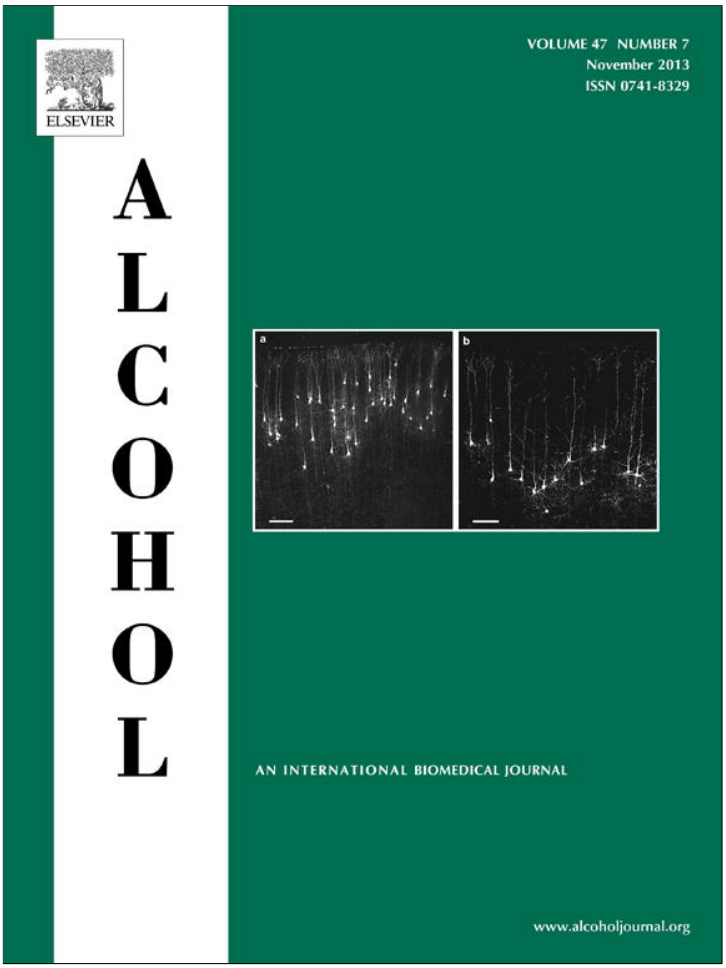

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Stress–response pathways are altered in the hippocampus of chronic alcoholics

Jeanette N. McClintick [a], Xiaoling Xuei [a], Jay A. Tischfield [b], Alison Goate [c], Tatiana Foroud [d], Leah Wetherill [d,e], Marissa A. Ehringer [f,g], Howard J. Edenberg [a,d,*]

[a] Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[b] Department of Genetics, Rutgers University, Piscataway, NJ 08854, USA
[c] Department of Psychiatry, School of Medicine, Washington University in St. Louis, St. Louis, MO 63110, USA
[d] Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[e] Department of Psychology, IUPUI School of Science, Indianapolis, IN 46202, USA
[f] Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309, USA
[g] Department of Integrative Physiology, University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

The chronic high-level alcohol consumption seen in alcoholism leads to dramatic effects on the hippocampus, including decreased white matter, loss of oligodendrocytes and other glial cells, and inhibition of neurogenesis. Examining gene expression in post mortem hippocampal tissue from 20 alcoholics and 19 controls allowed us to detect differentially expressed genes that may play a role in the risk for alcoholism or whose expression is modified by chronic consumption of alcohol. We identified 639 named genes whose expression significantly differed between alcoholics and controls at a False Discovery Rate (FDR) ≤ 0.20; 52% of these genes differed by at least 1.2-fold. Differentially expressed genes included the glucocorticoid receptor and the related gene FK506 binding protein 5 (*FKBP5*), UDP glycosyltransferase 8 (*UGT8*), urea transporter (*SLC14A1*), zinc transporter (*SLC39A10*), Interleukin 1 receptor type 1 (*IL1R1*), thioredoxin interacting protein (*TXNIP*), and many metallothioneins. Pathways related to inflammation, hypoxia, and stress showed activation, and pathways that play roles in neurogenesis and myelination showed decreases. The cortisol pathway dysregulation and increased inflammation identified here are seen in other stress-related conditions such as depression and post-traumatic stress disorder and most likely play a role in addiction. Many of the detrimental effects on the hippocampus appear to be mediated through NF-κB signaling. Twenty-four of the differentially regulated genes were previously identified by genome-wide association studies of alcohol use disorders; this raises the potential interest of genes not normally associated with alcoholism, such as suppression of tumorigenicity 18 (*ST18*), BCL2-associated athanogene 3 (*BAG3*), and von Willebrand factor (*VWF*).

Introduction

Alcohol dependence (alcoholism) is a complex disorder with a 40–60% genetic contribution to risk (Edenberg & Foroud, 2006; Heath et al., 1997; McGue, 1999). Although several genes in which variants affect the risk for alcohol dependence have been identified (Rietschel & Treutlein, 2012), their overall effect accounts for only a small portion of the vulnerability to alcohol dependence. Many studies are underpowered, and determining which modest association results are true positives can be difficult. Studies of gene expression in the human brain can reveal differences between alcoholics and controls that might be either risk factors or sequelae of excessive drinking; in either case, this increases the likelihood that such genes are relevant to the disease.

Prior studies have compared gene expression between alcoholics and controls using human post mortem brains (Flatscher-Bader, Harrison, Matsumoto, & Wilce, 2010; Flatscher-Bader et al., 2005; Iwamoto et al., 2004; Kryger & Wilce, 2010; Lewohl et al., 2000; Liu, Lewohl, Harris, Dodd, & Mayfield, 2007; Liu et al., 2006; Mayfield et al., 2002; Sokolov, Jiang, Trivedi, & Aston, 2003; Zhou, Yuan, Mash, & Goldman, 2011). Others have examined brain regions from animal models (Edenberg et al., 2005; Kerns et al., 2005; Kimpel et al., 2007; McBride et al., 2010; Mulligan et al., 2006, 2008; Saito et al., 2004; Tabakoff et al., 2008; Wolen et al., 2012; Worst et al., 2005). The human studies have examined superior frontal cortex (Lewohl et al., 2000; Liu et al., 2006; Liu et al., 2007), frontal cortex (Liu et al., 2007), prefrontal cortex (Flatscher-Bader

* Corresponding author. Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Dr., MS4063, Indianapolis, IN 46202, USA. Tel.: +1 317 274 2353; fax: +1 317 274 4686.
E-mail address: edenberg@iu.edu (H.J. Edenberg).

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et al, 2005; Iwamoto et al., 2004), temporal cortex (Sokolov et al., 2003), nucleus accumbens and ventral tegmental area (Flatscher-Bader et al, 2005, 2010), basolateral amygdala (Kryger & Wilce, 2010), and hippocampus (Zhou & Yuan et al., 2011). These studies have found down-regulation of myelin-related genes (Liu et al., 2006; Mayfield et al., 2002) and mitochondrial dysfunction (Liu et al., 2007; Sokolov et al., 2003), and dysregulation of genes involved in ubiquitination (Liu et al., 2006; Sokolov et al., 2003) and apoptosis and cell survival (Liu et al., 2004, 2006, 2007).

The hippocampus is a key region related to learning, for which neurogenesis is required (Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006). Chronic, excessive consumption of alcohol leads to dramatic effects on the hippocampus. Hippocampal size is decreased with chronic drinking (Agartz, Momenan, Rawlings, Kerich, & Hommer, 1999; Laakso et al., 2000), and abstinence leads to a recovery of this volume loss (Crews & Nixon, 2009). The decrease in hippocampal size is due to a combination of neurodegeneration and decreased neurogenesis (Crews & Nixon, 2009; Morris, Eaves, Smith, & Nixon, 2010; Richardson et al., 2009). While neurodegeneration is noted in alcoholism, post mortem studies of the hippocampus have found glial cell loss but no neuronal loss. A post mortem study of the hippocampus found a loss of white matter, including oligodendrocytes, but with no significant loss of neurons (Harding, Wong, Svoboda, Kril, & Halliday, 1997). Alcoholics who had been abstinent before death did not show a significant loss of white matter, implying that recovery from this loss is possible (Harding et al., 1997). A second post mortem examination of the hippocampus showed a 37% loss of glial cells (astrocytes, oligodendrocytes, and to a lesser extent microglia) in alcoholics (Korbo, 1999). Part of the neurodegeneration in brain is related to ethanol-induced inflammation through the Toll-like receptors and induction of the NF-κB pathway (Alfonso-Loeches et al., 2012; Crews & Nixon, 2009; Qin & Crews, 2012). Neuroinflammation may also play a part in the addiction process because alcohol and stress induce innate immune genes via the NF-κB pathway that lead to changes in behavior that mimic addiction (Blednov et al., 2011, 2012; Crews, Zou, & Qin, 2011; Mayfield, Ferguson, & Harris, 2013). Inflammation has been seen to block neurogenesis through the NF-κB pathway in depression (Koo, Russo, Ferguson, Nestler, & Duman, 2010), and neurogenesis can be restored by blocking inflammation (Monje, Toda, & Palmer, 2003).

To obtain a global picture of changes in gene expression in the hippocampi of alcoholics, we conducted a microarray study of post mortem hippocampi from 20 alcoholics and 19 controls. We report the differences in gene expression between alcoholics and controls and the pathways affected. We compare our results with genes identified in other human brain expression studies and in genome-wide association studies (GWAS) for alcohol dependence or phenotypes associated with alcohol use disorders to look for genes in common and the pathways they delineate.

Materials and methods

Hippocampal tissue from 20 alcoholics and 19 controls, all of European background (6 females in each group), was obtained from the New South Wales Tissue Resource Centre at the University of Sydney, Australia (Sheedy et al., 2008). Supplemental Table S1 describes the samples used. Total RNA was extracted using TRIzol® Reagent (Invitrogen; Carlsbad, CA) following a modified protocol with twice as much TRIzol® per gram of tissue (Edenberg et al., 2005). RNA was further purified using the Qiagen RNeasy mini-kit (Qiagen; Valencia, CA). Quality of the RNA, determined using the Agilent Bioanalyzer (Agilent; Santa Clara, CA), did not significantly differ between the 2 groups (mean RIN 6.8, SD 1).

RNA was labeled and hybridized to Affymetrix Gene 1.0 ST arrays, following the standard WT protocol (GeneChip® Whole Transcript [WT] Sense Target Labeling Assay, rev. 5, www.affymetrix.com). Samples were processed in 2 groups, balanced by phenotype and sex. Arrays were scanned and data were imported into Partek Genomics Suite version 6.2 (Partek, Inc.; St. Louis, MO).

Robust Multichip Average signals (RMA) (Irizarry et al., 2003) were generated for the core probe sets using the RMA background correction. Quantile normalization and summarization was done by Median Polish analysis using the Partek Genomics Suite. Summarized signals for each probe set were $\log_2$ transformed. These data are deposited in the NCBI Gene Expression Omnibus under series number GSE44456. The $\log_2$ transformed signals were used for principal components analysis, hierarchical clustering, and signal histograms to determine if there were any outlier arrays; none were found. We have previously shown that removing probe sets not reliably detected above background in any experimental condition improves analysis by reducing the multiple testing burden (McClintick & Edenberg, 2006). The signal histogram (not shown) indicated that probe sets with $\log_2$ values <4 were at background level. Therefore, probe sets with mean $\log_2$ values <4.0 in both alcoholics and controls were removed. The remaining probe sets were analyzed using a 3-way ANOVA with the factors of *phenotype* (control/alcoholic), *sex* (male/female), and *processing batch* (for potential technical variations). Interaction between sex and phenotype was not significant after correcting for multiple testing (Storey & Tibshirani, 2003) and was removed from the analysis. Fold changes were calculated using the untransformed RMA signals. False discovery rates (FDR) were calculated using q value (Storey & Tibshirani, 2003).

We collected lists of differentially expressed genes from 10 other gene expression studies of post mortem brain tissue comparing alcoholics to controls (Flatscher-Bader et al, 2005, 2010; Iwamoto et al., 2004; Kryger & Wilce, 2010; Lewohl et al., 2000; Liu et al., 2006, 2007; Mayfield et al., 2002; Sokolov et al., 2003; Zhou & Yuan et al., 2011). Similarly, we assembled lists of genes identified in 12 recent GWAS studies of risk for alcoholism or related traits (Bierut et al., 2010; Edenberg et al., 2010; Foroud et al., 2007; Hack et al., 2011; Johnson, Drgon, Walther, & Uhl, 2011; 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., 2012). We annotated the list of differentially expressed genes from our study (Supplemental Table S2) to show these overlaps. We also created a list of genes identified by 2 or more studies (including the present one) in Supplemental Table S4; these will be referred to as "multiply-identified genes" in the rest of the text.

To identify transcripts enriched in different cell types we used 3 files from Cahoy et al. (2008): astrocytes (Cahoy Supplemental Table S4), oligodendrocytes (Cahoy Supplemental Table S5), and neurons (Cahoy Supplemental Table S6). These were matched by the official gene symbol (HUGO Gene Nomenclature Committee) to our data set.

Ingenuity Pathway Analysis (IPA, www.Ingenuity.com) was performed using probe sets with an FDR $\leq$ 0.20 to examine Canonical Pathways. For all of our analyses the Ingenuity knowledge base was used as the reference set to insure all analyses used similar parameters. We analyzed the list of probe sets identified at FDR $\leq$ 0.20 from our study, the list of multiply identified genes described above, and the cell-type enriched sets of genes described above. We also carried out an IPA Upstream Regulator report to identify transcription factors, cytokines, and chemicals, etc. that are predicted to be activated or inactivated based on the direction of change in their downstream targets; a positive Z-score indicates likely activation and a negative Z-score indicates likely inactivation in alcoholics relative to the controls.

Quantitative Real-Time PCR (qRT-PCR) was used to confirm differences in 4 genes: *FKBP5*, *GRM3*, *NR3C1*, and *NR4A2*. Primers were selected from Life Technologies™ (Carlsbad, CA) catalog of Taqman® Gene Expression Assays (http://bioinfo.appliedbiosystems.com/genome-database/gene-expression.html). One μg of total RNA from each sample was used for reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Life Technologies™, Carlsbad, CA). Each gene of interest was measured in duplicate using TaqMan® Fast Advanced Master Mix (Life Technologies). Primers for *POL2RA* (Taqman® primer: Hs00172187_m1) were included in each well as a control. The C_T of the *POL2RA* run in the same well was subtracted from the C_T of the target gene to yield the Delta C_T (relative expression). The Delta C_T from 2 replicates for each sample was used in a 3-way ANOVA using phenotype, sex, and sample ID as factors.

Results

We analyzed RNA extracted from the hippocampi of 20 alcoholics and 19 controls (6 females in each group) using Affymetrix Gene 1.0 ST microarrays. Supplemental Table S1 describes the samples. Subject age and RNA integrity (RIN) did not significantly differ between alcoholics and controls (all $p > 0.4$). The single factor that most affects microarray measurement of gene expression from post mortem brain tissue is the pH (Atz et al., 2007); pH (mean 6.5, SD 0.3) did not significantly differ between alcoholics and controls. A total of 22,987 probe sets (80% of the core probe sets on the Affymetrix Gene 1.0 ST array) were expressed (detected above background) in at least 1 of the 2 groups (alcoholics or controls). A 3-way ANOVA using factors for phenotype (alcoholic/control), sex, and microarray-processing batch detected 743 probe sets that significantly differed between alcoholics and controls at a False Discovery Rate (FDR) ≤ 0.20. This represented 639 named genes (46 of which were measured twice) plus 58 unnamed probe sets (Supplemental Table S2). Among the significant probe sets, 50% (52% of the named genes) showed absolute fold changes ≥1.2 (Fig. 1). Slightly over half the changes (53%) reflected lower expression in the alcoholics.

Large fold changes were found among genes associated with inflammatory and immune response (GO: 0006954 and GO: 0006955), particularly interleukin receptors (Table 1A). Twenty-one genes involved in hypoxia (GO: 0001666) were differentially expressed, with two-thirds of them showing higher expression in the brains of alcoholics (Table 1B). The expression of most genes in the glucocorticoid pathway, including the glucocorticoid receptor (*NR3C1*) and 2 FK506 binding proteins (*FKBP4*, *FKBP5*), differed significantly between alcoholics and controls. *NR3C1* expression was 30% lower in alcoholics, whereas *FKBP5*, which functions as a negative regulator of the pathway, was increased over 2-fold (Table 1C). Genes related to myelination and oligodendrocytes demonstrated decreased expression in the alcoholic hippocampi (Table 1D). Fourteen of 16 significantly changed genes in this group were expressed at lower levels in alcoholics, averaging 74%, whereas only 2 were at higher levels. Eight metallothioneins (MT) with an FDR ≤ 20% were expressed at higher levels in the hippocampus of alcoholics (mean 1.44-fold), and 9 more (20% < FDR ≤ 40%) were also expressed at higher levels in alcoholics (mean 1.2-fold; Table 1E).

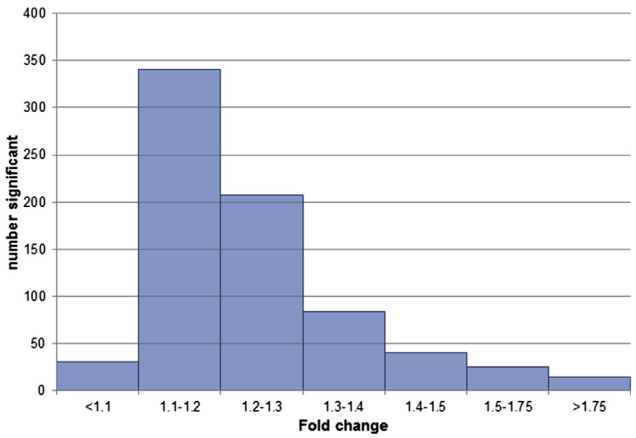

Fig. 1. Distribution of fold changes for the 743 transcripts significant at FDR ≤ 0.20.

Ingenuity Pathways Analysis (IPA) of genes with FDR ≤ 0.20 revealed many canonical pathways that differed between alcoholics and controls (Table 2). Signaling pathways predominated, along with stress or immune responses. Acute phase response signaling, IL-6 signaling, IL-8 signaling, IL-10 signaling, LPS/IL-1 mediated inhibition of RXR function, mTOR signaling, hypoxia signaling, p38 MapK signaling, EIF2 signaling (eukaryotic translation initiation factor 2), and glucocorticoid signaling were up. GADD45 (growth arrest and DNA-damage-inducible) signaling, p38 signaling, and Her2 signaling, were mixed or down. Many of the pathways shared key genes. *ATM* (ataxia telangiectasia mutated; down 20%) is in 39 of the 60 pathways and *AKT1* (v-*akt* murine thymoma viral oncogene homolog 1; increased 8%) is in 32 of the pathways. *TRAF6*, *PRKD1*, *MAP2K3*, *RHOB* and *RHOC*, *CREB1*, *CCND*, and the guanine binding proteins *GNAI1*, *GNB2*, and *GNG5* were each in at least 12 of the pathways.

To see whether the alcoholics differed in expression of genes enriched in particular cell types, we examined the sets of genes whose expression is known to be enriched in astrocytes, oligodendrocytes, or neurons (Cahoy et al., 2008), noted in Supplemental Table S2. The vast majority of these cell-enriched genes were not differentially expressed: about 95% have FDR > 0.20. However, for those genes that were differentially expressed, the fraction up and down was skewed compared to the overall results. Eighty-three percent of the differentially expressed transcripts enriched in oligodendrocytes were expressed at lower levels in alcoholics ($p = 3.9 \times 10^{-9}$), as were 83% of the differentially expressed transcripts in neurons ($p = 2.1 \times 10^{-4}$), whereas only 53% of the total probe sets were down. The differentially expressed genes expressed in astrocytes demonstrated the opposite trend, with 61% at higher levels in alcoholics ($p = 0.003$), including hypoxia response genes.

Analyzing upstream regulators can clarify the pathway findings by looking for commonalities in their regulation, i.e. it may be possible to identify sets of differentially expressed genes that are downstream targets of specific transcription factors, cytokines, signaling cascades, and endogenous and exogenous chemicals. Both the glucocorticoid and aldosterone pathways were significantly altered in alcoholic brains, and the upstream effectors analysis indicated that their receptors, *NR3C1* and *NR3C2*, are in an activated state (Supplemental Table S3). Other genes identified as activated include many regulators related to immune function (including cytokines IL1B, IL10, IL11, IL15, IL17A, and EDN1), other regulators, including hypoxia-related gene HIF1A and Endothelial PAS domain-containing protein 1 (EPAS1), and 2 genes that are general indicators of stress, TP53 and TGFB1. The expression of downstream targets for the Wnt/β catenin pathway and the *ERBB4* pathways involved in neurogenesis (Lazarov & Marr, 2010), including *TCF4* and cyclin D1, provide evidence that both of these pathways were less active in the alcoholics (Supplemental Table S3).

Bioinformatic analysis found 386 genes that were identified in 2 or more studies (GWAS or gene expression, including the present study), which we refer to as multiply identified genes (listed in Supplemental Table S4). One hundred seven of these genes were identified by our study and at least one other (noted in

Table 1
Functional categories of selected genes that significantly differ between alcoholics and controls (FDR ≤ 0.20).

Gene symbol	Gene title	Fold	*p* value	FDR
A. Inflammatory/immune response GO (0006954 & 0006955)				
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	−1.55	1.1E-02	0.21
TAC1	Tachykinin, precursor 1	−1.53	2.9E-02	0.27
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	−1.37	1.5E-02	0.23
LIPA	Lipase A, lysosomal acid, cholesterol esterase	−1.34	1.4E-03	0.13
HDAC9	Histone deacetylase 9	−1.31	7.8E-03	0.19
PXK	PX domain containing serine/threonine kinase	−1.31	5.8E-03	0.18
IKBKAP	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	−1.23	3.8E-04	0.08
SEMA4D	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain,	−1.22	1.2E-02	0.21
KLRG1	Killer cell lectin-like receptor subfamily G, member 1	−1.21	5.6E-04	0.10
BLNK	B-cell linker	−1.20	3.2E-02	0.28
OAS1	2′,5′-oligoadenylate synthetase 1, 40/46 kDa	−1.20	2.6E-03	0.15
PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator	−1.20	2.7E-02	0.27
PLA2G4C	Phospholipase A2, group IVC (cytosolic, calcium-independent)	−1.18	3.6E-02	0.29
IGKC	Immunoglobulin kappa constant	−1.13	1.6E-02	0.23
TRAF6	TNF receptor-associated factor 6	−1.12	4.1E-03	0.16
ITCH	Itchy E3 ubiquitin protein ligase homolog (mouse)	−1.11	2.7E-02	0.27
ADORA1	Adenosine A1 receptor	−1.11	3.4E-03	0.16
AKT1	v-akt murine thymoma viral oncogene homolog 1	1.08	7.0E-03	0.19
GTPBP1	GTP binding protein 1	1.10	3.1E-03	0.15
KIR2DL3	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	1.10	2.7E-02	0.27
FCGRT	Fc fragment of IgG, receptor, transporter, alpha	1.12	2.7E-02	0.27
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	1.13	3.7E-02	0.29
LTB4R	Leukotriene B4 receptor	1.13	6.5E-03	0.18
FTH1	Ferritin, heavy polypeptide 1	1.13	3.0E-02	0.28
SMAD1	SMAD family member 1	1.14	1.5E-02	0.23
MR1	Major histocompatibility complex, class I-related	1.15	3.2E-02	0.28
PROK2	Prokineticin 2	1.15	3.5E-02	0.29
ULBP2	UL16 binding protein 2	1.18	2.8E-02	0.27
S1PR3	Sphingosine-1-phosphate receptor 3	1.20	1.4E-02	0.22
TGFBR3	Transforming growth factor, beta receptor III	1.25	1.2E-02	0.21
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	1.25	2.6E-03	0.15
KIR2DL3	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	1.27	3.1E-02	0.28
C1R	Complement component 1, r subcomponent	1.27	7.8E-03	0.19
PNP	Purine nucleoside phosphorylase	1.27	2.9E-03	0.15
TARP	TCR gamma alternate reading frame protein	1.39	3.0E-04	0.08
IFITM2	Interferon induced transmembrane protein 2 (1-8D)	1.46	1.7E-03	0.14
SLC11A1	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	1.51	2.1E-03	0.14
IL4R	Interleukin 4 receptor	1.57	2.1E-04	0.08
IFITM3	Interferon induced transmembrane protein 3 (1-8U)	1.60	6.2E-05	0.05
IL1R1	Interleukin 1 receptor, type I	1.71	1.7E-05	0.03
CD163	CD163 molecule	1.80	3.7E-03	0.16
S100A8	S100 calcium binding protein A8	1.85	7.8E-03	0.19
IL1RL1	Interleukin 1 receptor-like 1	1.87	7.9E-04	0.11
SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	2.28	4.0E-03	0.16
B. Hypoxia GO (0001666)				
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	−1.40	1.5E-02	0.23
PYGM	Phosphorylase, glycogen, muscle	−1.29	2.8E-02	0.27
VLDLR	Very low density lipoprotein receptor	−1.28	3.7E-03	0.16
PRKCQ	Protein kinase C, theta	−1.21	2.8E-02	0.27
HSP90B1	Heat shock protein 90 kDa beta (Grp94), member 1	−1.17	1.5E-02	0.23
ADAM17	ADAM metallopeptidase domain 17	−1.14	3.0E-02	0.28
BIRC2	Baculoviral IAP repeat-containing 2	−1.13	1.7E-02	0.24
EGLN2	egl nine homolog 2 (*C. elegans*)	1.10	8.0E-03	0.19
PLD2	Phospholipase D2	1.11	2.9E-02	0.28
ECE1	Endothelin converting enzyme 1	1.14	3.7E-02	0.29

HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	1.15	2.0E-02	0.25
SOD2	Superoxide dismutase 2, mitochondrial	1.16	1.7E-02	0.24
SDC2	Syndecan 2	1.18	2.5E-02	0.27
ADM	Adrenomedullin	1.25	2.2E-02	0.26
SOCS3	Suppressor of cytokine signaling 3	1.31	7.6E-03	0.19
TGFB3	Transforming growth factor, beta 3	1.32	3.7E-02	0.29
DDIT4	DNA-damage-inducible transcript 4	1.39	7.9E-03	0.19
HIF3A	Hypoxia inducible factor 3, alpha subunit	1.40	1.5E-03	0.13
PDLIM1	PDZ and LIM domain 1	1.42	1.1E-02	0.21
ANGPTL4	Angiopoietin-like 4	1.57	8.6E-05	0.06
EDN1	Endothelin 1	1.65	3.3E-04	0.08
C. HPA axis				
HSPA1A	Heat shock 70 kDa protein 1A	−1.47	7.7E-03	1.9E-01
HSP90AA1	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	−1.37	3.7E-04	8.5E-02
HSPA5	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	−1.29	1.9E-03	1.4E-01
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	−1.29	1.8E-05	2.7E-02
HSPA8	Heat shock 70 kDa protein 8	−1.26	7.7E-03	1.9E-01
FKBP4	FK506 binding protein 4, 59 kDa	−1.16	3.4E-03	1.6E-01
FKBP5	FK506 binding protein 5	2.21	4.6E-06	2.1E-02
D. Myelination				
UGT8	UDP glycosyltransferase 8	−1.73	1.4E-04	0.07
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	−1.55	1.1E-02	0.21
KLK6	Kallikrein-related peptidase 6	−1.46	3.0E-03	0.15
MOG	Myelin oligodendrocyte glycoprotein	−1.46	3.6E-03	0.16
TF	Transferrin	−1.44	6.0E-03	0.18
ASPA	Aspartoacylase	−1.28	6.6E-02	0.33
PLP1	Proteolipid protein 1	−1.26	1.6E-03	0.14
OMG	Oligodendrocyte myelin glycoprotein	−1.25	3.9E-03	0.16
PLLP	Plasmolipin	−1.23	1.9E-02	0.25
MAG	Myelin associated glycoprotein	−1.23	8.1E-02	0.35
CNP	2′,3′-cyclic nucleotide 3′ phosphodiesterase	−1.20	4.2E-02	0.30
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	−1.20	4.3E-02	0.31
PMP2	Peripheral myelin protein 2	−1.18	5.8E-02	0.33
MYEF2	Myelin expression factor 2	−1.09	3.6E-02	0.29
MYT1	Myelin transcription factor 1	1.16	2.4E-02	0.26
MPZL2	Myelin protein zero-like 2	1.84	1.5E-03	0.14
E. Metallothioneins				
MT1X	Metallothionein 1X	1.98	1.8E-05	0.03
MT1M	Metallothionein 1M	1.50	1.1E-03	0.13
MT1A	Metallothionein 1A	1.48	2.6E-04	0.08
MT2A	Metallothionein 2A	1.47	1.1E-03	0.13
MT1G	Metallothionein 1G	1.38	2.1E-02	0.25
MT1L	Metallothionein 1L (gene/pseudogene)	1.37	2.0E-03	0.14
MT1JP	Metallothionein 1J (pseudogene)	1.26	1.6E-02	0.23
MT1P3	Metallothionein 1 pseudogene 3	1.25	3.3E-03	0.16
MT1DP	Metallothionein 1D (pseudogene)	1.24	7.1E-03	0.19
MT1E	Metallothionein 1E	1.22	5.7E-02	0.33
MT1B	Metallothionein 1B	1.21	1.1E-02	0.21
MT1P2	Metallothionein 1 pseudogene 2	1.21	6.9E-03	0.19
MT1F	Metallothionein 1F	1.19	1.1E-01	0.37
MT3	Metallothionein 3	1.16	6.1E-02	0.33
MT1H	Metallothionein 1H	1.15	6.4E-02	0.33
MT1IP	Metallothionein 1I (pseudogene)	1.14	4.6E-02	0.31
MT4	Metallothionein 4	1.13	7.4E-02	0.34

Table 2
Ingenuity pathway analysis using genes differentially expressed in hippocampi of alcoholics. Pathways in Section A are common to genes identified in multiple studies. Section B lists pathways identified only in this study.

Canonical pathways	*p* value	Significant genes in the pathway
A. Pathways common to multiple studies		
Acute phase response signaling	1.1E-04	*SOCS3, TCF4, SERPING1, TNFRSF1A, MAP3K1, VWF, SERPINA3, IL1R1, NR3C1, TRAF6, C1R, AKT1, TF, CFB, MAP2K3, OSMR*
Aldosterone signaling in epithelial cells	1.1E-04	*HSPA1A/HSPA1B, HSPH1, SLC12A2, DNAJA1, HSPA5, HSPA1L, PLCD1, HSPA8, HSPE1, HSP90AA1, HSPB7, DNAJB6, PLCD4, PRKD1, ATM*
Axonal guidance signaling	2.1E-02	*PXN, PAPPA, C9orf3, GNAI1, DPYSL5, SLIT2, ADAMTS9, TUBA1B, PLCD1, SEMA6D, AKT1, GNB2, ADAM10, RTN4, GNG5, ERBB2, SEMA4B, PLCD4, MYL3, FARP2, PRKD1, ATM*
Cell cycle: G1/S checkpoint regulation	4.9E-02	*HDAC9, CCND3, PAK1IP1, CCND1, ATM*
CXCR4 signaling	2.6E-02	*PXN, AKT1, RHOB, RHOC, GNB2, GNAI1, GNG5, MYL3, PRKD1, ATM*
Cyclins and cell cycle regulation	4.4E-02	*CCNA2, HDAC9, CCNA1, CCND3, CCND1, ATM*
EIF2 signaling	2.8E-05	*RPL24, RPS2, RPL23A, RPS17/RPS17L, RPLP0, RPL7, RPL10A, RPL35, RPS3A, AKT1, RPL7A, RPL39, RPL19, RPL12, RPS5, RPL29, ATM, RPSA*
Estrogen-mediated S-phase entry	4.1E-02	*CCNA2, CCNA1, CCND1*
Glioma invasiveness signaling	3.8E-02	*TIMP4, RHOB, TIMP1, RHOC, ATM*
HGF signaling	4.2E-02	*PXN, AKT1, MAP3K6, MAP3K1, CCND1, PRKD1, ATM*
ILK signaling	4.0E-02	*PXN, CDH1, AKT1, RHOB, TNFRSF1A, RHOC, CREB1, ITGB4, CCND1, MYL3, ATM*
Inhibition of matrix metalloproteases	7.9E-03	*TIMP4, TIMP1, THBS2, ADAM10, MMP24*
mTOR signaling	3.5E-03	*NAPEPLD, DDIT4, RHOC, RPS2, PRR5L, RPS17/RPS17L, PLD1, AKT1, RPS3A, RHOB, RPS5, PRKD1, ATM, RPSA*
p70S6K signaling	4.0E-02	*PLCD1, IL4R, AKT1, GNAI1, PLCD4, PLD1, PRKD1, ATM*
Protein ubiquitination pathway	1.8E-02	*USP28, MED20, HSPA1A/HSPA1B, HSPH1, USP19, DNAJA1, HSPA5, HSPA1L, HSPA8, TRAF6, UBE2G1, HSPE1, HSP90AA1, HSPB7, DNAJB6*
Reelin signaling in neurons	4.2E-02	*AKT1, ARHGEF2, PAFAH1B1, VLDLR, ATM, APP*
RhoGDI signaling	2.4E-02	*CDH1, PPP1R12C, RHOB, RHOC, GNB2, GNAI1, GNG5, ARHGEF17, ARHGEF2, DLC1, MYL3*
Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	3.8E-02	*SOCS3, TCF4, IL1RL1, TNFRSF1A, CEBPD, IL1R1, CCND1, PLCD1, TRAF6, AKT1, CREB1, MAP2K3, PLCD4, PRKD1, TCF7L2, ATM*
Signaling by Rho family GTPases	7.6E-03	*SEPT8, PPP1R12C, RHOC, SEPT7, GNAI1, ARHGEF17, PLD1, CDH1, RHOB, GNB2, GNG5, ARHGEF2, PARD3, MYL3, ATM*
TR/RXR activation	1.9E-02	*KLF9, AKT1, NXPH2, ACACA, THRA, TBL1XR1, ATM*
Type II diabetes mellitus signaling	3.4E-02	*SOCS3, AKT1, TNFRSF1A, MAP3K1, ACSL5, SLC27A3, PRKD1, ATM*
B. Additional significant pathways		
Activation of IRF by cytosolic pattern recognition receptors	1.5E-02	*DHX58, IFIH1, TRAF6, ZBP1, IKBKAP, IFIT2*
Acute myeloid leukemia signaling	1.2E-02	*TCF4, AKT1, CCNA1, MAP2K3, CCND1, TCF7L2, ATM*
Aryl hydrocarbon receptor signaling	1.9E-03	*TGM2, ALDH4A1, CCNA2, ALDH1L1, CCNA1, CCND3, HSP90AA1, HSPB7, DHFR, CCND1, PTGES3, ATM*
ATM signaling	4.4E-02	*MDM4, GADD45A, CREB1, BLM, ATM*
Biotin-carboxyl carrier protein assembly	5.9E-03	*ACACB, ACACA*
Cardiac hypertrophy signaling	1.4E-02	*MAP3K6, RHOC, MAP3K1, GNAI1, PLCD1, AKT1, RHOB, CREB1, GNB2, GNG5, MAP2K3, PLCD4, MYL3, ATM*
Colorectal cancer metastasis signaling	4.9E-02	*TCF4, TNFRSF1A, RHOC, CCND1, MMP24, CDH1, AKT1, MSH2, RHOB, GNB2, GNG5, TCF7L2, ATM*
Complement system	2.0E-02	*C1R, SERPING1, CD59, CFB*
Endometrial cancer signaling	2.5E-02	*CDH1, AKT1, ERBB2, CCND1, ATM*
eNOS signaling	9.8E-03	*HSPA8, CCNA2, AKT1, CCNA1, HSPA1A/HSPA1B, HSP90AA1, HSPA5, NOSTRIN, HSPA1L, ATM*
GADD45 signaling	3.4E-03	*GADD45A, CCND3, CCND1, ATM*
Germ cell-sertoli cell junction signaling	4.6E-03	*PXN, CDH1, AKT1, MAP3K6, RHOB, TNFRSF1A, RHOC, MAP3K1, MTMR2, MAP2K3, TUBA1B, ATM*
Glucocorticoid receptor signaling	4.7E-03	*HSPA1A/HSPA1B, MAP3K1, HSPA5, CD163, NR3C1, TAF13, TSC22D3, PTGES3, HSPA1L, HSPA8, TRAF6, AKT1, CREB1, FKBP4, HSP90AA1, FKBP5, ATM*
HER-2 signaling in breast cancer	1.1E-02	*AKT1, ITGB4, ERBB2, PARD3, CCND1, PRKD1, ATM*
Hereditary breast cancer signaling	3.4E-02	*HDAC9, AKT1, MSH2, GADD45A, BLM, CCND1, FANCL, ATM*
HIF1α signaling	4.8E-02	*EGLN2, AKT1, EDN1, MAPK4, HSP90AA1, MMP24, ATM*
HMGB1 signaling	3.0E-02	*AKT1, RHOB, TNFRSF1A, RHOC, MAP2K3, IL1R1, ATM*
Huntington's disease signaling	5.8E-03	*HDAC9, HSPA1A/HSPA1B, DNM3, HSPA5, HSPA1L, ZDHHC17, HSPA8, TGM2, DYNC1I2, AKT1, CREB1, GNB2, GNG5, PRKD1, ATM*
Hypoxia signaling in the cardiovascular system	1.9E-02	*AKT1, EDN1, UBE2G1, CREB1, HSP90AA1, ATM*
IL-1 signaling	3.0E-02	*TRAF6, MAP3K1, GNB2, GNAI1, GNG5, MAP2K3, IL1R1*
IL-10 signaling	2.2E-02	*TRAF6, SOCS3, IL4R, IL1RL1, MAP2K3, IL1R1*
IL-6 signaling	1.3E-02	*TRAF6, SOCS3, AKT1, TNFRSF1A, IL1RL1, HSPB7, MAP2K3, IL1R1, ATM*
IL-8 signaling	3.0E-03	*NAPEPLD, RHOC, GNAI1, CCND1, PLD1, TRAF6, CDH1, AKT1, CCND3, RHOB, GNB2, GNG5, PRKD1, ATM*
LPS/IL-1 mediated inhibition of RXR function	2.7E-02	*ALDH4A1, TNFRSF1A, IL1RL1, MAP3K1, IL1R1, FMO5, TRAF6, ALDH1L1, UST, ACSL5, NR5A2, SLC27A3, HS3ST5*
LXR/RXR activation	4.2E-02	*SCD, TF, TNFRSF1A, IL1RL1, MYLIP, ACACA, S100A8, IL1R1*
Melanoma signaling	4.5E-02	*CDH1, AKT1, CCND1, ATM*
P2Y purigenic receptor signaling pathway	6.0E-03	*PLCD1, AKT1, CREB1, GNB2, GNAI1, P2RY12, GNG5, PLCD4, PRKD1, ATM*

p38 MAPK signaling	3.6E-02	*TRAF6, TNFRSF1A, IL1RL1, DUSP10, CREB1, HSPB7, MAP2K3, IL1R1*
Phospholipase C signaling	4.0E-02	*HDAC9, NAPEPLD, RHOC, ARHGEF17, PLD1, TGM2, RHOB, CREB1, GNB2, GNG5, ARHGEF2, MYL3, PRKD1*
Phospholipases	3.5E-02	*PLCD1, NAPEPLD, PLA1A, PLCD4, PLD1*
Protein kinase A signaling	3.9E-02	*TCF4, PXN, PTPRD, MAP3K1, GNAI1, TTN, PDE8A, PLCD1, DUSP10, CREB1, GNB2, GNG5, DUSP7, PLCD4, MYL3, PDE6B, TCF7L2, PRKD1, DUSP16*
Role of BRCA1 in DNA damage response	4.4E-02	*MSH2, GADD45A, BLM, FANCL, ATM*
Role of NFAT in cardiac hypertrophy	3.3E-02	*PLCD1, HDAC9, AKT1, MAP3K1, GNB2, GNAI1, GNG5, MAP2K3, PLCD4, PRKD1, ATM*
Role of PKR in interferon induction and antiviral response	3.9E-02	*TRAF6, AKT1, TNFRSF1A, MAP2K3*
Superpathway of D-myo-inositol (1,4,5)-trisphosphate metabolism	4.1E-02	*INPP1, ITPKC, IMPA2*
Thrombin signaling	8.9E-03	*RHOC, GNAI1, PLCD1, AKT1, RHOB, CREB1, GNB2, GNG5, ARHGEF2, PLCD4, MYL3, PRKD1, ATM*
Thyroid cancer signaling	3.9E-02	*CDH1, TCF4, CCND1, TCF7L2*
Xanthine and xanthosine salvage	3.2E-02	*PNP*
γ-linolenate biosynthesis II (animals)	1.6E-02	*ACSL5, CYB5R3, SLC27A3*

Supplemental Tables S2 & S4). Twenty-four of these 107 were identified by at least one of the GWAS (Supplemental Table S2). The 386 multiply identified genes (Supplemental Table S4) were used for Ingenuity analysis, and 81 pathways were significantly altered ($p < 0.05$; Supplemental Table S5). There were 21 pathways in common between the multiply identified genes and our dataset (section A of Table 2 and of Supplemental Table 5).

We chose 4 genes to test by qRT-PCR, based upon their roles in pathways that are affected. *NR3C1* is the glucocorticoid receptor gene, the key transcription factor in the glucocorticoid pathway. *FKBP5* (FK506 binding protein 5) is an immunophilin gene important in that pathway that also interacts with 90 kDa heat shock protein and sequesters NR3C1 in the cytosol, increasing glucocorticoid resistance. *NR4A2* is a transcription factor in the steroid-thyroid hormone-retinoid receptor superfamily, mutations in which have been related to dopaminergic dysfunction. NR4A2 has been shown to repress inflammatory genes activated by NF-κB (Saijo et al., 2009) in microglia. *GRM3* (glutamate receptor, metabotropic 3) was chosen because L-glutamate is the major excitatory neurotransmitter in the central nervous system, and affects most aspects of brain function. All 4 genes showed similar fold-changes in qRT-PCR as they did in the microarrays (Table 3).

Discussion

This study presents a global picture of differences between alcoholics and controls in gene expression in the post mortem hippocampus. A major theme that emerges from the data is that the hippocampus in alcoholics shows dramatic signs of stress. Genes and pathways (Table 2) involved in stress responses are mostly increased in alcoholics. Metallothioneins, a large number of which are increased in the hippocampus (Table 1E), are increased in many stress conditions (Aschner & West, 2005). EIF2 signaling, which is increased, functions to resolve endoplasmic reticulum (ER) stress; if ER stress cannot be resolved, apoptosis can result (Lerner et al., 2012). *TXNIP* (1.7-fold higher in alcoholics) can be transcriptionally induced by TGFβ1 and glucocorticoids (Chen et al., 2010; Han et al., 2003), and can link oxidative stress to inflammation via the NLRP3 inflammasome (NLR family, pyrin domain containing 3) (Zhou, Tardivel, Thorens, Choi, & Tschopp, 2010), an upstream activator of NF-κB signaling that plays a role in the regulation of inflammation, the immune response, and apoptosis.

Signs of hypoxia are present, as evidenced by the increases in Angiopoietin-like 4, *EPAS1* (endothelial PAS domain protein 1, also known as *HIF2α*), *HIF3α*, and *HIF1α* (15% increase, FDR 0.26) shown in Supplemental Table S2. Analysis of upstream regulators (Supplemental Table S3) reinforces this, since the pattern of expression of the genes regulated by *EPAS1* and *HIF1α* also indicates that they are activated.

There is also evidence of involvement of the hypothalamus-pituitary-adrenal (HPA) axis, specifically the cortisol pathway

Table 3
Confirmation by qRT-PCR.

Gene symbol	RT-PCR *p* value	RT-PCR fold	Array *p* value	Array fold
FKBP5	2.6E-27	1.84	4.6E-06	2.21
NR3C1	8.6E-03	−1.26	1.8E-05	−1.29
NR4A2	3.9E-02	−1.79	3.5E-04	−1.95
GRM3	1.0E-18	−1.47	2.7E-05	−1.45

Primers used *FKBP5*, Hs01561010_m1; *NR3C1*, Hs00353740_m1; *NR4A2*, Hs00428691_m1; *GRM3*, Hs00168260_m1; *POL2RA*, Hs00172187_m1 used as control to normalize sample-to-sample variation.

(Table 1C), and particularly in astrocytes: 37% of the astrocyte-enriched genes that showed increased expression are downstream of the glucocorticoid signaling, and others are downstream of either IL1β or TGFβ1. Pathway and upstream analysis (Table 2, Supplemental Table S3) indicates that the glucocorticoid receptor is activated although its transcript level (*NR3C1*) is decreased. Cortisol-releasing-hormone (CRH) increases as a result of stress, ethanol abuse, chronic drinking, and the early stage of withdrawal (Armario, 2010; Gianoulakis, Dai, & Brown, 2003; Roy, Mittal, Zhang, & Pandey, 2002), which should activate the glucocorticoid receptor. The HPA and CRH are also activated by alcohol consumption (Clarke & Schumann, 2009), increasing the amount of adrenocorticotropic hormone (ACTH) produced, which in turn stimulates the release of glucocorticoids (Mesotten, Vanhorebeek, & Van den Berghe, 2008). Glucocorticoids down-regulate the further release of CRH through a negative feedback loop to the hypothalamus, but increase the production of CRH outside the hypothalamus, e.g. in the central amygdala (Pastor et al., 2008). Dysregulation of the HPA axis is a known problem in alcoholism and other addictions (Armario, 2010; Koob & Kreek, 2007; Sorocco, Lovallo, Vincent, & Collins, 2006) as well as in at-risk individuals (Sorocco et al., 2006). The increased levels of CRH may lead to increased alcohol consumption as the brain tries to adapt to its increasingly dysregulated state (Koob & Le Moal, 2005). Increased CRH levels also lead to increased sensitivity of stress-induced alcohol consumption (Ciccocioppo et al., 2009; Clarke, Krause, Li, & Schumann, 2009). Glucocorticoids mediate the development of sensitization to drugs such as ethanol (Roberts, Lessov, & Phillips, 1995) in a feed-forward fashion.

FKBP4 and *FKBP5* (over 2-fold higher) and *NR3C1* itself are downstream targets of glucocorticoid signaling. *FKBP5* functions as a negative regulator of the pathway by lowering the cortisol affinity of the glucocorticoid receptor and keeping it in the cytoplasm, which increases cortisol resistance and short-circuits the glucocorticoid feedback circuit (Binder, 2009; Binder et al., 2008). Mice with chronic exposure to corticosterone (the rodent equivalent of cortisol) develop anxiety and have decreased expression of *Nr3c1* and *Hsp90* and increased expression of *FKBP5* in many tissues (Lee et al., 2010). Increased *FKBP5* expression due to known polymorphisms leads to increased risk of affective and anxiety disorders (Binder et al., 2008) and bipolar disorder (Willour et al., 2009).

Our data show decreased myelination (Table 1D) in the hippocampus. Decreased hippocampal volume (Agartz et al., 1999; Laakso et al., 2000; Tyan et al., 2012) and decreases in hippocampal neurogenesis have been observed in alcoholism (Crews & Nixon, 2009; Morris et al., 2010; Richardson et al., 2009). Pathways (WNT/β catenin, reelin signaling in neurons, and *ERBB4*) and genes (*APP*, *PSEN1*, *ADAM10*, *ERBB2*, and reelin) that play a role in neurogenesis (Lazarov & Marr, 2010) have decreased activity or expression in the hippocampi of the alcoholics. Both inflammation (Monje et al., 2003) and stress with increased cortisol production (Schoenfeld & Gould, 2012) can inhibit neurogenesis. Chronic cortisol decreases neurogenesis and treatment with the glucocorticoid antagonist mifepristone reverses this reduction (Mayer et al., 2006).

Are these stresses and dysfunctional changes related? Most of these stresses can be linked to NF-κB (Fig. 2), which is connected to 25 of the differentially expressed genes in this dataset, including genes related to hypoxia, inflammation, neurogenesis, and myelin.

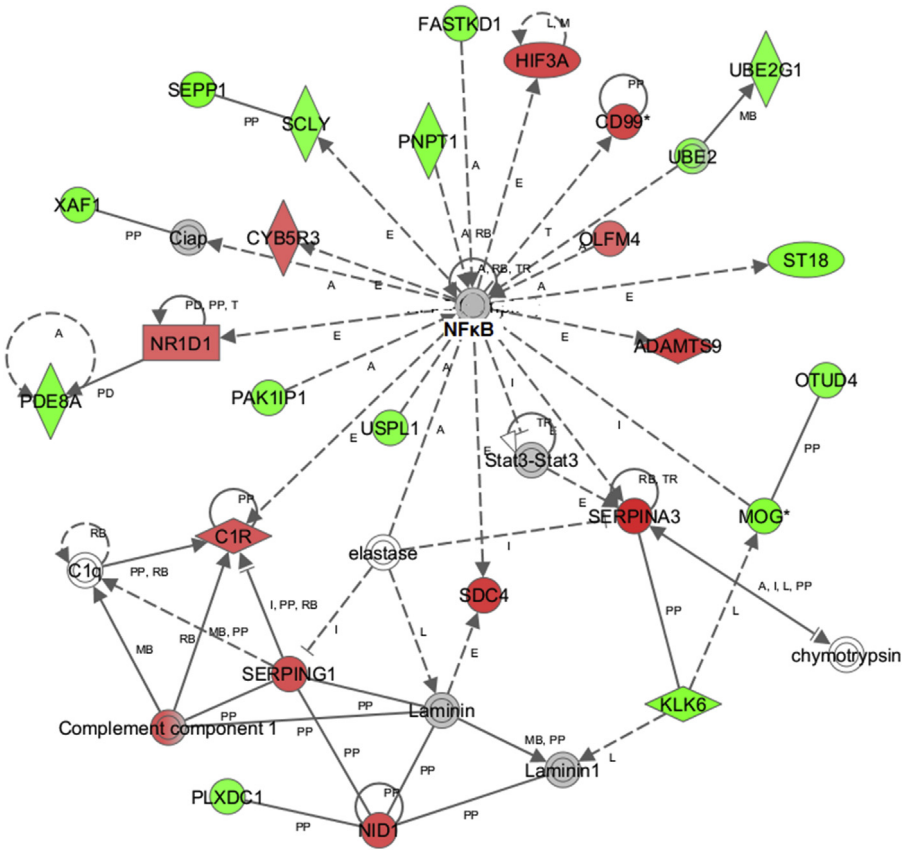

Fig. 2. Ingenuity Pathway Analysis network with NF-κB as central hub. Red: genes with increased expression; green: genes with decreased expression; gray: gene in dataset but was not significantly changed; white: not in the data set used for analysis.

Variations within *NFKB1*, a subunit of NF-κB, have been associated with alcoholism (Edenberg et al., 2008). One can conceptualize the inter-relationships as in Fig. 3. Ethanol activates inflammation via the TLR4 pathway and NF-κB. Increased inflammation, via the toll-like receptor 4 (*TLR4*), can play a role in the loss of white matter seen in alcoholics (Alfonso-Loeches et al., 2012). Wild-type mice chronically treated with ethanol for 5 months had decreased expression of several myelin-related genes in multiple brain regions, and also a reduced number of oligodendrocytes, but *Tlr4* knockout mice similarly treated did not show decreased expression of the myelin genes. The ER stress we have found, if unresolved, can also increase inflammation via *TXNIP* (strongly increased) and NF-κB.

One goal of examining gene regulation in the brain is to inform the analyses of genes that may influence risk for alcoholism. Toward that end, we compiled data from 10 previously published gene expression studies (Flatscher-Bader et al., 2005, 2010; Iwamoto et al., 2004; Kryger & Wilce, 2010; Lewohl et al., 2000; Liu et al., 2006, 2007; Mayfield et al., 2002; Sokolov et al., 2003; Zhou & Yuan et al., 2011), from this study, and from 12 GWAS for risk of alcoholism or alcoholic traits (Bierut et al., 2010; Edenberg et al., 2010; Foroud et al., 2007; Hack et al., 2011; Johnson et al., 2011; 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., 2012). There were 386 genes identified by at least 2 of these collected studies (Supplemental Table S4). Five genes were identified in 4 studies, and are thus strong candidates for further study: selenoprotein P (*SEPP1*), heterochromatin protein 1 binding protein 3 (*HP1BP3*), transferrin (*TF*), EGF-like repeats and discoidin I-like domains 3 (*EDIL3*), and contactin associated protein-like 2 (CNTNAP2). *SEPP1* binds selenium and has antioxidant activity and is down-regulated by both inflammatory cytokines like IL1β (Dreher, Jakobs, & Köhrle, 1997) and glucocorticoids (Rock & Moos, 2009); it is decreased in the hippocampi of alcoholics (Supplementary Table S2). Transferrin is an iron transporter and is also a negative acute phase response protein; it is also decreased. HP1BP3 has been identified as a biomarker for postpartum depression (Guintivano, Arad, Gould, Payne, & Kaminsky, 2013). *EDIL3* can stimulate cerebral angiogenesis (Fan et al., 2008) and was down-regulated in mouse embryos exposed to ethanol (Zhou & Zhao et al., 2011). CNTNAP2 is an extremely large protein in the neurexin family, polymorphisms in which were recently found to be associated with depression and schizophrenia in a Han Chinese population (Ji et al., 2013). Several pathways identified using this list of genes overlap with the pathways identified by our study (Supplemental Table S5, Section A) which include stress-related pathways EIF2 and mTOR signaling. IPA also identified NF-κB as significantly altered for this group of multiply identified genes.

Twenty-four of the genes identified by our study were previously identified by GWAS (Supplemental Table S2, GWAS column). This list includes several genes with large fold changes, such as *SLC39A10* (a zinc transporter), suppression of tumorigenicity 18 (*ST18*), protein tyrosine phosphatase receptor type D (*PTPRD*), BCL2-associated athanogene 3 (*BAG3*), and von Willebrand factor (*VWF*). Although these genes might not be thought of as related to alcoholism, their differential expression in alcoholic brains, together with their genetic connection, suggests they might be. The IPA analysis of the 107 genes in our study that were identified in other studies indicated that 38 of these genes are related to cell death, including ST18 and BAG3.

This study demonstrates many differences in gene expression between the hippocampi of alcoholics and controls, and highlights interrelated insults to the hippocampus: stress, hypoxia, inflammation, and excess cortisol (Figs. 2 and 3). These may play roles in the demyelination, loss of glial cells, and decreased neurogenesis seen with chronic alcohol abuse. NF-κB appears to be a key player in these processes (Fig. 3). Some of these differences in gene expression may be due to genetic variations that precede the addiction process and may play an active role in the addiction process. Others may be the result of years of excessive alcohol consumption, and still others may be altered due to the interaction of genetic variation with excessive alcohol consumption. A post mortem study such as this cannot distinguish among these possibilities. The modifications seen here in gene expression in these pathways could be part of the allostatic change suggested by Koob and Kreek (2007). In the hippocampus, resetting the cortisol pathway may be one way to break this chain of events. Decreased neurogenesis and increased inflammation are also seen in major depressive illness (Koo et al., 2010), but antidepressant treatment has had mixed results in the treatment of alcoholism *per se* (Kranzler, Feinn, Armeli, & Tennen, 2012). Animal and human post mortem research indicate the innate immune function induced by TLRs and NF-κB signaling creates negative affect and stress, which with repeated cycles of ethanol abuse leads to addiction (Crews et al., 2011). This study demonstrates that this increase in the innate immune system and NF-κB signaling is still present after years of chronic drinking. With multiple stressors increasing NF-κB signaling, it may take a multi-pronged approach to normalize the brain of chronic drinkers.

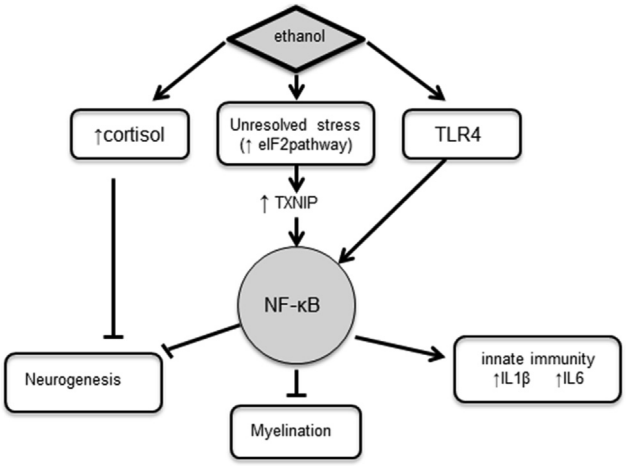

Fig. 3. Key pathways affected by ethanol. Ethanol intake increases cortisol and activates NF-κB via Toll-like receptor 4 (TLR4). NF-κB activation increases innate immune activity. Hippocampal neurogenesis is inhibited via NFκB. NR4A2 represses NF-κB transactivation of other genes. When stress cannot be resolved by the eIF2 pathway, transcription of TXNIP is increased which also increases NF-κB transactivation. Vertical arrows indicate pathways, genes, or signaling molecules that have increased/decreased expression or activity in the hippocampus of alcoholics.

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Appendix. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.alcohol.2013.07.002.

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