Molecular Profiles of Drinking Alcohol to Intoxication in C57BL/6J Mice

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Background: Alcohol addiction develops through a series of stages, and mechanistic studies are needed to understand the transition from initial drug use to sustained controlled alcohol consumption followed by abuse and physical dependence. The focus of this study was to examine the effects of voluntary alcohol consumption on brain gene expression profiles using a mouse model of binge drinking. The main goal was to identify alcohol-responsive genes and functional categories after a single episode of drinking to intoxication.

Methods: We used a modification of a "Drinking In the Dark" (DID) procedure (Rhodes et al., 2005) that allows mice to experience physiologically relevant amounts of alcohol in a non-stressful environment and also allows for detection of alcohol-sensitive molecular changes in a dose-dependent manner. C57BL/6J male mice were exposed to either 20% ethanol solution or water (single bottle) starting 3 hours after lights off for 4 hours and brains were harvested immediately after the drinking session. cDNA microarrays were used to assess the effects of voluntary drinking on global gene expression in 6 brain regions. We employed three statistical approaches to analyze microarray data.

Results: A commonly used approach that applies a strict statistical threshold identified the eight top statistically significant genes whose expression was significantly correlated with blood ethanol concentration (BEC) in one of the brain regions. We then used a systems network approach to examine brain region-specific transcriptomes and identify modules of co-expressed (correlated) genes. In each brain region, we identified alcohol-responsive modules, i.e., modules significantly enriched for genes whose expression was correlated with BEC. A functional over-representation analysis was then applied to examine the organizing principles of alcohol-responsive modules. Genes were clustered into modules according to their roles in different physiological processes, functional groups, and cell types, including blood circulation, signal transduction, cell–cell communication, and striatal neurons. Finally, a meta-analysis across all brain regions suggested a global role of increasing alcohol dose in coordination of brain blood circulation and reaction of astrocytes.

Conclusions: This study showed that acute drinking resulted in small but consistent changes in brain gene expression which occurred in a dose-dependent manner. We identified both general and region-specific changes, some of which represent adaptive changes in response to increasing alcohol dose, which may play a role in alcohol-related behaviours, such as tolerance and consumption. Our systems approach allowed us to estimate the functional values of individual genes in the context of their genetic networks and formulate new refined hypotheses. An integrative analysis including other alcohol studies suggested several top candidates for functional validation, including *Mt2, Gstm1, Scn4b, Prkcz,* and *Park7*.

Key Words: Drinking In the Dark, Gene Expression, Brain Region, Network Analysis.

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ALCOHOLISM IS A complex disorder affected by genetic, epigenetic, and environmental factors. Owing to fundamental differences between human and animal behaviours, no single animal model can capture all elements of the disease. However, partial models exist, each designed to replicate an individual feature of alcoholism, such as high alcohol intake. Excessive alcohol consumption is a prerequisite for the development of alcohol dependence in human populations. Many mouse models of high alcohol drinking have employed the two-bottle alcohol preference test, where access to both water and (usually a 10%) ethanol solution is available 24 hour per day (Green and Grahame, 2008). A limitation of this behavioural assay is that even mice genetically

predisposed to drink alcohol rarely display a pattern that leads to a high blood ethanol concentration (BEC) and physiological intoxication. Most genetic studies exploit the genetic predisposition of the C57BL/6J strain to consume high levels of alcohol solutions even when other non-alcohol solutions are available. The recently proposed Drinking-In-the-Dark (DID) model allows the alcohol-preferring C57BL/6J strain to reach physiologically relevant blood alcohol levels after drinking an ethanol solution for 2 to 4 hours during the dark phase of the light–dark cycle (Rhodes et al., 2005). One advantage of this drinking paradigm is that it allows animals to experience intoxicating amounts of alcohol in the non-stressful environment of their home cage.

Addiction to alcohol is thought to develop through a series of stages including acute intermittent alcohol use, chronic controlled consumption and transition to a loss of control over alcohol intake and dependence (Koob et al., 2004). Acute administration of ethanol results in behavioural responses associated with drug reward, sensitivity, and tolerance (Koob et al., 2004; Ponomarev and Crabbe, 2002b; Schuckit, 1994). These initial responses to acute ethanol in humans may predict the risk for alcohol abuse in the future (Schuckit, 1994), but the mechanistic link between the acute ethanol effects and alcohol addiction is not well understood. A fundamental assumption of addiction research is that some of the initial drug-induced molecular changes mediate the processes of cellular adaptation leading to later stages of addiction (Kalivas and Volkow, 2005; Koob et al., 2004; McBride et al., 2005; Mcclung and Nestler, 2003; Sommer et al., 2005). Mechanistic studies are needed to understand the transition from initial drug use to sustained high alcohol consumption followed by abuse and physical dependence.

Acute administration of alcohol, like many other drugs of abuse, induces changes in brain gene expression (Kerns et al., 2005; Treadwell and Singh, 2004). Gene expression is a sensitive measurement of the functional state of a cell or tissue, and the importance of gene expression in drug addiction has been demonstrated by numerous studies in humans and animal models (Liu et al., 2006; Mcclung and Nestler, 2008; Rhodes and Crabbe, 2005; Tabakoff et al., 2003). To identify the molecular targets affected by alcohol after drinking to intoxication has been initiated, we measured global gene expression in brains of high alcohol-consuming C57BL/6J mice after a single bout of voluntary consumption using a modified DID procedure. Our study provides novel insight into initial drug-induced molecular changes that constitute cellular adaptations to alcohol, which may be important for alcohol-related behavioural changes, such as tolerance and transition to sustained high alcohol intake.

MATERIALS AND METHODS

Alcohol Self-Administration and Brain Dissections

All animals were purchased from The Jackson Laboratory and allowed to acclimate to individual housing and a reverse light/dark cycle for 2 weeks. One hundred C57BL/6J male mice aged approximately 70 days were used in this study. Ten subjects were randomly selected to comprise the water group, and the remaining subjects were selected as the ethanol group and exposed to a modified DID protocol (Rhodes et al., 2005) as follows. One hour before the start of the dark cycle, the animals were weighed. Three hours after the start of the dark cycle, all water bottles were removed from the cages, and the animals in the water group and the ethanol group received a 10-ml cylinder containing tap water or 20% v/v ethanol respectively. The total period of access to the cylinders was 4 hours. Fluid levels were recorded at 2 and 4 hours. Immediately following the final fluid reading, the mice were removed from their cages, and periorbital blood samples were taken to measure blood ethanol concentration (BEC), and the brains were removed. The olfactory bulbs (OB), frontal cortex (FC), striatum (STR, including both dorsal and ventral regions), cerebellum (CB), ventral midbrain (VMB, including ventral tegmental area and substantia nigra pars compacta), and the hippocampus (HIP) were rapidly dissected from each brain on wet ice and brain parts immediately frozen on dry ice. FC, STR, VMB, and HIP contain structures and circuitry involved in both neuroadaptation to alcohol and the neurocognitive processes that underlie addiction behaviour (Koob and Le Moal, 2006). OB and CB are implicated in the initial preference drinking in rodents (Tabakoff et al., 2008) and initial sensitivity and acute tolerance to the ataxic effects of ethanol respectively (Wallace et al., 2007).

Subject Selection and Sample Preparation

Seven animals from the water group and 21 animals from the alcohol group were selected for gene expression analysis as control and experimental subjects respectively. Selection of the alcohol-drinking animals was based on responses in 4 drinking variables: the amount of ethanol consumed during the first 2 hours (DID-1), the amount of alcohol consumed during the final 2 hours (DID-2), total alcohol consumption over the entire 4-hour period (DID-T), and the BEC. Twenty-one animals were randomly selected from three groups representing 7 mice for low (L) alcohol consumption and BEC (DID-T: 4.24 to 5.38 g/kg/4 h, $\bar{x}$ = 4.76 g/kg/4 h; BEC: 0.47 to 1.09 mg/ml, $\bar{x}$ = 0.80 mg/ml), 7 for Medium (M) alcohol (DID-T: 5.72 to 6.58 g/kg/4 h, $\bar{x}$ = 6.27 g/kg/4 h; BEC: 1.16 to 1.50 mg/ml, $\bar{x}$ = 1.38 mg/ml) and 7 for High (H) alcohol (DID-T: 6.96 g/kg/4 h, $\bar{x}$ = 7.65 g/kg/4 h; BEC: 1.78 to 3.42 mg/ml, $\bar{x}$ = 2.32 mg/ml) respectively. Control animals were randomly selected from littermates of the alcohol-treated animals. They were not given access to alcohol, and their BEC is assumed to be 0 mg/ml. Total RNA was extracted from each of the 6 brain regions using a combination of QIAzol lysis reagent and RNeasy kit (QIAGEN, Valencia, CA) and assayed for gene expression using cDNA microarrays as described below.

Microarray Hybridization

cDNA-spotted arrays were printed in house as described elsewhere (Mulligan et al., 2006). Total RNA (1 to 3 ug) from each experimental sample was hybridized against the same concentration of reference sample according to the manufacturer's protocol (Genisphere Array 350 Kit; Genisphere, Inc., Hatfield, PA). The reference sample consisted of pooled whole-brain total mRNA from 100 adult C57BL/6J male mice (Mulligan et al., 2008). Hybridized microarrays were scanned using an Axon scanner, and the resulting red (r) and green (g) image files were processed using GenePix 6.1 software. Arrays were hybridized in small batches (4 to 18 samples), usually consisting of the same tissue by two experienced technicians.

Data Postprocessing

The Longhorn Array Database (LAD) was used for microarray experiment normalization storage and retrieval (Killion and Iyer, 2004; Killion et al., 2003). Loess normalization was performed in

LAD by print tip group based on mean *r* values. Filtering was performed in LAD as follows: mean log2 normalized r/g ratios were retrieved for each spot with both a regression correlation >0.2 and a sum of mean raw *r* and g intensities >100. Filtering was performed for each brain region individually. cDNA probes containing more than 30% missing values within a brain region were removed from further analysis. To remove outliers from the data, residuals were calculated for each gene based on linear regression of gene expression on each of the drinking variables (DID-1, DID-2, and DID-T) and the BEC variable. As the distribution of the residuals was expected to be normal, samples whose residuals deviated from the mean by more than 2.5 standard deviations were considered to be outliers, and the corresponding normalized mean log2 ratios were removed from further analysis. After data postprocessing, the total number of cDNA probes reliably expressed in each brain region was 7624, 12,530, 6445, 6920, 8226, and 8000 for the cerebellum, striatum, frontal cortex, hippocampus, olfactory bulbs, and ventral midbrain, respectively.

Correlation Analysis

Pearson's correlation (*r*) was used to measure the linear relationship between gene expression and the BEC within each of the 6 brain regions after data postprocessing. Animals from control group were included in the analysis and were assigned BEC values of 0. Significance was determined from *r* based on *r* to *t* conversion: $t = r/\sqrt{(1\text{-}r^2)/(df)}$, where df = *n*-2. The resulting *p*-values from *t* distributions were used to select genes in each brain region that were significantly correlated with the BEC and to estimate the false discovery rate (FDR) using the *q*-value package in R (Storey and Tibshirani, 2003).

Principal Component Analysis (PCA)

A second data filtering was performed as described earlier with the following exceptions: data for all 6 brain regions were filtered simultaneously and only genes detected on at least 95% of all arrays (156 arrays) were further analyzed. PCA was performed on 3692 genes that passed the filter using STATISTICA software (Version 6.1; StatSoft, Inc., Tulsa, OK). Mean values for each gene were substituted for missing values.

Weighted Gene Co-expression Network Analysis (WGCNA)

Data for each brain region were analyzed separately using WGCNA. For computational reasons, only unique known genes were included for brain region WGCNA. This reduced the size of the data set in each brain region to 3349, 5493, 2839, 3015, 3610, and 3585 in the cerebellum, striatum, frontal cortex, hippocampus, olfactory bulbs and ventral midbrain respectively. Weighted gene co-expression networks were constructed from log2 normalized *r/g* ratios after the "data postprocessing" procedure described above, using custom functions available at http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/ and the free software environment for statistical computing and graphics, R, available at http://www.r-project.org/. WGCNA involves the construction of: (i) an unsigned similarity matrix based on pair-wise Pearson correlations between gene expression profiles; (ii) a weighted adjacency matrix of connection strengths based on the similarity matrix and a power adjacency function; (iii) a topological overlap matrix from the adjacency matrix that measures the relative interconnectedness of the genes in the network; and (iv) average linkage hierarchical clustering of the topological overlap dissimilarity matrix to identify clusters of co-expressed genes, hereby referred to as modules (Zhang and Horvath, 2005). Based on the number of experiments used to construct the network, the average number of connections per node within each brain region network and the estimated fit of the network with the scale-free topology criterion proposed by Zhang and Horvath, 2005, the power was set to $\beta = 5$ for each brain region–weighted network. Module identification after hierarchical clustering was performed using the Dynamic Tree Cut package for R using the "dynamic" and "deep split" option and a minimum module size of 30 genes (Langfelder et al., 2008). Dendrogram cut height varied between 0.994 and 0.996 for each brain region and was selected based on the largest height that would create modules spanning the entire length of the dendrogram. Singular value decomposition (SVD) was used to identify the 1st principal component, or module eigengene, and associated variance for each module using the R package moduleColor for R.

Identification of Alcohol-Responsive Modules

The hypergeometric distribution was used to evaluate whether a cluster (module) of co-expressed genes determined by WGCNA was alcohol-responsive, i.e., significantly enriched for genes whose expression was correlated with the BEC at $p < 0.05$. A module was said to be alcohol-responsive if it contained more genes whose expression was correlated with the BEC than was expected by chance (hypergeometric $p < 0.05$). Alcohol-responsive modules were further subjected to functional and cell/tissue type over-representation analyses.

Functional Group Over-Representation Analysis

The NIH Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resource was used to determine functional group over-representation (Huang Da et al., 2007, 2009). All genes from alcohol-responsive modules were imported into DAVID based on Genebank Accession IDs. Genebank Accessions were later converted to gene symbols using the Gene ID conversion tool within the DAVID database. The background list for each brain region consisted of all genes associated with the cDNA probe sets used to construct the corresponding brain region gene networks.

Cell/Tissue Type Over-Representation Analyses

Alcohol-responsive modules were examined on whether they were enriched with genes that are preferentially expressed in a particular cell type (astrocyte, oligodendrocyte, and neuron) or tissue (blood). Genes enriched in cell types were determined using previously published data (Cahoy et al., 2008). A cutoff of >3-fold enrichment was used to assign cell-type enrichment. Genes enriched in blood were determined from an experiment for a separate project by Dr. Mayfield at the Waggoner Center. Briefly, blood from 9 mice on mixed C57BL/6J x C3H/HeJ genetic background was repeatedly profiled for gene expression. Whole-blood samples (300 ul) were obtained from the peri-orbital sinus of individual mice, and total RNA was extracted (RiboPure™-Blood RNA kit; Ambion, Austin, TX). Globin transcripts were cleared from total RNA using the GLOBINclear kit (Applied Biosystems, Foster City, CA). RNA samples were stored at −80°C until use. A total of 4 blood samples were obtained from each animal during the study (3, 6, 9, and 12 months of age), resulting in 36 samples. Gene expression profiles were obtained using the same cDNA microarrays as used for brain profiling. Microarray hybridizations were performed using the Genisphere Array900 Kit (Genisphere Inc.). All samples were hybridized to a common reference sample (Universal Mouse Reference RNA, Ambion). The *r/g* ratios were calculated, and transcripts enriched more than 50-fold in blood vs the common reference were selected as "enriched in blood" (Table S1). Genes from alcohol-responsive modules were matched by either gene symbols (for cell type) or GenBank IDs (for blood) to determine genes enriched in different cell types or blood respectively, and the hypergeometric distribution

was again used to determine deviation from chance (module over-representation).

Meta-Analysis Across Brain Regions

To detect consistently regulated genes, data were combined across all 6 brain regions by matching cDNA probes across the brain regions. Fisher's method was used to combine *p*-values for the correlation between gene expression and the BEC across brain regions (Belknap and Atkins, 2001). The FDR was estimated for each combined *p*-value as described earlier. The criteria for consistent regulation across all brain regions were detection in all 6 brain regions and Fisher combined *p*-value <0.002, $q < 0.1$ (FDR $< 10\%$). Hierarchical clustering of consistently regulated transcripts was carried out to detect co-expression clusters. Data were normalized by brain region prior to hierarchical clustering to account for differences in intensity between brain regions and print group. The average log2 normalized ratio for each group was used for hierarchical clustering. Only unique transcripts annotated with gene symbols were included in the cluster analysis. Redundant probe sets having duplicate gene symbols were removed leaving only the probe set with the lowest Fisher combined *p*-value. Average linkage hierarchical clusters based on euclidean distance were calculated and visualized using the freely available software Cluster 3.0 and JavaTreeView respectively (De Hoon et al., 2004; Saldanha, 2004).

Quantitative RT-PCR

RT-PCR was conducted using total RNA from the same samples used for microarray experiments. Control and high alcohol groups were compared across several brain regions for 2 selected genes: *Hba-a1* and *Mt2*. Expression of *Hba-a1* was examined in the cerebellum, hippocampus, striatum and olfactory bulb and expression of *Mt2* was examined in the striatum, frontal cortex and ventral midbrain. All real-time TaqMan® assays are predesigned by Applied Biosystems and labeled with FAM as a reporter and a non-fluorescent quencher. Detailed TaqMan® protocols are available on the manufacturer's website: http://www.appliedbiosystems.com/index.cfm.

Mm00845395_s1 and Mm00809556_s1 TaqMan® Gene Expression Assays were used for *Hba-a1* and *Mt2* respectively. Control and high alcohol groups in each brain region were compared using one-tail *t*-test and, the overall significance for each gene across brain regions was estimated using the Fisher's method to combine *p*-values (Belknap and Atkins, 2001).

RESULTS

Alcohol Self-Administration

Modification of the DID paradigm (Rhodes et al., 2005) to a single-day procedure during which mice are given access to 20% (v/v) ethanol for a 4-hour period during the dark cycle resulted in a wide range of responses (Fig. 1). Most mice consumed enough alcohol to achieve a BEC of at least 0.8 mg/ml, which results in quantifiable signs of intoxication (Crabbe et al., 2009). The amount of ethanol consumed was significantly correlated with the BEC (Fig. 1*A*). Twenty-one mice showing a wide response range of alcohol consumption and BEC were selected for microarray expression analysis (Fig. 1*B*). We used this wide range of drinking responses to identify alcohol-responsive genes, i.e., genes whose expression is significantly correlated with BEC (see methods for details).

Global Brain Gene Expression

We first examined the underlying structure of the whole microarray data set by conducting PCA using ~3700 genes that were reliably detected in all 6 brain regions (see Methods for details). PCA is a dimensionality reduction procedure that is often used to identify latent variables that may reflect biological principles underlying relationships among variables in large-scale data sets. The first several new variables or principal components (PCs) account for most of the data variance. Plotting all data in a multidimensional space of the first 2 to 3 PCs usually helps determining the main source of data variability as well as detecting technical outliers or procedural artifacts, such as the batch effect. Data for all 164 arrays plotted in a three-dimensional space of the first 3 PCs revealed clustering according to brain region (Fig. 2). Although a batch effect cannot be completely ruled out (see Methods), it is much more likely that this type of clustering reflects true biological variation in gene expression among brain regions, a result that would be consistent with findings from previous research (Hovatta et al., 2007; Kimpel et al., 2007; Sugino et al., 2006).

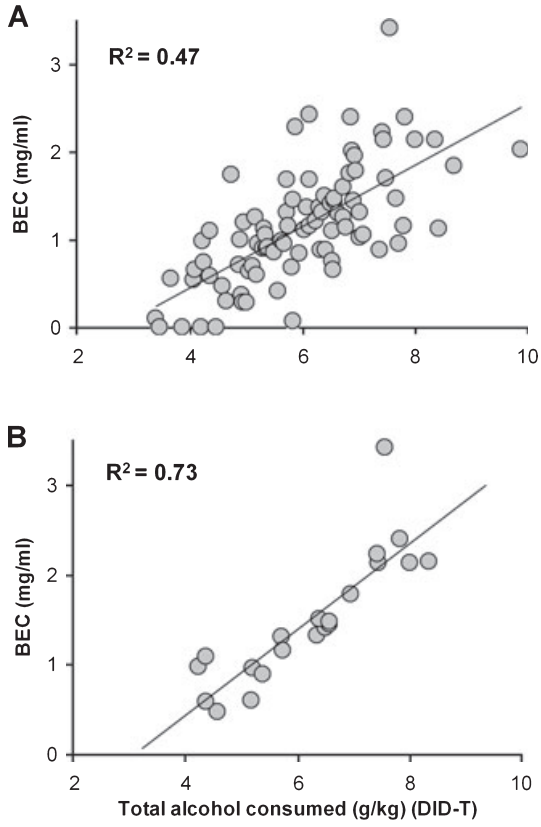

Fig. 1. Correlation plots showing relationships between total alcohol consumed during the 4-hour session at the 4th hour for all animals (**A**; mean ± SEM; drinking in the dark-T: 5.97 ± 0.14; blood ethanol concentration: 1.15 ± 0.07) and a subset of 21 animals selected for the microarray experiment (**B**).

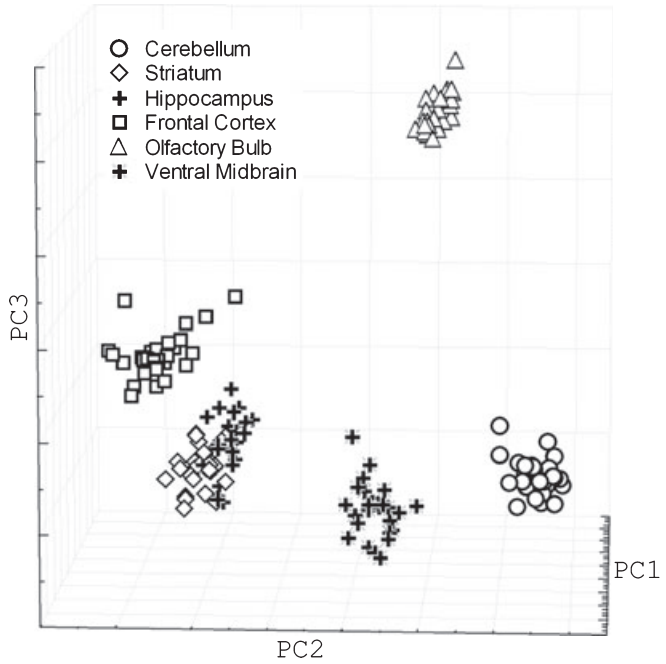

Fig. 2. Results of principal component analysis. A total of 164 arrays are shown in the three-dimensional space of the first three PCs that account for >50% of total variance in gene expression.

Brain Region Dependent Gene Expression

Acute drinking produced moderate effects on global gene expression in different brain regions. Although many transcripts were significantly correlated with the BEC, there were only eight known genes that passed the strict statistical threshold of $p < 0.0005$; FDR = 10% (Table 1). Six of these genes were regulated in cerebellum, suggesting that this brain region is more sensitive to acute alcohol, compared to other regions. These genes represented various molecular and cellular processes including oxidoreductase activity (ferritin heavy chain 1, *Fth1*), blood circulation (hemoglobin alpha, adult chain 1, *Hba-a;* hemoglobin, beta adult minor chain, *Hbb-b1*), lipid metabolism (phospholipid transfer protein, *Pltp*), and signal transduction (S100 calcium binding protein A10, calpactin, *S100a10*; phytanoyl-CoA hydroxylase interacting protein, *Phyhip*; glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A, *Grinl1a*; tubulointerstitial nephritis antigen-like 1, *Tinagl1*). This approach applies conservative multiple test correction to limit the possibility of false positives and ignores information about relationships among individual genes, limiting discovery to a handful of candidates.

To identify alcohol-responsive molecular patterns generated by interactions between many genes, we used a WGCNA for each brain region (Zhang and Horvath, 2005). In the first step, all genes that passed the expression detection filter in a given brain region were clustered into modules of co-expressed genes based on weighted gene-gene correlations across all samples. We then identified alcohol-responsive modules, i.e., modules significantly enriched for genes whose expression was correlated with BEC. All genes correlated with BEC at $p < 0.05$ are listed in Table S2. Organizing principles and biological significance of each alcohol-responsive module were assessed via functional over-representation (enrichment) analysis using the DAVID Bioinformatics Resources (Dennis et al., 2003; Huang et al., 2009) and the published literature. Identification of over-represented functional groups provides an additional level of statistical validity that mitigates effects of moderate false-positive rates for individual genes. This approach provides contextual information about biological function that may not be available when examining single genes and assists in the formulation of novel refined hypotheses.

The number of modules detected by WGCNA ranged from 45 to 84 in the ventral midbrain and striatum respectively, and the number of alcohol-responsive modules ranged from

Table 1. Top Annotated Statistically Significant Genes After Correction for Multiple Comparisons ($p < 0.0005$; false discovery rate = 10%)

Brain region	Clone ID GB ID	Gene symbol	Gene name	Relevant function cell type enrichment	Correlation with BEC
CB	H3008B06 BG063515	*Fth1*	Ferritin heavy chain 1	Iron ion binding Oxidoreductase activity	0.66
CB	AI843797 AI843797	*Hba-a1*	Hemoglobin alpha, adult chain 1	Oxygen transport enriched in blood cells	0.72
CB	H3032F06 CK334464	*Hbb-b1*	Hemoglobin, beta adult minor chain	Oxygen transport enriched in blood cells	0.68
CB	H3019C02 CK334355	*Grinl1a*	Glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A	Receptor activity transcription	−0.74
CB	AI848202 AI848202	*Phyhip*	Phytanoyl-CoA hydroxylase interacting protein	Protein binding	0.79
CB	AI842653 AI842653	*Pltp*	Phospholipid transfer protein	Lipid transport	0.73
OB	AI844800 AI844800	*S100a10*	S100 calcium binding protein A10 (calpactin)	Interaction with 5-HT signaling enriched in neurons	-0.87
STR	600866 AI449650	*Tinagl1*	Tubulointerstitial nephritis antigen-like 1	Immune response. Proteolysis	0.83

Brain regions: CB, cerebellum; STR, striatum; OB, olfactory bulb. BEC, blood ethanol concentration.

5 to 12 in the hippocampus and striatum respectively. All genes in alcohol-responsive modules are listed in Table S3. The top statistically significant results of subsequent over-representation analysis are presented in Table 2, and all over-represented functional and structural categories in alcohol-responsive modules, detected at FDR < 30%, are listed in Table S4. The most over-represented functional category was Blood Circulation, as four of 6 brain regions had modules over-represented with genes enriched in blood. The majority of genes in these modules were positively correlated with BEC, suggesting that alcohol dose-dependently increases blood circulation in brain. Several other general functional processes were affected by alcohol drinking across multiple brain regions, including signal transduction and cell–cell communication. Although the exact genes and pathways were largely different between regions (see Tables S3 and S4), these shared higher-order functions are likely common biological responses to alcohol. Several pathways and integrative functions, such as ATP-dependent processes, and enzymatic activity were identified specifically in ventral midbrain and striatum, suggesting coordinated responses to acute alcohol in these regions.

Of particular interest was the detection of turquoise module in striatum, which, in addition to be alcohol-responsive, was enriched with genes highly expressed in neurons. (Table 2, Table S3). To visualize gene–gene relationship in this module, we built a correlation-based network showing highly correlated genes from the alcohol-responsive and neuron-enriched categories (Fig. 3). This analysis demonstrated that alcohol-related and neuron-enriched networks are highly overlapping in the striatum. Ninety to 95% of all striatal neurons, a region implicated in drug reward and drug reinforcement, are GABAergic medium spiny neurons (MSNs) (Berke and Hyman, 2000; Maldve et al., 2002). A survey of striatal gene expression using Allen Brain Atlas (http://mouse.brain-map.org) revealed that most of the 26 neuronally expressed genes from this module show a dense pattern of expression, which is consistent with the distribution of MSNs. Several neuronal genes, including Huntingtin-associated protein 1 (*Hap1*) and guanine deaminase (*Gda*), were predominantly expressed in ventral striatum, a part of the reward pathway, and examination of literature confirmed that these genes are expressed in MSNs (Paletzki, 2002; Tang et al., 2004). Furthermore, the turquoise module includes several genes previously identified as candidates for genetic regulation of two-bottle alcohol preference drinking in mice (Mulligan et al., 2006), including sodium channel beta4 subunit (*Scn4b*), protein kinase C zeta (*Prkcz*), and Parkinson's disease protein 7 (*Park7*) (see Fig. 3). Based on our data and literature, we hypothesize that drinking to intoxication produces changes in gene expression in MSNs, which may contribute to regulation of alcohol consumption and preference.

Meta-Analysis Across Brain Regions

Most genes correlated with BEC at $p < 0.05$ were specific for a given brain region (see Table S2), suggesting that different cell types use different strategies to adapt to acute alcohol.

Table 2. Results of Functional Group Over-Representation Analysis of Alcohol-Responsive Modules

General functional category	Functional term	Functional annotation resource	Brain region	Expression module	*p*-Value	FDR %
Blood circulation	Enriched in blood	Suppl. Table S1	CB	Blue	5.15E-19	<1
	Enriched in blood	Suppl. Table S1	STR	Grey60	1.93E-17	<1
	Enriched in blood	Suppl. Table S1	VMB	MediumPurple3	1.90E-27	<1
	Enriched in blood	Suppl. Table S1	HIP	Cyan	9.61E-22	<1
Signal transduction	Regulation of signal transduction	Gene Ontology	CB	Blue	3.03E-03	5
	Wnt receptor signaling pathway	Gene Ontology	HIP	Blue	4.17E-03	7
	Signal	Swiss-Prot Keywords	OB	Brown	3.23E-03	5
Cell–Cell communication	Cell junction/synapse	Gene Ontology	OB	Brown	3.41E-03	5
	Adherens junction	Gene Ontology	FC	Salmon	5.25E-03	7
	Cell–Cell Signaling	Gene Ontology	STR	Green	5.61E-03	10
ATP-Dependent processes	Nucleotide binding/ATP binding	Swiss-Prot Keywords	VMB	YellowGreen	2.46E-03	4
	ATP binding	Gene Ontology	STR	SteelBlue	2.06E-05	<1
Cellular component/transport	Localization	Gene Ontology	STR	Green	4.06E-03	7
	Receptor-mediated endocytosis	Gene Ontology	HIP	GreenYellow	4.39E-03	8
	Cation transport	Gene Ontology	HIP	GreenYellow	2.44E-03	4
	Nucleolar part	Gene Ontology	OB	DarkTurquoise	5.03E-03	7
	Organelle envelope	Gene Ontology	FC	RoyalBlue	6.80E-03	9
	Actin cytoskeleton	Gene Ontology	VMB	Orange	5.39E-03	8
	RNA-Protein Complex Biogenesis	Gene Ontology	FC	Salmon	3.33E-03	6
Enzymatic activity	Kinase activity	Gene Ontology	STR	SteelBlue	2.42E-04	<1
	Transferase	Swiss-Prot Keywords	STR	Grey60	2.15E-03	3
	Phosphoprotein	Swiss-Prot Keywords	VMB	MediumPurple3	1.06E-03	2
CNS development	CNS development	Gene Ontology	CB	Cyan	3.25E-03	6
Metabolism	Butanoate metabolism	KEGG Pathway	FC	Plum	7.72E-03	9
Brain cell type	Enriched in neurons	Cahoy et al., 2008	STR	Turquoise	2.05E-03	7

Only top results are shown [over-representation $p < 0.008$; false discovery rate (FDR) < 10%]. Brain regions: CB, cerebellum; STR, striatum; VMB, ventral midbrain; HIP, hippocampus; FC, frontal cortex; OB, olfactory bulb.

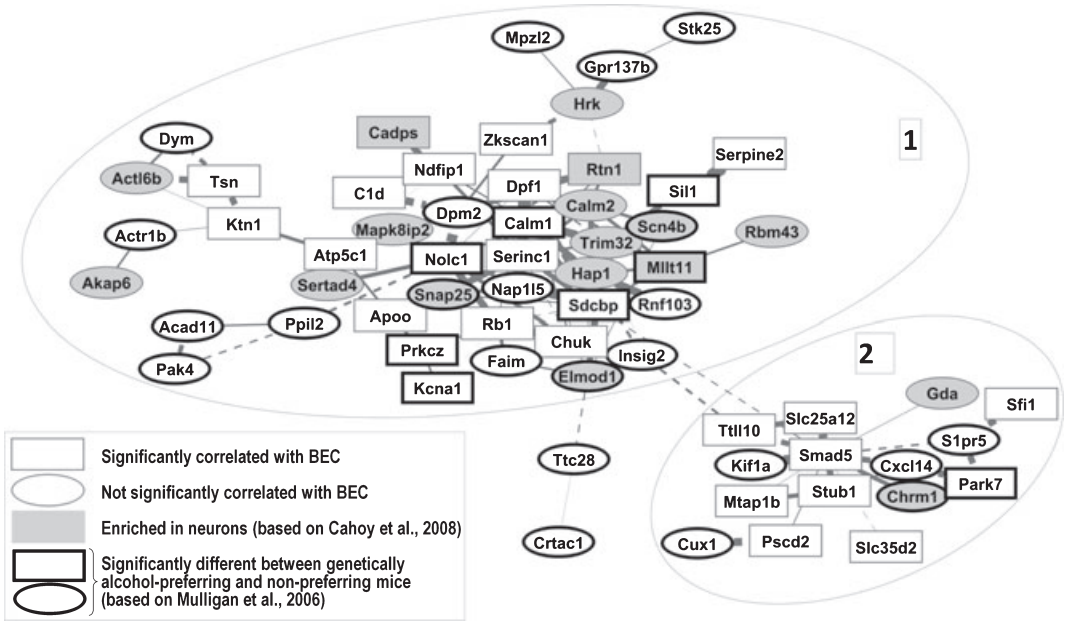

Fig. 3. A correlation-based network formed by a subset of genes from the turquoise module of striatum. Sixty-three highly intercorrelated genes (all $R > 0.50$; $p < 0.01$) of total 158 genes in the turquoise module are shown. Additional information for the genes shown, including gene names and correlations with blood ethanol concentration (BEC), is available in Supplementary Table S3. Solid lines between genes represent positive correlations, while dashed lines represent negative correlations and thickness of the lines is proportional to the correlation magnitude. Rectangular boxes represent genes regulated by acute drinking in the present study. Thick border lines represent genes differentially regulated between alcohol-preferring and non-preferring mice based on Mulligan and colleagues (2006). Grey background represents genes enriched in neurons based on Cahoy and colleagues (2008). Two separate modules detected by a layout algorithm are encircled with a dotted line. Functional over-representation analysis determined that Modules 1 and 2 are enriched with genes involved in protein binding ($p < 0.003$; FDR = 4%) and oxidative phosphorylation ($p < 0.002$; false discovery rate = 2%), respectively, suggesting that correlation-based gene networks are organized according to some known biological principles.

However, there was a subset of genes that were consistently regulated across multiple brain regions. These genes were identified using a meta-analysis. Top statistically significant transcripts ($p < 0.002$; FDR = 10%) representing 42 unique known genes are shown in Fig. 4. As expected, the four experimental groups clustered together across brain regions based on average linkage hierarchical clustering with the most separation observed between the control group and the high alcohol group. Clustering by gene separated genes with a positive vs negative correlation with BEC (clusters A and B respectively). Cluster A contained highly intercorrelated genes (Average $R > 0.5$) that could be further subdivided into two subclusters. Subcluster A1 was highly enriched for genes expressed in blood (hypergeometric $p <$ 1E-50), while three of the members of subcluster A2 (*Gstm1, Mt2 and Cst3*) were enriched for expression in astrocytes (hypergeometric $p <$ 3E-4). Analysis of pair-wise Pearson's correlations between astrocyte- and blood-enriched genes from the two subclusters resulted in a range of positive correlations, most of which were significant at $p < 0.05$ level (average $R = 0.55$; $p < 0.01$). This result suggests coordinated transcriptional responses in astroglia to alcohol-induced changes in brain blood circulation. Genes from Fig. 4 which had been previously identified by the Ethanol Related Gene Resource (ERGR) (Guo et al., 2009) as being associated with alcohol-related disease in humans or showing changes in gene expression after exposure to alcohol in mice, rats or humans are shown in Table S5. RT-PCR of control and high alcohol groups across several brain regions validated the expression of representative genes from each category, *Hba-a1* (blood) and *Mt2 (*astrocyte). Expression of both genes was greater in the high alcohol group compared to control (average fold change = 45%, combined $p = 0.008$ for *Hba-a1*; average fold change = 23%, combined $p = 0.0001$ for *Mt2*).

DISCUSSION

Initial administration of addictive substances results in changes in brain gene expression that may contribute to sustained drug intake and the development of tolerance and dependence. To our knowledge, the present study is the first to examine the effects of alcohol dose on global brain gene expression in a mouse model of voluntary drinking to intoxication. Compared to previous microarray studies examining the effects of acute alcohol on global gene expression (Daniels and Buck, 2002; Kerns et al., 2005; Treadwell and Singh, 2004), our approach took advantage of the DID paradigm, which allowed animals to experience intoxicating amounts of alcohol in the non-stressful environment of their home cages. In this experiment, age, sex and strain were held constant, and environment effects were minimized, yet there was remarkable variation in BECs after a single four-hour access period, most of which could be explained by differences in alcohol consumption. Some of the variation in initial alcohol

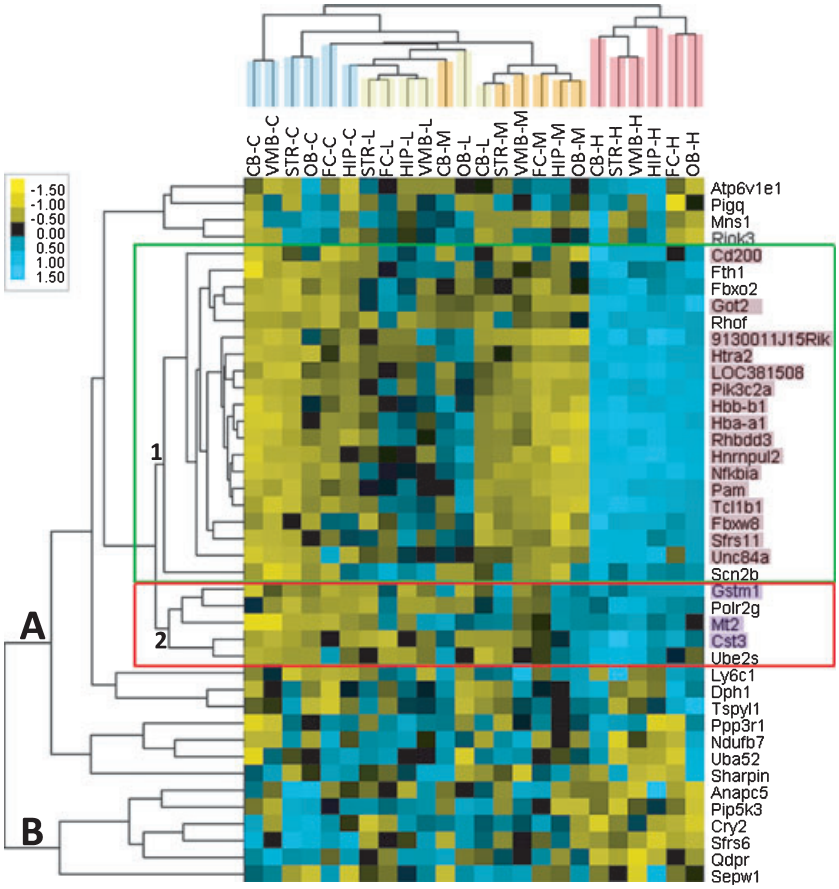

Fig. 4. A clustergram showing alcohol-responsive genes consistently detected across all brain regions using a meta-analysis. Four groups, one control (C) and three showing relative low (L), medium (M), and high (H) levels of alcohol intake and blood ethanol concentration (BEC) were clustered together across all brain regions (vertical clustering). Genes (horizontal clustering) formed two clusters: A and B corresponding to a positive or negative correlation between gene expression and BEC respectively. Two subclusters of cluster A were enriched in either blood (subcluster A1, green box) or astrocytes (subcluster A2, red box). Colors represent a relative increase (blue) or decrease (yellow) in gene expression. Brain regions: CB, cerebellum; STR, striatum; OB, olfactory bulb; FC, frontal cortex; VMB, ventral midbrain.

drinking may be because of underlying differences in prenatal environment, maternal care, and/or epigenetic factors not measured in this study. It is possible that these pre-existing environmental or epigenetic factors might affect drinking responses through differential expression of some genes. While understanding the role of these factors in alcohol consumption should be the focus of future work, the present study took advantage of this wide range of drinking responses to determine alcohol dose-dependent molecular patterns. These molecular changes, whether pre-existing or alcohol-induced, are related to differential alcohol drinking and should be considered for generating novel hypotheses.

Eight unique known genes were identified as significantly correlated with BEC using a genome-wide correction for false discovery rate. Half of these genes were identified by previous large-scale studies as alcohol related. Brain expression of *Hba-a1* and *Hbb-b1* is altered after acute alcohol administration in mice (Kerns et al., 2005). *Fth1* is differentially expressed in the brains of inbred rat strains that differ in alcohol preference (Edenberg et al., 2005; Kimpel et al., 2007). Pltp was previously found to be involved in changes in cholesterol metabolism after ethanol consumption (Lagrost et al., 1996; Liinamaa et al., 1997; Makela et al., 2008; Riemens et al., 1997). Of the remaining genes, *S100a10* and *Grinl1a* are involved in modulating serotonergic and glutamatergic neurotransmitter systems, respectively, and *Phyhip* and *Tinagl1* are not well characterized. *S100a10* is enriched in neurons (Cahoy et al., 2008) and directly regulates the trafficking of ion channels and serotonin receptors including, sodium channel, voltage-gated, type X (Scn10a) (Okuse et al., 2002), amiloride-sensitive cation channel 2 (Accn2) (Donier et al., 2005), transient receptor potential cation channel, subfamily V, members 5 (Trpv5) and 6 (Trpv6) (Van De Graaf et al., 2003), potassium channel, subfamily K, member 3 (Kcnk3) (Girard et al., 2002), and serotonin receptor 1B (Htr1b) (Svenningsson et al., 2006). Expression of *S100a10* has been shown to be altered in response to stress (Zhang et al., 2008) and depression (Svenningsson et al., 2006; Tzang et al., 2008). Grinl1a is likely to be directly involved in the regulation of glutamatergic signaling via association with the Grin1 subunit (Roginski et al., 2008) and has also been shown to be down-regulated in Alzheimer's disease

progression along with other glutamatergic receptors (Jacob et al., 2007).

Selecting a small number of candidate genes based on strict statistical thresholds has been widely used in microarray studies. However, there is growing evidence that statistical significance does not necessarily translate into functional significance and a selection strategy based on overall holistic evidence and biological relevance may be more appropriate. Recent advances in genomics and bioinformatics have allowed researchers to increase statistical power at a reduced cost per experiment. Combining these global gene expression assays with network and pathway analyses based on gene co-expression relationships has taken genomic discovery to a new level. These types of analyses will assist in prioritization of candidate genes for functional validation and formulation of specific hypotheses at the systems level (Oldham et al., 2008; Ponomarev et al., 2010). We used this systems-based approach in our study to identify alcohol-responsive modules of co-expressed genes. This analysis revealed alcohol-responsive functional and structural groups that were either specific to a brain region or detected in multiple regions. A striking example of the latter category was blood circulation, as transcript levels of many genes enriched in blood were correlated with BEC and clustered together in several brain regions. In principle, a change in expression of a particular gene reflects one of two distinct processes: an actual change in mRNA copy number or a change in the abundance of tissue or the number of cells where this gene is preferentially expressed. Because small doses of acute ethanol have been shown to increase cerebral blood flow (Mathew and Wilson, 1991), we hypothesize that the alcohol-induced increase in mRNA levels of genes enriched in blood reflects changes in blood flow because of BEC in different brain regions. Although this finding is not unexpected, it is reassuring to be able to make predictions at the systems level and validate physiological findings using genomics approaches.

One hypothesis that evolved from our modular network analysis is that striatal medium spiny neurons may react to acute alcohol consumption with transcriptional changes that may underlie subsequent changes in behaviour, including alcohol preference, tolerance and dependence. Medium spiny neurons are sensitive targets of acute ethanol, as they can be affected by the drug directly through inhibition of postsynaptic NMDA receptors or indirectly via ethanol-induced increase in synaptic dopamine (Gonzales et al., 2004; Maldve et al., 2002). Repeated alcohol induces cellular adaptation in these neurons in nucleus accumbens, possibly through interplay between dopamine and glutamate systems (Chandler, 2003; Gonzales et al., 2004; Maldve et al., 2002). Based on the modular analysis and convergent evidence, we nominate 3 genes (*Scn4b*, *Prkcz,* and *Park7)* as functional candidates for ethanol-induced plasticity in medium spiny neurons. Sodium channel beta4 subunit (*Scn4b*) is an auxiliary subunit with a role in regulation of neuronal activity (Grieco et al., 2005) and pathogenesis of Huntington's disease (Oyama et al., 2006). Protein kinase C zeta (*Prkcz*) is a serine/threonine kinase involved in a variety of cellular processes such as proliferation, differentiation, and secretion. A recent study implicated striatal expression of this gene in cocaine-induced sensitization (Chen et al., 2007). Parkinson's disease protein 7 (*Park7*) is a peptidase with a role in pathogenesis of Parkinson's disease and neuronal protection against oxidative stress (Aleyasin et al., 2010). All three of these genes differ in expression between genetic mouse models of high and low alcohol preference (Mulligan et al., 2006), *Park7* shows differential expression in a similar rat model (Kimpel et al., 2007), and we found both *Park7* and *Prkcz* to be alcohol dose-responsive. In addition, all 3 are candidate genes for 1 of the 2 behavioural quantitative trait loci (bQTLs) for alcohol consumption (Belknap and Atkins, 2001) because their chromosomal locations overlap with bQTLs on mouse chromosomes 9 (*Scn4b*) or 4 (*Park7*, *Prkcz*) and their expression in nucleus accumbens is regulated by closely linked genetic polymorphisms (genenetwork.org). Different validation strategies can now be used to determine alcohol-related roles of these genes in medium spiny neurons including cell type–specific deletion, downregulation or overexpression of selected genes using a combination of Cre driver lines, viral microinjections and RNAi (Lobo, 2009).

Finally, we used a meta-analysis of gene expression across all brain regions to identify consistent alcohol-responsive molecular patterns. Blood circulation was enriched in the top genes from the meta-analysis, an expected result given the identification of alcohol-responsive and blood-enriched modules in most brain regions. Additionally, this analysis detected an alcohol-responsive molecular signature specific to astroglia. These two patterns were highly correlated, suggesting a coordinated response of cerebral blood flow and gene expression in astrocytes to an increasing dose of alcohol. Astrocytes play active roles in brain physiology. They coordinate neurovascular coupling by regulating cerebral blood flow to supply neurons with energy metabolites in an activity-dependent manner (Koehler et al., 2009; Takano et al., 2006). Astrocytes sense glutamatergic synaptic activity and signal blood vessels about the need for regional increases in blood flow (Allen and Barres, 2009; Koehler et al., 2009). Ethanol produces biphasic effects at molecular, physiological and behavioural levels. Low doses lead to increases in the levels of glutamate in brain (Moghaddam and Bolinao, 1994), cerebral blood flow (Mathew and Wilson, 1991) and stimulate locomotor activity in mice (Ponomarev and Crabbe, 2002a; Shen et al., 1995), while high doses result in decreases in these responses. This cumulative evidence suggests that astrocytes may play an active role in ethanol-induced changes in cerebral blood flow.

Exposure to ethanol has been shown to produce oxidative stress in the brain (Nordmann, 1987; Reddy et al., 1999; Renis et al., 1996), especially in astrocytes and neurons (Montoliu et al., 1995; Rathinam et al., 2006). Several of the genes upregulated by ethanol and enriched in astrocytes may play a protective role in response to cellular stress. For example, glutathione S-transferase mu 1 (*Gstm1*) is expressed in the brain where its function is to detoxify electrophilic

compounds such as ethanol, drugs, and carcinogens (Hayes and Pulford, 1995). Metallothionein 2 (*Mt2*) synthesis is induced in the central nervous system in response to a variety of pathogens and disorders. This gene is involved in host defense reactions and neuroprotection during neuropathological conditions, in which it decreases inflammation and oxidative stress and promotes postinjury repair and regeneration (Pedersen et al., 2009). Several of the genes involved in blood circulation (*Hba-a1, Hbb-b1, Nfkbia, Pam, Sfrs11,* and *Unc84a*) as well as the astrocyte enriched genes (*Gstm1, Mt2 and Cst3*) have been detected by independent studies as alcohol-responsive or associated with different ethanol traits (ERGR, http://bioinfo.vipbg.vcu.edu/ERGR/). Several genes (*Gstm1*, *Mt2*, *Fth1, Qdpr,* and *Nfkbia*) displayed an association with responses to ethanol in two different species or in multiple independent studies. In many cases, the direction of change is similar across studies; however, it is important to note that brain region sampled, ethanol dose, ethanol administration paradigm and sampling time after ethanol exposure were not identical across studies. For this reason, expression changes are not expected to be directly comparable but simply to provide additional support for the nomination of these genes as strong candidates underlying the initial response to intoxicating doses of alcohol.

A recurrent theme in the progression from acute to chronic alcohol intake is an initial change in mRNA or protein levels that likely represent normal adaptive processes invoked to maintain system homeostasis under the increasing burden placed on the system by alcohol. Chronic exposure to alcohol may cause dysregulation of these homeostatic mechanisms leading to alcohol dependence and abuse. In this study, we generated candidate genes and pathways targeted by initial alcohol self-administration by measuring global brain gene expression in alcohol-preferring C57BL/6J mice after a single bout of voluntary drinking using a modified DID procedure and by employing several novel approaches and convergent evidence. Consistent with other global gene expression studies, we found that acute alcohol consumption results in small but consistent changes in brain gene expression. We identified both common and region-specific changes and generated hypotheses linking candidate genes to functions of specific cell types. Future studies will use genetic mouse models for high alcohol consumption, such as "High Drinking In the Dark" selectively bred lines (Crabbe et al., 2009) and advanced cell targeting techniques to validate the roles of selected genes in regulation of alcohol drinking.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Transcripts enriched in blood

Table S2. All genes correlated with BEC at $p < 0.05$

Table S3. All genes in alcohol-responsive modules

Table S4. Over-represented functional groups

Table S5. Genes from Fig. 4 that had been previously identified by the Ethanol Related Gene Resource (ERGR) (Guo et al., 2009) as alcohol-related

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