNA13</i>
Netrin Signaling	1.3E-02	<i>RAC1, PRKAR1B, NCK1, ABLIM1</i>
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1.6E-02	<i>ARPC1A, RAC1, PRKCE, RAB11A, NCK1, LCP2</i>
Tec Kinase Signaling	1.7E-02	<i>JAK1, RHOQ, RHOT2, GNG2, GNA11, PRKCE, GNA13, TNFRSF10A</i>
Paxillin Signaling	1.8E-02	<i>ITGB2, ARFIP2, SOS2, RAC1, NCK1, ITGAL</i>
Telomerase Signaling	1.8E-02	<i>ELF2, HSP90AB1, SOS2, HDAC7, PTGES3, ELF1</i>
Ephrin B Signaling	2.0E-02	<i>GNG2, GNA11, RAC1, GNA13, CTNNB1</i>
Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	2.0E-02	<i>DLAT, DLD</i>
Huntington's Disease Signaling	2.1E-02	<i>HSPA8, ARFIP2, BDNF, SOS2, HDAC7, GNG2, GNA11, PRKCE, CASP4, POLR2B</i>
Integrin Signaling	2.1E-02	<i>RAP2B, ITGB2, ARPC1A, RHOQ, RHOT2, SOS2, RAC1, NCK1, ITGAL</i>
Semaphorin Signaling in Neurons	2.2E-02	<i>SEMA4D, RHOQ, RHOT2, RAC1</i>
Cholecystokinin/Gastrin-mediated Signaling	2.2E-02	<i>RHOQ, RHOT2, SOS2, PRKCE, MEF2C, GNA13</i>
Cleavage and Polyadenylation of Pre-mRNA	2.5E-02	<i>PABPN1, CPSF4</i>
Role of NFAT in Regulation of the Immune Response	3.1E-02	<i>CALM1 (includes others), SOS2, GNG2, GNA11, CSNK1D, MEF2C, GNA13, LCP2</i>
CREB Signaling in Neurons	3.6E-02	<i>CALM1 (includes others), SOS2, GNG2, GNA11, PRKAR1B, PRKCE, POLR2B, GNA13</i>
Sertoli Cell-Sertoli Cell Junction Signaling	3.8E-02	<i>TUBB3, TJAP1, PPAP2B, RAC1, PRKAR1B, MLLT4, YBX3, CTNNB1</i>
Tight Junction Signaling	3.9E-02	<i>RAC1, PRKAR1B, MLLT4, YBX3, ARHGEF2, CTNNB1, CPSF4</i>
ERK5 Signaling	4.5E-02	<i>YWHAG, YWHAE, MEF2C, GNA13</i>
ERK/MAPK Signaling	4.6E-02	<i>ELF2, YWHAG, SOS2, RAC1, PRKAR1B, PRKCE, PPP1R11, ELF1</i>
Germ Cell-Sertoli Cell Junction Signaling	4.7E-02	<i>TUBB3, RHOQ, PPAP2B, RHOT2, RAC1, MLLT4, CTNNB1</i>

Ingenuity Canonical Pathways	pvalue	Molecules
PI3K/AKT Signaling	4.9E-02	<i>YWHAG, JAK1, YWHAE, HSP90AB1, SOS2, CTNNB1</i>
CXCR4 Signaling	4.9E-02	<i>RHOQ, RHOT2, GNG2, GNA11, RAC1, PRKCE, GNA13</i>

Genes with FDR 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.

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